Phosphorus mobilization and localisation in the roots and nodules of a Cape Floristic Region legume, and its impact on nitrogen fixation

by

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Summary

During phosphorus (P) deficiency, plants can exhibit a wide array of morphological, physiological and biochemical responses. Legume plants are vulnerable to P deficiency, because it affects their ability to fix atmospheric nitrogen (N_2) via their symbiotic interaction with rhizobial bacteria. In particular, legumes from nutrient poor ecosystems, such as the fynbos in the Cape Floristic Region (CFR) would have evolved on P deficient soils and may therefore display unique modifications. Moreover, since P distribution in soils is heterogenous, even less is known about the recovery from P deficiency responses in nodules. The aim of this research was to investigate P recycling and distribution in the nodules of the fynbos legume *Virgilia divaricata*, during low P supply and its recovery from P deficiency.

The legume species was inoculated with a locally compatible N_2 fixing bacterial strain, *Burkholderia*, isolated from *V. divaricata* nodules grown in fynbos soil. Plants were grown under glasshouse conditions, using a modified Long Ashton Nutrient Solution (LANS) to simulate the low nutrient conditions of the fynbos ecosystem. Plants were subsequently analysed for growth kinetics, nutrient acquisition and distribution, nodule anatomy, P recycling and P metabolite composition.

The results indicated that *V. divaricata* can experience P deficiency during exposure to low P supply. Under low P conditions, plants experienced lower biomass and nodule production. Although biological N_2 fixation (BNF) was lower during P deficiency as compared to during conditions of optimal P supply, the nodules of plants grown under P deficient conditions had a greater BNF per nodule mass and unit P. In addition, low P nodules also showed homogenous P tissue localisation and a greater concentration of Fe. The total P level was lower in nodule tissues, and the activities of phosphohydrolases (APase, RNase and phytase)

higher. In addition, there was also a possible remobilization of membrane phospholipids, in order to release additional Pi.

Although *V. divaricata* experiences P deficiency in its biomass and P nutrition, it also has a remarkable physiological ability to recover from P deficiency during P resupply. In contrast to the observed perturbations in biomass and nutrition during P stress, the impact on the nodules was different to that of the roots. The underlying mechanisms for functional maintenance of the nodules during low P seems to be associated with an internal mechanism, related to P mobilization from organic sources, metabolic bypass mechanisms to conserve P and a re-allocation of Fe to the infected cells. The higher enzyme activity of the internal phosphohydrolases (APase, RNase and phytase) favours the liberation of cellular P for metabolic reactions and internal P turnover.

This research has generated knowledge regarding the physiological impact and flexibility of mechanisms involving below-ground P recycling in legumes. It has demonstrated that a legume from a nutrient poor ecosystem, favours internal mechanisms of P recycling and conservation, in order to maintain the efficient functioning of nodules under P stress rather than improve acquisition from external sources.

Opsomming

Gedurende fosfor (P) korting, kan plante 'n wye verskeidenheid van morfologiese, fisiologiese en biochemiese reaksies uit stal. Peulplante is sensitief vir P vermindering, deur hul vermoë om atmosferiese stikstof (N_2) te bind saam hul simbiotiese interaksie met rhizobiale bakterieë. In die besonder, sou peulplante van voedingstowwe swak ekosisteme, soos die fynbos in die Kaapse Floristiese Streek ontwikkel het op P gebrekkige grond en kan dus unieke wysigings vertoon. Verder, aangesien P verspreiding in gronde heterogene is, is nog minder bekend oor die herstel van P vermindering in wortel-knoppies . Die doel van hierdie navorsing was om P herwinning en verspreiding in die wortel-knoppies van *Virgilia divaricata* tydens lae P aanbod en sy herstel van P –tekort te bepaal.

Virgilia divaricata is ingeënt met 'n lokale versoenbaar bakterië, *Burkholderia*. Plante is gekweek in gesteriliseerde sand, onder glasshuis voorwaardes, met 'n aangepaste Long Ashton voedingsoplossing om die lae voedingstowwe van die fynbos ekosisteem te simuleer. Plante is daarna ontleed vir groei kinetika, voedingstof verkryging en verspreiding, wortel-knoppies anatomie, P herwinning en P metaboliet samestelling.

Die resultate dui dat *V. divaricata* P-tekort kan ervaar tydens blootstelling aan lae P aanbod. Onder lae P voorwaardes, ervaar plante laer biomassa en wortel-knoppie produksie. Hoewel biologiese N_2 fiksasie laer was in P-tekort plante, is biologiese N_2 fiksasie per wortel-knoppie massa en eenheid P, meer. Die totale P vlak was laer in wortel-knoppie weefsel, en die aktiwiteite van phosphohydrolases (APase, RNase en fytase) hoër. Daarbenewens was daar ook 'n moontlike remobilization van membraan fosfolipiede, ten einde bykomende Pi vry te stel.

Hoewel *V. divaricata* P-tekort ervaar in biomassa en P voeding, het dit ook 'n merkwaardige fisiologiese vermoë om te herstel van P-tekort. In teenstelling met die waargeneem versteurings in biomassa en voeding tydens P stres, was die impak op die wortel-knoppies anders as dié van die wortels. Die onderliggende meganismes vir funksionele instandhouding van die wortel-knoppie tydens lae P is in verband met 'n interne meganisme. Die hoër ensiemaktiwiteit van die interne phosphohydrolases bevoordeel die bevryding van sellulêre P vir metaboliese reaksies en interne P omset.

Hierdie navorsing het kennis van die fisiologiese impak en buigsaamheid van meganismes wat onder-grond P herwinning gegenereer. Dit het getoon dat *V. divaricata* interne meganismes van P herwinning en bewaring, ten einde die doeltreffende funksionering van wortel-knoppies onder P stres te handhaaf eerder as verbeter verkryging van eksterne bronne.

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%	percentage
˚C	degrees Celsius
ADP	adenosine 5’-diphosphate
ANOVA	analysis of variance
APase	acid phosphatase
ATP	adenosine 5’-triphsophate
BNF	biological nitrogen fixation
CFR	Cape Floristic Region
CO_2	carbon dioxide
d	day
dw	dry weight
Fe	iron
fw	fresh weight
g	grams
GOGAT	glutamate synthase
GS	glutamine synthase
K	potassium
M	molar
MDH	malate dehydrogenase
Mg^{2+}	magnesium
m^2	square meter
ml	millilitre
mg	milligram
mM	millimolar

NAD	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
N	nitrogen
NH^3	ammonia
P	phosphorus
PEPC	phosphoenolpyruvate carboxylase
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
Pi	inorganic phosphate
Ppm	part per million
PSI	phosphorus starvation inducible
RGR	relative growth rate
RNase	ribonuclease
s	seconds
SS	sucrose synthase
SNAR	specific nitrogen acquisition rate
SPAR	specific phosphate acquisition rate
SNUR	specific nitrogen utilisation rate
SPUR	specific phosphate utilisation rate
TCA	tricarboxylic acid cycle
T-test	statistical student's t distribution
µmol	micromole
µl	microliter
µM	micromolar
$\delta^{15}N$	nitrogen isotopic ratio

Chapter 1: Literature Review

1. Legumes

1.1 Description and biogeography

The Leguminosae, generally known as the legume, bean or pea family is the third largest flowering plant family, comprising approximately 700 genera (Lewis et al. 2005). The order Fabales to which the legumes belong is part of a "nitrogen (N_2) fixing clade" that includes eight other flowering plant families known to form N_2 fixing symbioses with phylogenetically diverse soil bacteria called rhizobia (Lewis et al. 2005). Legumes have a worldwide distribution, except they are absent from the high arctic and Antarctica (Sprent 2009). They occur across four biomes described as 1) semi-arid, fire-intolerant, succulent-rich and grass-poor, dry tropical forest, thicket and bush land 2) a fire-tolerant, succulent-poor and 3) a tropical wet forest biome and 4) a temperate biome including both the Northern and Southern Hemispheres. Many legume clades have distinctive geographical and ecological phylogenetic structures, which in turn predict phylogenetic relatedness amongst terminal taxa (Lewis et al. 2005). In addition, the four biomes in which they are found could also be viewed as a result of dispersal assembly, where taxa with similar ecological preferences can disperse to similar ecological settings worldwide.

The fact that legumes occur across the world illustrates their broad ecological amplitude and adaptation. For example, many indigenous legumes (e.g. *Aspalathus, Cyclopia, Virgilia, Psoralea*, *Podalyria*, *Hypocalyptus* and *Wiborgia*) in the Cape Floristic Region (CFR) of South Africa seem to have adapted to the nutrient-poor fynbos soils and depend heavily on N_2 fixation for their N nutrition (Schutte 2000, Spriggs and Dakora 2007).

1.2. Legume use and value

Thousands of legume species exist, many of which are important grain, pasture, and agroforestry species. Grain and forage legumes account for 27% of the world's primary crop production (Graham and Vance 2000). Legumes are also an increasingly invaluable food source for humans as well as farm animals (Graham and Vance 2003). In 2004, legumes were grown on more than 13% of the total arable land under cultivation (Gepts et al. 2005). Many legume species provide valuable timber, tannins, resins, gums, insecticides, and fibers. Industrially, legumes are used in the production of biodegradable plastics, oils, dyes, and biodiesel fuel (Morris 1997). Traditionally, legumes are used in folk medicines, but their role in modern medicine has also been demonstrated (Gepts et al. 2005).

2. Nitrogen Fixation

Approximately 80% of the atmosphere is N_2 which cannot be used by most living organisms. This is because all organisms use the form ammonia (NH_3) to manufacture amino acids, proteins, nucleic acids and other N-containing components necessary for life (Lindemann and Glover 2008). An important characteristic of the plants of the Leguminosae is their ability to develop root nodules and fix N_2 in symbiosis with rhizobia (Graham and Vance 2003). Legumes receive the bulk of N_2 fixed by rhizobia as NH_3, which is incorporated into organic form before being exported from nodules. NH_3 assimilation within nodules also requires carbon compounds and amino acids that play an important role in fuelling N_2 fixation and assimilation as well as exporting products to the rest of the plant (Udvardi and Poole 2013).

The process of N_2 fixation is denoted by the equation $\mathbf{N_2 + 8H^+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi}$. The reaction is performed exclusively by prokaryotes, using the nitrogenase enzyme, which is highly sensitive to oxygen. The enzyme is inactivated when exposed to oxygen, because this reacts with the iron component of the proteins (Abrol et al.

2007). In symbiotic N_2 fixing organisms such as *Rhizobium*, the root nodules can contain leghaemoglobin, which shows as a pink colour when the active N fixing nodules of legume roots are cut open. Leghaemoglobin may regulate the supply of oxygen to the nodule tissues in the same way as haemoglobin regulates the supply of oxygen to mammalian tissues (Abrol et al. 2007).

3. Legume-rhizobia interaction and nodule development

The symbiotic association between legumes and rhizobia results from the infection of the legume by rhizobia; this group of prokaryotes encompass members of the α-Proteobacteria (*Rhizobium, Sinorhizobium, Ensifer, Mesorhizobium, Bradyrhizobium Azorhizobium, Methylobacterium, Ochrobacterium* and *Phyllobacterium*) (Lodwig and Poole 2003), and β-Proteobacteria (*Burkholderia* and *Cupriavidus*) (Sprent 2009). Rhizobia have the unique capacity to induce the formation of root nodules in legumes by the production of specific signal molecules called Nod factors (Lerouge et al. 1990). The interaction between rhizobia and the legume is mediated by the Nod factor (Figure 1.1) and trans-membrane receptors on the cells of the root hairs of the legume (Perret et al. 2000). Different strains of rhizobia produce different Nod factors and different legumes produce receptors of different specificity. With the correct combinations, the bacteria enter the epithelial cells of the root and migrate to the cortex, forming an infection thread. Once the infection thread reaches a cell deep in the cortex, the cell undergoes several rounds of mitosis without cytokinesis, and the cortex cells divide rapidly forming a nodule (Figure 1.1). The rhizobia in the nodule cells also undergo multiplication and start losing motility and change in shape, and are therefore then referred to as bacteroids. This differentiated form of the rhizobia (bacteroids), is able to convert atmospheric dinitrogen into NH_3 and supply the plant with “fixed” N in exchange for other nutrients and carbohydrates (Mergaert et al. 2006). The relationship between the

rhizobium and the legume is mutualistic. The legume supplies carbon resources and nutrients (Vance 2002) to the bacteroids and this is used to synthesize large amounts of ATP needed to convert N_2 into NH_3. In addition, the legume supplies one critical component, molybdenum, which is part of the nitrogenase enzyme complex that is essential for N_2 fixation (Kim and Reese 1994). The bacteroids need oxygen to make their ATP (by cellular respiration). However because nitrogenase is strongly inhibited by oxygen, the bacteroids walk a fine line between too much and too little oxygen.

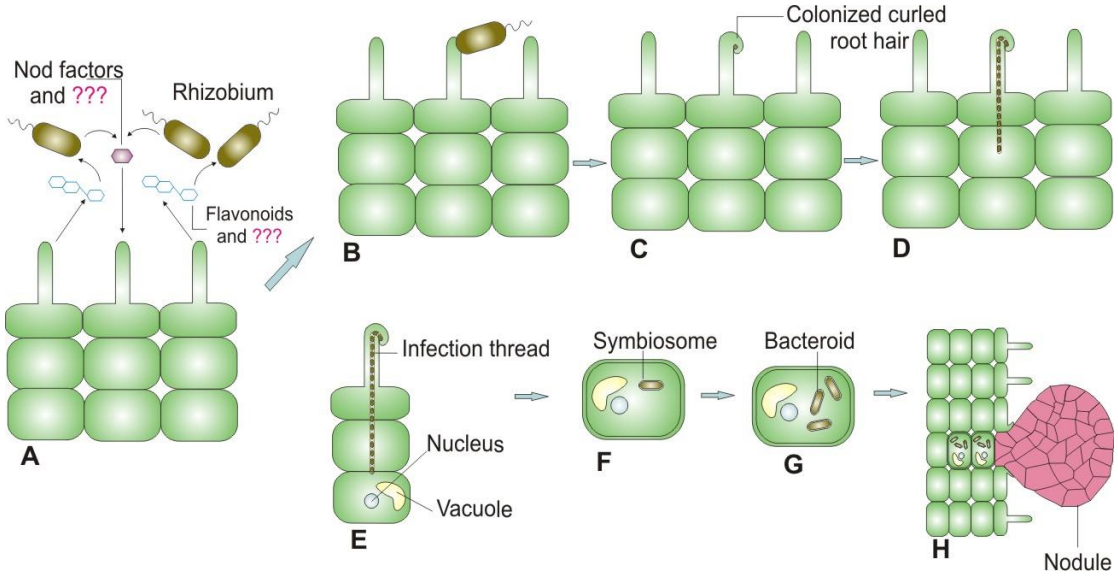

Figure 1.1 A) The initial signaling in Rhizobium-legume interaction. B-C) Attachment of rhizobium to the root hair and curled root hair formation. D) Root hair invasion by development of the infection thread. E) Endocytosis of bacteria into plant cell. F) Formation of symbiosome as an individual bacterium with surrounding endocytic membrane. G) Differentiated bacteroid. H) Nitrogen fixing nodule. (Adapted from Jensen 2015).

3.1 Exchange of metabolites between the legume host and bacteria during BNF

During N_2-fixation, a complex exchange of metabolites (Figure 1.2) between the bacteroid and legume occurs. Bacteroids use nitrogenase to reduce N_2. The first product of the reaction is NH_3 which was always thought to be the sole N source secreted by the bacteroid because

the levels of bacteroid NH_3 assimilatory enzymes (GS-GOGAT) are insufficient for the rates of glutamate/ glutamine synthesis required for N_2 fixation by the plant (Temple et al. 1998). However, soybean bacteroids isolated on sucrose gradients synthesized and secreted alanine as the sole N product (Waters et al. 1998). In pea nodules, the secretion products of bacteroids isolated on Percoll gradients were cell density dependent (Allaway et al. 2000). At low bacteroid densities only NH_3 was produced, while at high bacteroid density alanine and NH_3 were secreted. Alanine accumulates from N reduction by nitrogenase more rapidly at high bacteroid densities.

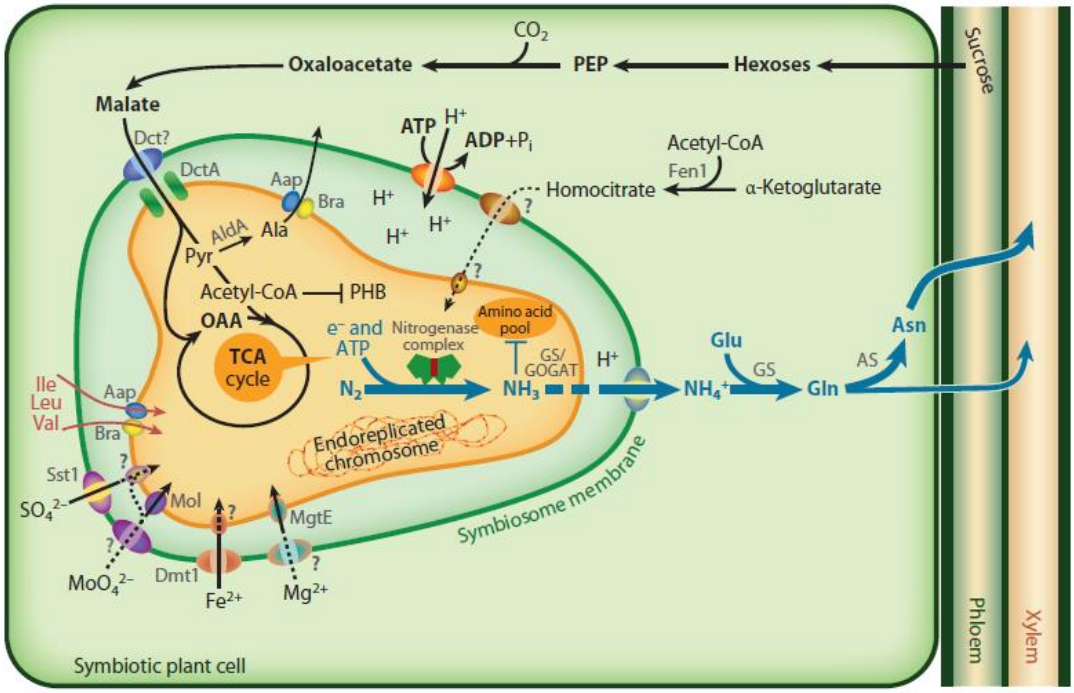

Figure 1.2 Transport and metabolism in an infected nodule cell. Sucrose from the shoot is converted to malate in the plant and imported across the symbiosome membrane and into bacteroids, where it fuels N_2 fixation. The product is then exported back to the plant, where it is assimilated into asparagine (Asn) for export to the shoot (blue arrows). In many legumes, such as soybean, the export products are ureides instead of Asn. The plant must provide metals and ions to the bacteroid, although only some of the transport systems on the symbiosome and bacteroid membranes are defined. (Adapted from Udvardi and Poole 2013).

Glutamine is the primary amino compound synthesized from the NH_3 within the plant cytosol by the coupled activities of glutamine synthetase and glutamate synthase (GS-GOGAT). In the plant cytosol there are high levels of GS-GOGAT (Figure 1.2). Nodule glutamate synthase (GOGAT) activity is increased compared to other plant organs (Chen and Cullimore 1988). The high levels of nodule GS and GOGAT are achieved by the nodule-specific induction of a single enzyme or by the induction of nodule-specific isoenzymes (Roche et al. 1993).

Fixed N_2 is further transferred from glutamine to either asparagine or to purine derivatives known as ureides, depending on the legume species. Temperate legumes (e.g. pea, clover and alfalfa) which form indeterminate nodules, export mainly asparagine, formed by glutamine dependent asparagine synthetase (Vance 2000). Tropical legumes (e.g. soybean and common bean) which form determinate nodules, mainly export ureides such as allantoin and allantoic acid (Atkins and Smith 2000).

The carbon supplied to the bacteroid to fuel N_2 fixation originates from photosynthate transported to the nodule as sucrose via the phloem (Streeter 1981). In nodule tissue sucrose is cleaved by sucrose synthase (SS) to uridine diphosphate (UDP) -glucose and fructose. SS activity is nodule enhanced suggesting that it is the main enzyme of sucrose cleavage in the nodule (Lodwig and Poole 2003). Following cleavage by SS, the hydrolyzed products are used as substrates for cellulose or starch biosynthesis and/or are metabolized by glycolytic enzymes to produce phosphoenolpyruvate (PEP). PEP is further metabolized to produce malate via phosphoenolpyruvate carboxylase (PEPc) and malate dehydrogenase (MDH) (Lodwig and Poole 2003).

4. Biological nitrogen fixation constraints

Three major factors influence biological nitrogen fixation. These are factors relating to climate, biology and nutrition which will be discussed in this section.

4.1 Climatic Factors

Climatic factors influence all aspects of nodulation and symbiotic N_2 fixation. In some instances, these factors can reduce rhizobial survival and diversity in the soil. Factors that are important include temperature, waterlogging and drought.

4.1.1 Temperature

Temperature has a marked influence on the survival and persistence of rhizobia, and nodule development (Mohammadi et al. 2012). Every legume-rhizobia interaction has an optimum temperature relationship which is approximately 30 °C for clover and pea, between 35 to 40 °C for soybean; peanut; and cowpea, and between 25 to 30 °C for common bean (Long 2001). The optimal temperature for nodulation is very species specific. Nodule formation in arrow-leaf clover occurs at a temperature as low as 7 °C whereas for the majority of tropical and subtropical legumes 15 to 18 °C is a more common minimum (Haque and Jutzi 1984). In soybean on the other hand, nodulation occurs at 25 °C (Lindemann and Ham 1979).

Nitrogenase activity is also affected by temperature. The minimum range for nitrogenase function is between 2 to 10 ˚C, with maximum functioning at 20 to 25 ˚C and an upper limit of 35 to 40 ˚C (Liu et al. 2011). Tropical and subtropical legumes tend to exhibit higher minimum temperatures for optimal nitrogenase activity, when compared to temperate legumes. The effect of temperature on the nitrogenase enzyme is also species specific (Serraj and Adu-Gyamfi 2004, Liu et al. 2011).

4.1.2 Drought and water excess

Both water stress (drought) and excess may adversely affect nodulation and N_2 fixation. Nodules function is tremendously sensitive to drought stress (Galves et al. 2005). Drought stress may cause structural changes in nodules including folding and dehydration of the cell wall, damage to the bacteroid- and peribacteroid membranes, and decreased air spaces in the bacteroids often leading to no senescence of bacteroids (Guerin et al. 1990). Some legume species, such as *Arachis hypogea* and groundnut crop *Vigna subterranean* are however, quite drought tolerant (Basu et al. 2007).

Permanently or temporarily waterlogged soils are common in the highlands and tropical areas of Africa. Since legumes tolerant to waterlogging are known, adaptation to waterlogging is possible (Sprent 2009). For example *Sesbania rostrata*, which forms stem nodules, has five to ten times more nodules than the best nodulated crops, and has outstanding potential for N_2 fixation in flooded soils (Dreyfus and Dommergues 1981). Similarly, *Discolobium pulchellum* will only nodulate when submerged (Lourerio et al. 1994). Peas and beans are unable to endure extended periods in waterlogged soil though (Sprent 2009). This is a function of the whole plant, not just the nodules.

4.2 Biological factors

Legumes are targeted by many pests and pathogens. Insect pests and plant pathogens have no direct effect on symbiotic N_2 fixation, but they can indirectly affect fixation through their effect on the growth and persistence of the legume plant (Sprent 2009). Plants growing under P deficiency also show enhanced levels of root colonization by mycorrhizal fungi. Some mycorrhizal fungi can access forms of N and P that are unavailable to non-mycorrhizal infected or associated plants, particularly organic forms of these nutrients (Morgan et al. 2005). Mycorrhizal fungi have the ability to produce mycotoxins which may result in decreased rhizobial populations in the soil and consequently less nodulation on legume roots

(Al-Falih 2002), hence these fungi can have a competitive advantage when occurring simultaneously with rhizobia.

4.3 Nutritional factors

The legume-rhizobia symbiosis imposes additional nutrient requirements apart from those generally needed for growth and function (Serraj and Adu-Gyamfi 2004). Legumes supplied with adequate concentrations of nutrients generally nodulate and fix N_2 better (Mohammadi et al. 2012). Specific elements such as phosphorus (P), molybdenum (Mo) and iron (Fe) are essential for the process of nodulation and N_2 fixation and limited availability of these elements may negatively impact the growth, survival, and metabolic activity of the legume and rhizobia (Werner and Newton 2005).

4.3.1 Iron and Molybdenum

Iron is essential for both the legume and the rhizobium (Tang et al. 1990). Iron deficiency has been reported to cause poor nodulation in chickpea (Rai et al. 1982) and lentil (Rai et al. 1984). The element is also important for N_2 fixation as it is a component of several key proteins such as nitrogenase, leghaemoglobin and ferredoxin (Tang et al. 1990). In peanut Fe deficiency resulted in the delay or prevention of nitrogenase production (O'Hara et al. 1988). The role of Fe as a component of leghemoglobin is that it functions in the regulation of oxygen supply to bacteroids (Tang et al. 1992).

The nitrogenase enzyme consists of two subunits, one of which is the Mo-Fe protein directly involved in reducing N_2 to NH_3 (Kaiser et al. 2005). Molybdenum availability is closely correlated with nodule development (Kaiser et al. 2005). Legumes seem to maintain Mo concentrations in nodules as the partitioning of molybdenum in common bean and soybean favours both nodules and developing seeds relative to other tissues (Brodrick and Giller

1991). In pasture legumes, Mo deficiency, associated with the inability to fix N_2, results in stunting and leaf yellowing (Fageria 2009).

4.3.2 Phosphorus

Phosphorus plays an important role in legume growth. Limited P restricts root growth, the process of photosynthesis, translocation of sugars, and other such functions which directly or indirectly influence N_2 fixation (Fatima et al. 2006). Nodules are strong sinks for P (Al-Niemi et al. 1997) and nodule formation and function require large amounts of P for maintenance (Tang et al. 2001). Phosphorus fertilization usually results in enhanced nodule number and mass and greater N_2 fixation per plant and per gram of nodules (Valentine et al. 2010). Studies of barrel medic, *Medicago truncatula*, showed that P stress delayed: (1) leaf development and leaf expansion along the main and axillary shoots; (2) axillary shoot emergence and elongation, resulting in stunted plants; and (3) timing and frequency of flower emergence (Bucciarelli et al. 2006). In alfalfa, architectural root changes occur under P stress (Tesfaye et al. 2006).

5. Phosphorus availability and uptake

Phosphorus is taken up by plants as inorganic phosphate (Pi) which occurs in soil solutions at low concentrations of 0.1 to 10 μM (Hinsinger 2001). Inorganic P usually accounts for 35-70% of total P in soil (Shen et al. 2011). Inorganic P availability is controlled by soil solution pH, ionic strength, concentrations of P and metals (Fe, Al and Ca) and the presence of competing anions, including organic acids (Sanyal and DeDatta 1991). Inorganic P is mainly supplied to plant roots by diffusion rather than mass flow. Uptake of Pi requires large amounts of energy. Kinetic analysis of Pi uptake shows that plants have both a low- and high-affinity uptake system. The high-affinity system operating at low Pi concentrations has an apparent K_m ranging from 3 to 10 μM while the low-affinity system operating at high Pi

concentrations has a K_m (Michaelis-Menten constant reflecting the affinity of an enzyme for its substrate) ranging from 50 to 300 μM. The high-affinity uptake process is induced when Pi is deficient whereas the low-affinity system appears to be constitutive in plants (Raghothama 1999).

6. The Pi- starvation response

In order to cope with the scarce availability of Pi, plants are thought to have evolved various morphological, physiological, and biochemical strategies to enhance Pi uptake, often also termed the Pi-starvation response (Tran et al. 2010, Vance et al. 2003). Morphological strategies include changes in root architecture, increasing the root: shoot growth ratio, shifting from primary to lateral root growth, and increasing root hair growth and density (Vance et al. 2003). Reorganizing cellular metabolism in a manner that conserves the limited pools of adenylates and Pi is an alternative and significant biochemical adaptation of Pi deprived plants. This is accomplished by altering the organization of glycolysis, mitochondrial respiration, and tonoplast H+-pumps allowing adenylate and Pi-dependent reactions to be bypassed during Pi starvation (Plaxton 2004, Plaxton and Podestá 2006). Several of these bypasses facilitate respiration and vacuolar pH maintenance during extended periods of Pi starvation by using pyrophosphate in performing cellular work, while simultaneously conserving ATP and recycling Pi (Figure 1.3). Glycolytic bypass enzymes include pyrophosphate-dependent phosphofructokinase (PFK) and PEP. The PEPc catalyzed bypass of cytosolic pyruvate kinase also results in the synthesis of organic acids from glycolytic metabolites which is critical for the anaplerotic replenishment of TCA cycle intermediates, as well as the root secretion of organic acids, a common response to Pi starvation (Tran et al. 2010).

Organic acid secretion may increases the ability of secreted acid phosphatases to scavenge Pi from soil localized organic-Pi-esters. However, the effectiveness of different organic acids in

soil Pi mobilization depends upon the form and amount of the particular anion being released. Citrate appears to be the most effective relative to other organic anions, such as malate. Plants also increase the efficiency of Pi use during Pi deficiency via upregulation of Pi-starvation inducible hydrolases that scavenge Pi from non-essential P-esters. Classical Pi-deficiency inducible hydrolases include non-specific phospholipases, ribonucleases (RNases), and acid phosphatases (APases) (Barriola et al. 1999, Plaxton 2004). See Figure 1.3.

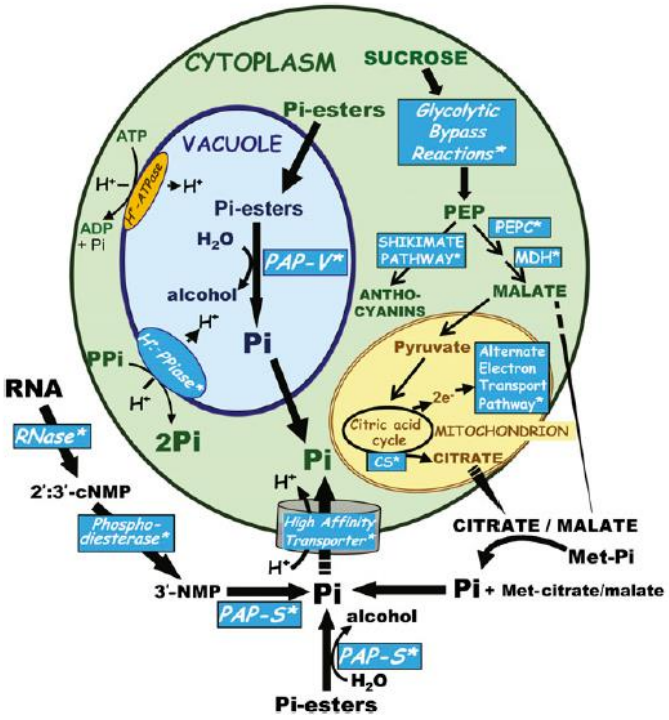

Figure 1.3 A model suggesting various adaptive metabolic processes (indicated by asterisks) that are believed to contribute to the ability of plants acclimate to nutritional Pi deficiency. Alternative pathways facilitate respiration and vacuolar pH maintenance by Pi-starved plant cells because they negate the dependence on adenylates and Pi, the levels of which become markedly depressed during severe Pi starvation. Large quantities of organic acids produced by PEP carboxylase (PEPC), malate dehydrogenase (MDH), and citrate synthase (CS) may also be secreted by roots to: (i) increase the availability of mineral bound Pi (by solubilizing Ca-, Fe- and Al-phosphates = 'Met–Pi'), and (ii) increase the availability of organic-Pi and its amenability to hydrolysis by secreted acid phosphatases. Adapted from Tran et al. 2010.

7. Phosphorus scavenging enzymes (hydrolases)

During Pi deficiency, plants may increase the efficiency of Pi use by up-regulating a wide range of phosphorus starvation inducible (PSI) hydrolases that scavenge and recycle Pi from intra and extracellular organic P compounds (Vance et al. 2003, Tran et al. 2010). The induction of secreted RNases, nucleases, phosphodiesterases, and APases function in the systematic catabolism of soil-localized nucleic acids and their degradation products to mobilize Pi, which roots obtain via high-affinity Pi transporters (Plaxton and Tran 2011). The upregulation of intracellular (vacuolar) and secreted APases, enzymes that hydrolyze Pi from a broad range of Pi monoesters is also a well-documented PSI response (Tran et al. 2010).

PSI APases are secreted into the extracellular matrix and intercellular spaces (apoplast) of plant tissues (Kaida et al. 2009). It is thought that extracellular APases function in Pi recycling, from organic P compounds leaked from Pi-deficient cells while intracellular (vacuolar) APases scavenge and remobilize Pi from expendable intracellular Pi monoesters and anhydrides. This is accompanied by marked reductions in levels of cytoplasmic P metabolites during extended Pi deprivation (Vance et al. 2003). Intracellular APases appear to be much less stable than extracellular forms, which remain stable for hours to days (Miller et al. 2001).

It has frequently been reported that the activity of APases increase under low Pi conditions (Ciereszko et al. 2011, Wyszołmirska et al. 2006). However, in a study with *Arabidopsis* tissues, several APase isozymes were present yet only a subset of these isozymes was induced by Pi deficiency (Trull et al. 1997). Conversely, there are other studies that found a negative relationship between APase activity and Pi uptake/use efficiency under phosphate starvation (McLachlan 1980, Yan et al. 2002).

Furthermore, APases have been implicated in providing P during seed germination from stored phytate (Biswas and Cundiff 1991); release of P from soil organic P-esters by exudation of enzymes into the rhizosphere (Lefebvre et al. 1990, Miller et al. 2001); and the synthesis of glycolate from P-glycolate (Christeller and Tolbert 1978) as well as glycerate from 3-PGA during photorespiration. Characteristically, phytases and root-secreted APases have little substrate specificity, while APases involved in carbon metabolism (i.e. phosphoglycolate phosphatase, 3-PGA phosphatase and PEP phosphatase have much stricter substrate specificities (Duff et al. 1991, Miller et al. 2001).

8. Mechanisms for improved P uptake

8.1 In non-legumes

The foundation of contemporary P deficiency research comes from studies on the model plant, *Arabidopsis*, in which several P deficiency responses have been documented. These studies have shown that root hair density (Ma et al. 2001) and elongation are regulated by P availability (Bates and Lynch 2000). Root hair density was fivefold greater in low-P than in high P-media. Grierson et al. (2001) reported that at least 40 genes in *Arabidopsis* affect root hair initiation and development and that many of these may be responsive to P-deficiency.

For *Arabidopsis*, it has been shown that P deficiency responses are regulated at the transcriptional level with a highly coordinated gene expression program. Transcriptional-level studies have identified many genes differentially regulated by P deficiency. Transcription factors (TFs) are master control proteins and in *Arabidopsis*, the expression of several TFs is regulated in a cell- or tissue-specific manner in response to a specific stress (Chen et al. 2007). The initial response to P stress internally in *Arabidopsis*, is considered to be general or non-specific and includes the induction of genes related to oxidative stress and pathogen responses (Franco-Zorrilla et al. 2004). The expression of these genes (e.g.

monogalactosyl diacyl glycerol synthase) may decrease with extended P deficient periods and may trigger the formation of root modification that lead to acclimation to P deficiency (Wu et al. 2003). During early P stress, the expression of certain genes may also be repressed- for example, glutamine synthase which is involved in nitrogen assimilation (Lodwig and Poole 2003).

After the initial period of exposure to P deficiency, specific responses can be observed in gene expression (Wu et al. 2003, Misson et al. 2005), and includes the PEPc and MDH genes that participate in the TCA pathways and whose products promote the synthesis of organic acids that are secreted for P remobilization in the soil, the genes involved in P remobilization (phosphatases, RNAses), and in P transport (Lopez and Hernandez 2008). Because TFs regulate expression, it is important to delineate the functions of TFs. The TFs involved in P stress in *Arabidopsis*, belong to different gene families including MYB, SCARECROW, AP2, F-box, HOMEOBOX, WRKY and Zinc-finger members (Wu et al. 2003, Misson et al. 2005, Muller et al. 2007). Despite all this knowledge on gene expression in response to P stress, there is not a lot of information on the regulation of gene expression changes for legume species.

8.2 In legumes

The main focus of P deficiency research in legumes has been directed at white lupin (*Lupinus albus*) and common bean (*Phaseolus vulgaris*), and to a lesser extent in *Medicago truncatula* (a model legume system) and soybean (*Glycine max*) (Graham and Vance 2003, Vance et al. 2003, Tesfaye et al. 2007). Phosphorus deficiency in white lupin is correlated with the formation of proteoid/cluster roots which increase the root surface area enormously and secrete organic acids to aid in P remobilization (Johnson et al. 1996), as well as the enhanced expression of many genes, such as secreted acid phosphatase (LaSAP1) and Pi transporters such as LaPT1 (Vance 2001, Vance et al. 2003). In white lupin, most of the genes induced in

cluster root formation were found to be involved in the metabolic bypassing of phosphate use (organic acid biosynthesis (PEPc, MDH), P remobilization, phytohormone metabolism, and proteoid root development (Uhde-Stone et al. 2003). Similarly, beans form adventitious and shallow roots, and modify their root growth axis in response to P deficiency to enhance P uptake (Ochoa et al. 2006).

However, the previous studies on legumes largely document general changes associated with the root organ (Penheiter et al. 1997, Araujo et al. 2008, Li et al. 2011, Bargaz et al. 2012), and less is known about the impacts on the nodules. Furthermore, most of these studies (Vance 2001, Vance et al. 2003, Uhde-Stone et al. 2003, Ochoa et al. 2006) have been conducted on P deficient and sufficient plants, where the responses were documented in comparison to the control plants. With the exception of recent work in lupins (Thuynsma et al. 2014), it is largely unknown what the functional plasticity of these P deficincy responses are, when P is resupplied to the plant. The recent work by Thuynsma et al. (2014) has shown that during P resupply, *Lupinus albus* can alter its biomass allocation to cluster roots and nodules. The implication of such findings to legumes from nutrient-poor ecosystems is very important from a functional and evolutionary perspective. This is because the flexibility may reveal functional traits, in order to recover from the P stressed physiological syndrome, if a P enriched patch of soil is encountered by sections of the nodulated root system. It is well-known that nutrient distribution in natural soils is heterogenous and that roots can display some plasticity in their responses to this (Hodge 2004).

The evolutionary effect of a flexible response to variable levels of P for legumes that have evolved in nutrient-poor ecosystems is important. This is because the degree of flexibility may impact expansion of the species and may benefit the species where there are both spatial and temporal variations in P availability. Since this is not currently known for any legume species from a nutrient-poor ecosystem, the use of *Virgilia divaricata* (Adamson) from the

fynbos ecosystem in the Cape Floristic Region, may serve as a model for local fynbos species.

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Chapter 2: General Introduction

Nitrogen (N) makes up 78% of the Earth's atmosphere but is the critical limiting element for growth of most plants due to its unavailability (Vance 2001). With the advent of the green revolution, the industrial process of converting N_2 to NH_3 was crucial as it successfully guaranteed N fertilizer for food crops (Valentine et al. 2010). Nitrogen is however a major pollutant in eutrophied regions causing detrimental impacts to freshwater and marine creatures, as well as disturbing the ecological equilibrium of terrestrial food webs (Valentine et al. 2010, Vance 2001). This has led to the development of alternative methods to increasing soil N. In agricultural systems, legumes that symbiotically fix N_2 with rhizobia, is considered the main natural contributor for usable N inputs. Legumes are incorporated into the soil using tillage to increase soil N and is a major source of N in areas where the cost of fertilizer is too high.

The ability of legumes to obtain atmospheric N is due to their symbiotic relationship with several species of certain soil bacteria. Nitrogen fixation by legume-rhizobium symbiosis adds approximately 40 million tonnes of N into agricultural systems annually (Herridge et al. 2008). There are however factors that may limit legumes from fixing N_2 (Lynch and Smith 1993, Hardarson and Atkins 2003) and it is important to understand these limiting factors in order to optimize the amount of N obtained though BNF.

Based on current evidence, one of the most limiting factors for biological N_2 fixation is phosphorus (P) availability. Although P plays a crucial role in plant growth and development, it is like N, often only present in growth limiting amounts. Nitrogen fixing legumes require more P than legumes growing on mineral N. Growing root nodules are strong P sinks in legumes. For example, P concentration in the nodules of soybean (Sa and Israel 1991) and white lupin (Schulze et al. 2006) from P deficient plants can reach up to 3-fold that of other

plant organs. Whilst this might be typical of agricultural systems, what about BNF constraints for legumes in nutrient poor natural ecosystems? In nutrient poor ecosystems such as the fynbos of South Africa and heathland of South Western Australia, both P and N are particularly limiting. Furthermore, the soils of the fynbos are acidic and acidity mobilizes iron and molybdenum which in turn exacerbates P deficiency. For legumes, P deficiency may limit the amount of N_2 fixed because of the role P plays in N_2 fixation (Al Niemi et al. 1997, Aono et al. 2001). Phosphorus deficiency has important implications for the Pi and adenylate pools of plants, which influence respiration and N_2 fixation (Theodorou and Plaxton 1996, Plaxton and Podesta 2006) and P forms part of important compounds such as ATP which is needed for N_2 fixation.

Legumes have evolved morphological and physiological responses (as briefly discussed in Chapter 1) to cope with P deficiency. The morphological responses involve the modification of root architecture, principally by decreasing primary root growth and increasing lateral root and root hair formation. The physiological and biochemical responses include modifications of carbon metabolism to bypass P requiring steps, synthesis and secretion of APases, exudation of organic acids, and enhanced expression of high affinity phosphate transporters.

Currently, there is little information concerning the regulation of P in nodules under P deficient conditions and how it relates to N_2 metabolism. Furthermore, most of these studies are based on agriculturally important legumes. In recent years, there has been an increase in studies examining P deficiency in roots, as roots are the primary source of mineral acquisition, although nodules are more important as the active site for biological N_2 fixation. The few studies on nodules are restricted to model legumes such as lupin, alfalfa and soybean which are crop species, and so the flexibility of these crop species to respond to P deficiency may differ from that of legumes from naturally nutrient poor habitats.

Since it is well-known that nutrient distribution in natural soils, is very heterogenous and that roots can display some plasticity in their responses to this, the implication of such findings to legumes from nutrient poor ecosystems is very important from a functional and evolutionary perspective. For this reason, the work presented in this thesis is based on a non-model legume, indigenous to South Africa. This would make a novel contribution to the understanding of the role of P in N_2 metabolism of local plants.

The research (outlined hereafter) is based on *Virgilia divaricata* (Adamson), commonly known as keurboom. The species is endemic to the fynbos ecosystem of South Africa and is typically found along the south-eastern coast from the Klein Swartberg Mountains to George, to Van Stadens Pass near Port Elizabeth. *V. divaricata* trees are small to medium in size, have colourful flowers, and produce seeds enclosed in a pod (Goldblatt and Manning 2000).

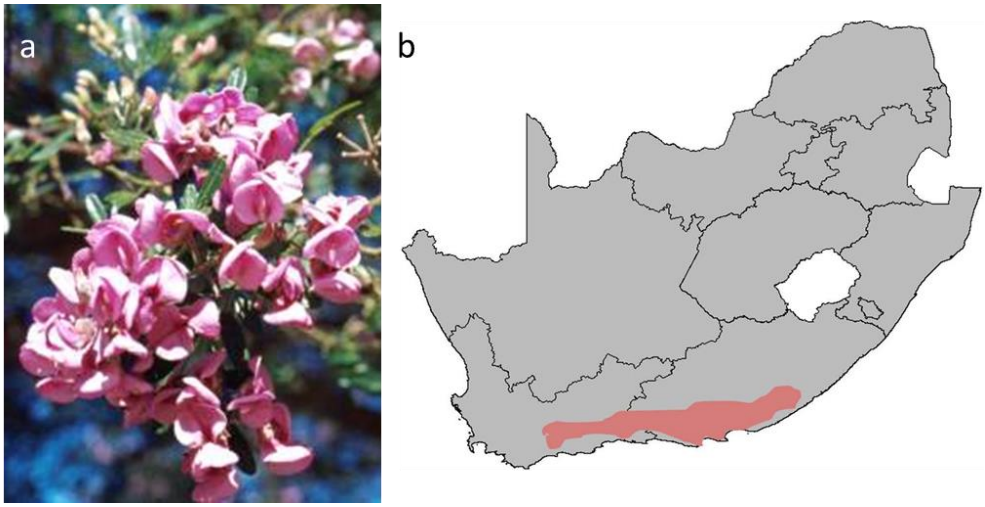

Figure 2.1 *Virgilia divaricata* (Adamson) (a) and the distribution thereof (b) in the Cape Floristic Region, South Africa.

The main hypothesis of this research was that variation of phosphorus supply at two extremes may have different functional impacts on the nodules of *V. divaricata* in terms of N_2-fixation capacity. The hypothesis was addressed via three specific aims:

a). To assess the growth, anatomy and mobilization of P and P- turnover during P stress (Chapter 3).

b). To determine the distribution of P (and other N_2 fixing elements) in the nodules and the associated costs of nutrient allocation and uptake and its effect on N_2 metabolism during variable P supply (Chapter 4).

c). To examine P recycling and P partitioning (the different forms or chemical fractions of P) in the nodules under variable P supply during N_2-fixation (Chapter 5).

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Chapter 3: Altered nodule anatomy drives P recycling and optimal nodule function of *Virgilia divaricata* during limited P –supply.

3.1 Abstract

Phosphorus (P) is one of the most limiting nutrients for plant growth. In legumes, P plays an important role in nodule initiation, bacteroid function and nitrogen (N) fixation. Although legumes can respond to P deficiency by increasing their biological N_2 fixation (BNF) per unit nodule, the underlying mechanisms remain unclear. The goal of this research was to investigate this underlying mechanism in the nodules of legumes from nutrient poor ecosystems, where they have evolved in P poor soils. The role of P nutrition in nodule function was tested in *Virgilia divaricata* (Adamson), indigenous to the nutrient-poor fynbos ecosystem in the Cape Floristic Region (CFR). Biomass allocation to nodules, nodule anatomy, nutrition and P recycling were assessed under P sufficient and deficient conditions. During P deficiency, biomass allocation to nodules was reduced but the ratio of infected cells per nodule was greater. The nodule recycling of organic P from internal and external pools, increased with P deficiency. Although P deficiency caused a lower BNF, BNF per nodule was higher and may be underpinned by altered nodule anatomy and efficient P recycling.

3.2 Introduction

Phosphorus (P) plays a pivotal role in plant cell metabolism but is one of the least accessible nutrients due to its low soil availability and the fact that it binds to metals such as Fe and Al rendering it insoluble (Aono et al. 2001). Generally, plants meet their P requirement by the uptake of P in inorganic form (Pi) and by employing an acquisition and/or recycling strategy (Vance et al. 2003). Plants possess various strategies for acquisition of P under limited availability, such as increased root growth or changes in root architecture (Lynch and Brown 1998) as well as the secretion of phosphohydrolases, enzymes that hydrolyze an organic phosphate group such as acid phosphatase (APase) and/ or ribonuclease (RNase) into the rhizosphere to improve P availability (Tadano et al. 1993, Duff et al. 1994, Rao et al. 1999). Furthermore, plants can also use P efficiently within the cell by making use of phosphohydrolases to recycle P internally. Bosse and Köck (1998) have shown that APase and RNase induction during P deficiency is associated with P turnover in plants. The induction of RNase during P deficiency has been reported for tomato cells (Nürnberger et al. 1990), *Arabidopsis* (Bariola et al. 1994), *Brachiaria* a tropical grass, as well as rice (Nanamori et al. 2004). Similarly, increased secretion of APases during P deficiency has been reported in barley (Ciereszko et al. 2011), cucumber (Ciereszko et al. 2002) as well as oat cultivars (Zebrowska et al. 2012).

The problem of low soil P availability is exacerbated in low-nutrient ecosystems especially for legume plants that require P to engage symbiotically in N_2 fixation with rhizobia. Previous studies have documented that P deficiency may have a negative effect on N_2 fixation because the process occurs in the nodule/ bacteroid fraction of the plant where NH_3 is assimilated into amino acids and ureides. This is an energy consuming process that depends on the energy status of the nodules (Sa and Israel 1991). Responses of legumes to P deficiency include the maintenance of a high P concentration in nodules (Pereira and Bliss

1987), increased absorption of P from the soil solution (Al-Niemi et al. 1997), and increased BNF per unit of nodule mass to compensate for reduced nodulation (Almeida et al. 2000). The underlying mechanisms for increased BNF per unit nodule mass, have not been elucidated, but may be underpinned by altered nodule anatomy and efficient P recycling.

In this study, we investigated BNF in the nodules of *V. divaricata*, a legume which is known to grow in the highly leached, P deficient soils of the CFR (Coetsee and Wigley 2013). Although the CFR has a rich legume diversity, very little is known about the mechanisms these legumes employ to cope with P limitation. Specifically we assessed BNF in response to altered nodule anatomy, nutrient metabolism and the exudation of APases and RNases in *V. divaricata.*

3.3 Materials and methods

3.3.1 Seed germination, bacterial inoculation, and growth

V. divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were placed in smoke solution (Smoke Plus, Kirstenbosch National Botanical Garden, South Africa) (Magadlela et al. 2014) and incubated in a water bath at 50 °C for 4 h. Thereafter seeds were surface sterilised, rinsed with distilled water and germinated in 5cm deep seed trays containing sterile sand under natural light conditions (midday irradiances between 600-800 μmol m^{-2} s^{-1}) in a temperature controlled (15-25 °C), north-facing greenhouse at Stellenbosch University, South Africa.

After the first fully expanded leaf emergence, seedlings were transferred to pots containing sterile sand and inoculated with *Burkholderia* (isolated from *V. divaricata* nodules grown in soil from Stellenbosch Mountain, Stellenbosch (natural fynbos inoculum). Inoculum was prepared by growing the bacterium on yeast mannitol agar (YMA) containing 0.5 g/L yeast extract (Biolab), 10 g/L mannitol (Saarchem), 0.5 g/L dipotassium hydrogen orthophosphate

(K_2HPO_4, Biolab), 0.2 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$, Biolab), 0.1 g/L sodium chloride (NaCl,

Biolab), 15 g/L bacteriological agar (Biolab) and 2.5 g/L Congo red (Saarchem) (Somasegaran and Hoben 1994). After incubation at 28 °C for 4 days, single colonies were selected and cultures prepared in Tryptone-Yeast medium containing 5 g/L tryptone (Biolab), 3 g/L yeast extract (Biolab) and 2 ml of a 440 g/L calcium chloride dihydrate ($CaCl_2.2H_2O$, Biolab) solution (Somasegaran and Hoben 1994). After incubation for 24 h at 28 °C, 50 ml of the bacterial culture were applied to each seedling.

Plants were separated into two treatment groups: group I (low phosphorus, LP) and group II (high phosphorus HP, control). The plants in these groups were supplied with 100 mL of a quarter strength Long Ashton nutrient solution (Hewitt, 1966) twice a week. The nutrient solution was modified to contain either 500 µM P (High P) or 5 µM P (Low P) (pH 5.8), and 500 µM NH_4NO_3. Plants were grown for 10 weeks under natural light conditions (midday irradiances between 600-800 µmol m^{-2} s^{-1}) in a temperature-controlled (15-25 °C), north-facing greenhouse at Stellenbosch University, South Africa.

3.3.2 Microscopy

Nodule samples from all three treatments were fixed under vacuum in 3% (v/v) glutaraldehyde made up in sodium (Na) cacodylate buffer (pH 7.2) at room temperature for 24 h. The samples were then rinsed in 0.05 M Na-cacodylate buffer, post-fixed in 2% (v/v) osmium tetraoxide (OsO_4) at 4 °C for 24 h and dehydrated in a graded (20-90%) ethanol series. The specimens were subsequently infiltrated in increasing concentrations of Spurr's resin (Spurr 1969), and embedded at 70 °C for 16 h. For light microscopy, thin transverse sections (1 mm in thickness) were prepared using a diamond cutter attached to a Reichert OMU3 ultramicrotome (C Reichert, Vienna, Austria). Sections were heat-fixed onto glass

slides, stained with Azur II and toluidine blue and examined with a Nikon Eclipse light microscope (Nikon, Tokyo, Japan).

3.3.3 Biomass parameters and nutrient concentrations

Upon harvesting, a subset of plants was separated into nodules, roots, and shoots. The harvested material was dried at 50 °C for 72 h and dry weights (dw) recorded. The latter were used to calculate growth parameters. The dried material was milled and analysed for their respective C and N concentrations at the Archeometry Department (University of Cape Town, South Africa) and P concentration at the Central Analytical Facility (Stellenbosch University, South Africa) using inductively coupled mass-spectrometry (ICP-MS).

3.3.4 Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} dw d^{-1}) reflects the net P absorption rate per unit root dry weight (Nielson et al., 2001) and was determined using the formula:

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

where M is the P content per plant, t is the time, and R is the root dry weight. This equation was however, modified to calculate the net P absorption rate for nodules. As such the nodule dry weight was used instead of root dry weight. Specific P utilization rate (SPUR) (g dw mg^{-1} P d^{-1}) is a measure of the dry weight gained for the P taken up by the plant (Nielson et al., 2001) and was estimated with the following formula:

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

where M is the P content of the plant and W is the plant dry weight. This equation was modified to calculate the dry weight gained for the P uptake by nodules, hence the nodule dry weight was used instead of plant dry weight.

3.3.5 Calculations of $\delta^{15}N$

The $\delta^{15}N$ analyses were also carried out at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000\% [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the samples and standards as defined by Farquhar et al. (1989). Approximately 2 mg of each sample was weighed into an 8 mm x 5 mm tin capsule (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the N gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. These values were used to determine the percentage N derived from the atmosphere (NDFA) according to the calculation by Shearer and Kohl (1986):

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

The reference plant was non-nodulated *V. divaricata*, grown under the same glasshouse conditions. The B value is the $\delta^{15}N$ natural abundance of the N derived exclusively from biological N fixation of nodulated *V. divaricata*, also grown under same conditions as the reference plants, but with an N-free nutrient solution.

3.3.6 RNase extraction and activity

The extraction followed the procedure described by Nanamori et al. (2004). For RNase analyses, 0.5 g of root and nodule tissue was homogenized in 2 ml of 100 mM sodium acetate buffer (pH 5.6), using an ice cold mortar and pestle. After centrifuging at 15,000×g for 20 min at 4 °C, the supernatant was collected as a crude extract and stored at –80° C until analysis. RNase activity was measured according to Bosse and Köck (1998). One unit of RNase was defined as the activity that liberated the amount of soluble nucleotide

corresponding to one unit of nucleotide per min. One unit is the amount of nucleotide that has an A260 of 1.0 in a volume of 1 ml according to Wilson (1975).

3.3.7 Extracellular acid phosphatase activity

To determine root and nodule surface APase activity quantitatively, fresh roots and whole nodules (approximately 3 g fresh weight [fw]) were washed in distilled water, blotted dry and placed into 5 ml of incubation medium with substrate mixture (6 mM p-nitrophenyl phosphate (pNPP) and 1 mM DTT in 50 mM Na-acetate buffer, pH 5.0), and incubated at 25 °C (Zebrowska et al. 2012, Ciereszko et al. 2002). After 30 min, 100 µl of the reaction medium was removed and added to 100 µl of 1 M NaOH to stop the reaction. Absorbance was then determined at 410 nm and enzyme was defined as the amount of p-nitrophenyl (pNP) released, relative to known pNP standards derived from a standard curve, expressed as µmol pNP min^{-1} g^{-1} fw (Ciereszko et al. 2002).

3.3.9 Statistics

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Kaleidagraph, Synergy Software, USA). Where the ANOVA revealed significant differences between treatments, the means were separated using a t-test (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences among treatments.

3.4 Results

3.4.1 Plant Biomass

Total plant biomass increased under P sufficient conditions. Root, nodule and shoot growth was greater (Table 3.1) compared to that of P deficient plants. The root to shoot ratio of P deficient plants exceeded that of P sufficient plants (Table 3.1).

Table 3.1 Biomass of *Virgilia divaricata* (Adamson) grown under adequate (High) and deficient (Low) phosphorus conditions for 10 weeks.

Dry weight (g)	High P 500µM, control	Low P 5µM
Total plant	1.187 ± 0.037 b	0.613 ± 0.023 a
Root	0.280 ± 0.007 b	0.174 ± 0.009 a
Shoot	0.655 ± 0.031 b	0.323 ± 0.009 a
Nodule	0.252 ± 0.015 b	0.116 ± 0.021 a
Root: shoot	0.431 ± 0.031 a	0.540 ± 0.041 b

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

3.4.2 Nodule formation and infection

All *V. divaricata* plants grown under P sufficient and deficient conditions formed nodules, in clusters, on their root system (Figure 3.1). Longitudinal sections of the root nodule depicted in Figure 3.2 shows that the nodules are determinate, as the inner tissue is homogenous with a mixture of infected and non-infected cells in the core, and scattered to a lesser extent throughout the tissue (Figure 3.2). These nodules lack the complex structure of indeterminate nodules which have defined zones. Nodules examined from the P sufficient treatment contained a greater coverage of infected tissue (Figure 3.2).

Figure 3.1 *Virgilia divaricata* (Adamson) (a) whole plants, (b) the root system containing (c) clusters of nodules.

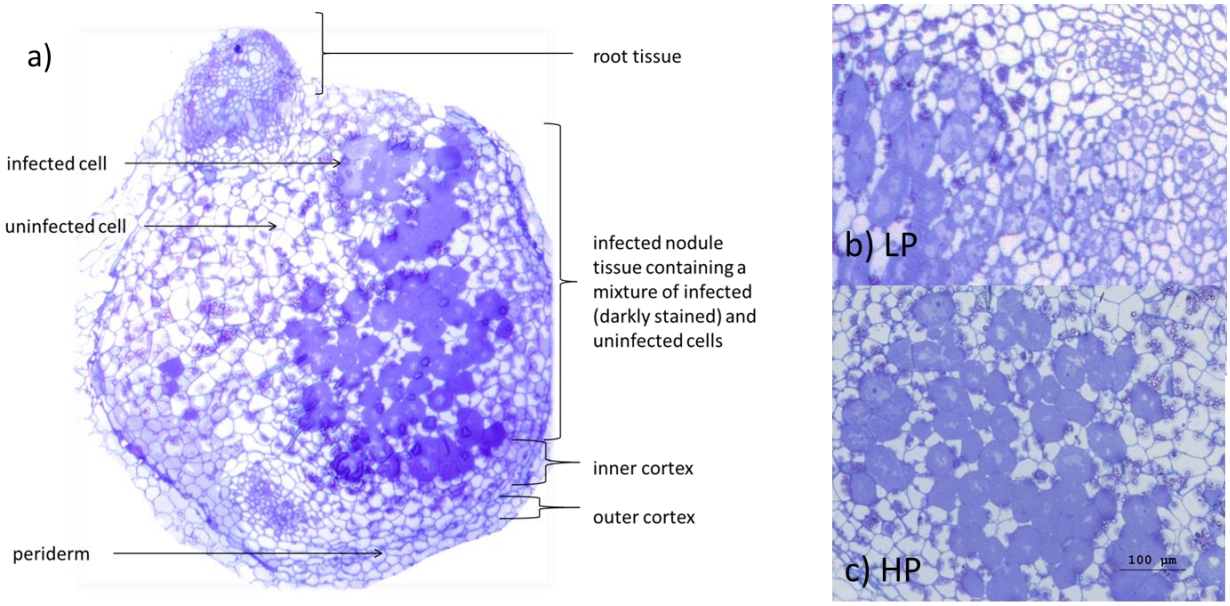

Figure 3.2 Nodule anatomy of *Virgilia divaricata* (Adamson). (a) Nodules consist of a mixture of infected and non-infected tissue, surrounded by the periderm and cortex. (b and c) Enlarged sections of a nodule grown under high P and low P conditions.

There was a two-fold increase in nodule production under P sufficient conditions compared to the P deficient treatment (Table 3.2). However, the ratio of infected cells to nodule number was greater under P deficient conditions (Table 3.2).

Table 3.2 Nodule parameters measured in *Virgilia divaricata* (Adamson) grown under adequate (High) and deficient (Low) phosphorus conditions for 10 weeks.

Nodule parameters	High P 500µM, control	Low P 5µM
Percentage (%) of infected cells	62.631 ± 2.546 b	47.000 ± 1.982 a
Number of nodules	51.000 ± 5.000 b	26.000 ± 7.000 a
Infected cells: nodule number	1.215 ± 0.090 a	1.807 ± 0.073 b

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

3.4.3 Nitrogen and phosphorus nutrition

The concentration of total P in the nodules was similar under both P treatments for but differed between roots, with higher values under P sufficient conditions (Table 3.3). The N concentration in both roots and nodules was greater under P sufficient conditions (Table 3.3). The decline in these elements under P deficient conditions coincided with reduced levels of uptake (SPAR) and utilization (SPUR) in the nodules (Table 3.4). Control plants obtained a greater percentage (62%) of N from the atmosphere compared to low P plants (46%) (Table 3.4). However, on a nodule-mass basis, P deficient plants showed a two-fold higher value in BNF (Table 3.4).

Table 3.3 Nitrogen and phosphorus concentration in the roots and nodules of *Virgilia divaricata* (Adamson) grown under adequate (High) and deficient (Low) phosphorus conditions for 10 weeks.

Nutrient concentration (mmol g^{-1} dw)	**High P 500µM, control**	**Low P 5µM**
Phosphorus (P)		
Root	4.825 ± 0.346 b	2.585 ± 0.123 a
Nodule	2.606 ± 0.257 a	2.039 ± 0.362 a
Nitrogen (N)		
Root	2.985 ± 0.088 b	1.935 ± 0.020 a
Nodule	3.459 ± 0.090 b	2.384 ± 0.085 a

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Table 3.4 Phosphorus absorption, utilization rates and nitrogen fixation in *Virgilia divaricata* (Adamson) nodules grown under adequate (High) and deficient (Low) phosphorus conditions.

Nutrition parameter	**High P 500µM, control**	**Low P 5µM**
Nodule Specific P Absorption Rate (SPAR) (mg P g^{-1} dw d^{-1})	0.092 ± 0.003 b	0.047 ± 0.002 a
Nodule Specific P Utilization Rate (SPUR) (g dw mg^{-1} P d^{-1})	0.030 ± 0.001 b	0.015 ± 0.002 a
Percentage N derived from the atmosphere (%NDFA)	62.610 ± 4.329 b	46.350 ± 3.507 a
Biological Nitrogen Fixing (BNF) (mmol N mg^{-1} nodule dw)	0.193 ± 0.074 a	0.384 ± 0.021 b

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

3.4.4 RNase and APase activity

Both RNase and APase root and nodule activity was greater under P deficient conditions (Figure 3.3). For roots, the activity of both enzymes, however, exceeded those recorded for nodules.

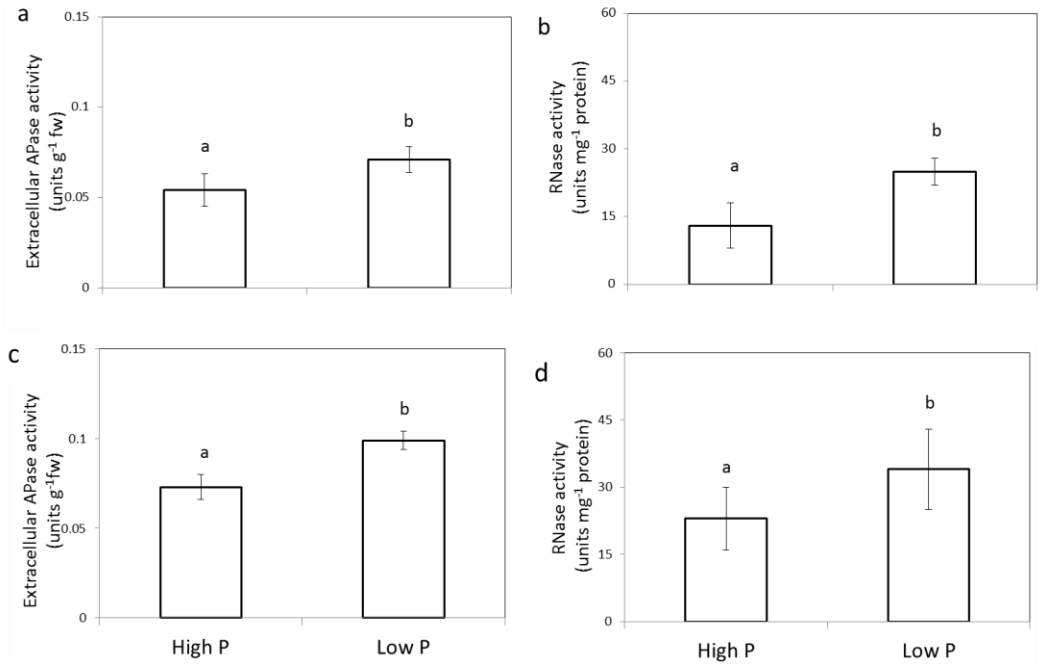

Figure 3.3 Extracellular APase enzyme activity in *Virgilia divaricata* (Adamson) nodules (a) and roots (c), and RNase activity in nodules (b) and (d) roots grown under adequate (High) and deficient (Low) phosphorus conditions. Values are presented as means ± SE of separate replicates (n = 5). Different letters indicate significant differences between treatments ($P \leq 0.05$).

3.5 Discussion

During low P supply, the percentage N derived from the atmosphere was lower and so the overall contribution of BNF is lower. When expressed per unit nodule dry weight however, BNF was higher. The implications of this to energy costs in a nutrient poor ecosystem and the underlying mechanism of altered nodule anatomy and increased P remobilisation are discussed.

Root, shoot and nodule biomass accumulation was lower while the root to shoot ratio was higher, a typical response of legumes limited by P availability as documented in lupins (Le Roux et al. 2006, Kleinert et al. 2014) as well as beans (Rychter and Mikulska 1990). The significantly lower amount of nodules formed under P deficiency is indicative of the high nodular requirement for P. This suggests that P deficiency was induced with rhizobial inoculation and although sufficient bacteria were present, perhaps there was not sufficient P for infection to occur to produce more nodules.

The maintenance of nodule P concentrations, while root P declined during P limitation, concurs with previous reports, of the nodule sink strength for soil and host P (Sa and Israel 1991, Le Roux et al. 2006, Kleinert et al. 2014). Compared to roots under P stress, nodules can scavenge and store the available P, as found in previous studies on *Glycine max* (Sa and Israel, 1991) and *Medicago truncatula* (Tang et al. 2001). Nodules can scavenge P from the roots and shoots but do not readily release P back to the roots (Al-Niemi et al. 1997), suggesting that nodules are strong sinks for P (Vadez et al. 1997). Therefore, a lower level of BNF during P deficiency may be related to an indirect effect of P stress on host function such as C allocation (Kleinert et al. 2014), which can indirectly affect nodule physiology.

In spite of the lower BNF during P deficiency, the production of almost 50% less nodules, may be compensated for by an increase in infected tissue per nodule, which may favour the acquisition of N at lower costs in a nutrient-poor ecosystem. This would serve as a distinct advantage for *V. divaricata*, in its indigenous low P soils. Although higher levels of BNF per nodule, concurs with Almeida et al. (2000), this is the first report to account for the possible mechanisms of such increased BNF.

Our findings suggest that this was underpinned by the alteration in nodule anatomy and its effect on P utilisation and recycling. In spite of the decline in nodule number, the high ratio of infected cells per nodule implies a redistribution of functional tissues within nodules. Two mechanisms identified in this study may be involved in the acquisition of P under P deficient conditions. The first associated mechanism to underpin the maintenance of P concentrations within nodules during P deficiency is the reduction in P utilisation for growth. Although roots also show a decline in P utilisation for growth, this appears to be a natural response to lower P levels in roots. However, compared to roots this is rather surprising in nodules, since roots showed a decline in P concentrations under P deprivation, while nodules did not. This P deficient response of nodules is possibly to conserve P within nodules. The second mechanism is an increase in P mobilisation from organic P pools within the nodule and associated with its surface. This is evidenced by the increased activities of cell wall associated APases and cellular RNases. Previous studies have shown that when these two phosphohydrolases are induced under low P conditions, that rapid P turnover enables the plant to use P more efficiently resulting in lower levels of P due to recycling within the cells (Nanamori et al. 2004). Vacuolar RNase is believed to contribute to the release of inorganic P by degradation of RNA (Bosse and Kock 1998).

P deficient roots and nodules exhibited greater APase activity compared to the P sufficient treatment. The greater activity in roots, however, suggests that roots scavenge for P and

transport P to nodules where nodules conserve P and typically do not exchange P with other organs (Schulze and Drevon 2005). Higher root APase activity may constitute an adaptive mechanism for N_2 fixing legumes to tolerate P deficiency (Kouas et al. 2008, Bargaz et al. 2012) e.g. white lupin secretes copious amounts of APases from its roots and proteoid roots when subjected to P starvation (Miller et al. 2001, Wasaki et al. 2008).

In conclusion, the higher BNF per nodule dry weight during P deficiency in the legume, *V. divaricata*, may serve as an advantage in the P poor soils of its fynbos ecosystem. The proposed physiological mechanism, which underpins this response, is the increased concentration of bacteroid-infected cells per number of nodules and the increased secretion of RNases and APases. Further investigation concerning the first mechanism is however required as this is not documented in the literature. We therefore surmise that the consequent sink demand for P by these infected cells was provided for by improved conservation and recycling strategies during P limitation. Although the increased BNF per nodule is achieved by integrated cellular, physiological and anatomical responses, the degree of flexibility of these responses is not known. This functional benefit of such flexibility in *V. divaricata* may be important in the short term recovery from P stress during the root system's encounter with the heterogenous distribution of P in soils.

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Chapter 4: Nodules from fynbos legume *Virgilia divaricata* have high functional plasticity under variable P levels

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Physiology

Nodules from Fynbos legume *Virgilia divaricata* have high functional plasticity under variable P supply levels

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4.1 Abstract

Legumes have the unique ability to fix atmospheric nitrogen (N_2) via symbiotic bacteria in their nodules but depend on phosphorus (P), which affects nodulation, and the carbon costs and energy costs of N_2 fixation. Consequently, legumes growing in nutrient-poor ecosystems (e.g., sandstone-derived soils) have to enhance P recycling and/or acquisition in order to maintain N_2 fixation. In this study, we investigated P recycling and distribution within the nodules and their effect on N nutrition in *Virgilia divaricata* (Adamson), an indigenous legume in the Cape Floristic Region of South Africa. Specifically, we assessed tissue elemental localization using micro-particle-induced X-ray emission (PIXE), measured N_2 fixation using nutrient concentrations derived from inductively coupled mass-spectrometry (ICP-MS), calculated nutrient costs, and determined P recycling from enzyme activity assays. Morphological and physiological features characteristic of adaptation to P deprivation were observed for *V. divaricata*. Decreased plant growth and nodule production with parallel increased root: shoot ratios are some of the plastic features exhibited in response to P deficiency. Plants resupplied with P resembled those supplied with optimal P levels in terms of growth and nutrient acquisition. Under low P conditions, plants maintained a higher N_2 fixation despite lower levels of inorganic phosphate (Pi) in the nodules. This can be attributed to two factors: (i) an increase in Fe concentration under low P, and (ii) greater APase activity in both the roots and nodules under low P. These findings suggest that *V. divaricata* is able to acquire N under P deficiency, owing to the plasticity of its nodule physiology.

4.2 Introduction

Soil P availability is the most limiting factor for plant growth, notably for legumes that symbiotically fix atmospheric nitrogen (N_2) with rhizobia (Vance et al. 2003). N_2 fixing legumes are autonomous at acquiring N, but depend on and require more P than legumes growing on mineral N (Drevon and Hartwig 1997). Phosphorus not only affects the energy costs of N_2 fixation (Schulze et al. 1999, Valentine et al. 2010), it is also important for nodule formation and function (Israel 1987). After N, however, P is often the most limited element in soils, especially in ancient sandstone-derived soils which are coarse grained and highly leached such as soils encountered in the Cape Floristic Region (CFR) of South Africa (Witkowski and Mitchell 1987). The legume tree, *V. divaricata* is native to the CFR, and is distributed over a wide range of variably P poor soils, from relatively richer forest margins to poorer fynbos soils (Coetsee and Wigley 2013).

Phosphorus is taken up as inorganic phosphate (Pi), but the chemistry of Pi in low Pi concentrations in the soil solution, thus limiting Pi diffusion to the root system (Morgan et al. 2005). Many studies have investigated P in rhizobium–legume symbiosis (Israel 1993, Høgh-Jensen et al. 2002, Olivera et al. 2004, Bucciarelli et al. 2006), and have demonstrated that improving P nutrition in legumes under P deficient conditions is generally based on two broad mechanisms (Raghothama 1999, Hammond et al. 2003). These are (i) increasing P acquisition that can be accomplished by increasing carbohydrate allocation to the roots, which increases the root: shoot ratio or causes a shift from primary to lateral root growth (Vance et al. 2003) and (ii) enhancing P utilization by increasing the abundance of Pi transport proteins and the exudation of organic acids, as well as phosphatases to mobilize P from organic or insoluble compounds (Plaxton 2004). An alternative way to attain P from the soil is through the secretion of APases to mobilize organic P (Duff et al. 1994). In plant

tissues, APases are mainly found in the cell wall and intracellular spaces (Yadav and Tarafdar 2001, Olczak et al. 2003). Extracellular APases are involved in breakdown of organic phosphate monoesters in the soil, whereas intracellular APases are thought to be pivotal in the remobilization and scavenging of Pi from intracellular phosphate monoesters (Duff et al. 1994, Marschner 1995). In common bean, the activities of APases increase in the nodules under low P conditions, indicating that N_2 fixing legumes can enhance P utilization within the nodules to tolerate P deficiency (Araújo et al. 2008).

The mechanistic effects of P limitation on N_2 fixation are not fully understood and not much is known of P metabolism in nodules. This is despite the fact that legumes occur worldwide where they thrive under a diversity of ecological conditions, including limited P and N availability. It is therefore reasonable to expect that even in nutrient poor ecosystems, legume species respond to low P conditions or make use of alternate strategies to obtain and recycle P (He et al. 2011). Most studies on the effect of P limitation on N_2 fixation have largely been confined to model legumes such as *Lupinus albus* (Schulze et al. 2006, Thuynsma et al. 2014) and *Medicago truncatula* (Tang et al. 2001, Sulieman et al. 2013). Despite the high legume diversity found on the P poor soils of the CFR (Goldblatt and Manning 2002), little is known about the functional mechanisms which affect N nutrition within the nodules of indigenous legumes. Our aim was therefore to investigate how P recycling and distribution in nodules affects the N nutrition of the indigenous legume *V. divaricata* during variable P supply.

4.3 Materials and methods

4.3.1 Seed germination, bacterial inoculation, and growth

V. divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were placed in smoke solution (Smoke Plus, Kirstenbosch National Botanical Garden, South Africa) (Magadlela et al. 2014) and incubated in a water bath at 50 °C for 4 h. Thereafter, the seeds were surface

sterilized, rinsed with distilled water and germinated in 5 cm deep seed trays containing sterile sand under natural light conditions (midday irradiances between 600–800 µmol m^{-2} s^{-1}) in a temperature-controlled (15–25 °C), north-facing greenhouse at Stellenbosch University, South Africa.

After the first fully expanded leaf emergence, seedlings were transferred to pots containing sterile sand and inoculated with *Burkholderia* (isolated from *V. divaricata* nodules grown in soil from Stellenbosch Mountain, Stellenbosch, South Africa (natural fynbos inoculum). Inoculum was prepared by growing the bacterium on yeast mannitol agar (YMA) containing 0.5 g/L yeast extract (Biolab), 10 g/L mannitol (Saarchem), 0.5 g/L dipotassium hydrogen orthophosphate (K2HPO4, Biolab), 0.2 g/L magnesium sulphate heptahydrate (MgSO4.7H2O, Biolab), 0.1 g/L sodium chloride (NaCl, Biolab), 15 g/L bacteriological agar (Biolab), and 2.5 g/L Congo red (Saarchem) (Somasegaran and Hoben, 1994). After incubation at 28 ◦C for 4 days, single colonies were selected and cultures prepared in tryptone–yeast medium containing 5 g/L tryptone (Biolab), g/L yeast extract (Biolab), and 2 mL of a 440 g/L calcium chloride dihydrate (CaCl2.2H2O, Biolab) solution (Somasegaran and Hoben, 1994). After incubation for 24 h at 28 °C, 50 mL of the bacterial culture was applied to each seedling.

Plants were separated into three treatment groups: group I (low P, LP), group II (high P, HP, control), and group III (resupplied phosphate, RP: 4 weeks of low P followed by 4 weeks of high P). The plants in these groups were supplied with 100 mL of a quarter strength Long Ashton nutrient solution (Hewitt 1966) twice a week. The nutrient solution was modified to contain either 500 µM P (HP) or 5 µM P (LP) (pH 5.8), and 500 µM NH_4NO_3. Plants were grown for eight weeks under the same conditions as described for germination after which they were harvested.

4.3.2 Specimen preparation for elemental analysis

Nodule samples were immediately frozen by immersion in liquid propane cooled by liquid nitrogen, using a Leica EM CPC cryoworkstation (Leica Microsystems AG, Vienna, Austria). Samples were subsequently freeze dried in a Leica EM CFD Cryosorption freeze dryer, following a 208 h programed cycle starting at −80 °C, and ending at ambient temperature. Transverse sections of the freeze-dried nodule samples were obtained by hand-sectioning under a stereomicroscope using a double-edge stainless steel razor blade, and mounted between two layers of 0.5% (w/v) Formvar film. To prevent charge build-up during measurements, the Formvar membrane facing the proton beam was coated with a thin layer of carbon. Light micrographs of each specimen were taken before and after proton irradiation.

4.3.3 Elemental analysis and data evaluation

Analyses were performed using the nuclear microprobe at the Materials Research Department, iThemba LABS, South Africa. A proton beam of 3 MeV energy from the 6 MV single-ended Van de Graaff accelerator was focused onto a 3 × 3 μm^2 spot and scanned over specimens using square or rectangular scan patterns with a variable number of pixels (up to 128 × 128). Scan sizes varied according to the sizes of nodules. Particle-induced X-ray emission (PIXE) and proton backscattering (BS) were used simultaneously. PIXE spectra were registered in the energy dispersive mode, using a Si (Li) detector. BS spectra were recorded with an annular Si surface barrier detector (100 mm thick) positioned at an average angle of 176°. Data were acquired in the event-by-event mode. The normalization of results was done using the integrated beam charge, collected simultaneously from a Faraday cup located behind the specimen and from the insulated specimen holder. A more detailed description of the nuclear microprobe set-up at iThemba LABS can be found in Prozesky et

al. (1995) and Przybylowicz et al. (1999, 2001, 2005). Data evaluation was performed using GeoPIXE II software (Ryan 2000). Quantitative elemental images were generated using the Dynamic Analysis method. The matrix composition and areal density were obtained from the analysis of corresponding BS spectra using a RUMP simulation package (Doolittle 1986) with non- Rutherford cross sections for C, O, and N. In addition to elemental images, average concentrations from nodules were also obtained. For this purpose, PIXE and BS spectra extracted from the nodule cross sections were used.

4.3.4 Biomass parameters and nutrient concentrations

Upon harvesting, a subset of plants was separated into nodules, roots, and shoots. The harvested material was dried at 50 °C for 72 h and dry weights (dw) recorded. The latter were used to calculate growth parameters such as biomass allocation and relative growth rate (RGR). The dried material was milled and analysed for their respective C and N concentrations at the Archeometry Department (University of Cape Town, South Africa) and P concentration at the Central Analytical Facility (Stellenbosch University, South Africa) using inductively coupled mass-spectrometry (ICP-MS).

4.3.5 Calculations of $\delta^{15}N$

The $\delta^{15}N$ analyses were also carried out at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope (15N: 14N) of the sample and standards as defined by Farquhar et al. (1989). Approximately 2 mg of each dried organ sample was put into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt

252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. %NDFA was calculated according to Shearer and Kohl (1986): %NDFA

$=100\ ((\delta^{15}\text{N reference plant} - \delta^{15}\text{N legume})/\ (\delta^{15}\text{N reference plant} - \text{B}))$.

The reference plant was non-nodulated *V. divaricata*, grown under the same glasshouse conditions. The B value is the δ^{15}N natural abundance of the N derived exclusively from biological N fixation of nodulated *V. divaricata*, also grown under same conditions as the reference plants, but with a N-free nutrient solution.

4.3.6 Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} dw d^{-1}) reflects the net P absorption rate per unit root dry weight (Nielson et al. 2001) and was determined using the formula:

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

where M is the P content per plant, t is the time, and R is the root dry weight. This equation was modified to calculate the net P absorption rate for nodules, where the nodule dry weight was used instead of root dry weight.

Specific P utilization rate (SPUR) (g dw mg^{-1} P d^{-1}) is a measure of the dry weight gained for the P taken up by the plant (Nielson et al., 2001) and was estimated with the following formula:

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

where M is the P content of the plant and W is the plant dry weight. This equation was modified to calculate the dry weight gained for the P uptake by roots and nodules, where the nodule and root dry weight was used instead of plant dry weight. The specific N absorption

and utilization rates (SNAR and SNUR, respectively) were adapted from this equation as well, to include N instead of P.

4.3.7 Enzyme activity assay: Intracellular acid phosphatase

To assess P recycling, fresh nodule and root samples detached upon harvesting were frozen at −80 °C. Nodule and root samples (approximately 40 mg fresh weight [fw]) were ground with an extraction buffer according to Araújo et al. (2008) consisting of 0.1 M Na-acetate and 1% β -mercaptoethanol. The material was centrifuged at 13,000 × g at 4 °C for 30 min, and the supernatant was taken for enzyme assay determinations. For APase activity, 200 µL of nodule or root crude protein extract was incubated for 30 min at 28 °C with a mixture of 50 mM Na-acetate buffer containing 5 mM p-nitrophenyl phosphate (pNPP). The reaction was stopped by the addition of 1.0 mL 0.5 M NaOH, and activity was measured spectrophotometrically at 410 nm. APase activity was defined as the amount of p-nitrophenyl (pNP) released relative to known pNP standards (derived from a standard curve) and expressed per unit protein.

4.3.8 Statistical analysis

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Kaleidagraph, Synergy Software, USA). Where the ANOVA revealed significant differences between treatments, the means (4–5) were separated using the post-hoc Tukey's LSD multiple range test (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences among treatments.

4.4 Results

4.4.1 Nodule formation

All 50 *V. divaricata* plants grown, formed nodules and all subsequent results are based on nodules that were functionally fixing N_2 (Figure 4.1a and c). Nodules were round-spherical in shape and often produced in clusters (Figure 4.1a and b). Most nodules formed at the top portion of the root system (Figure 4.1a).

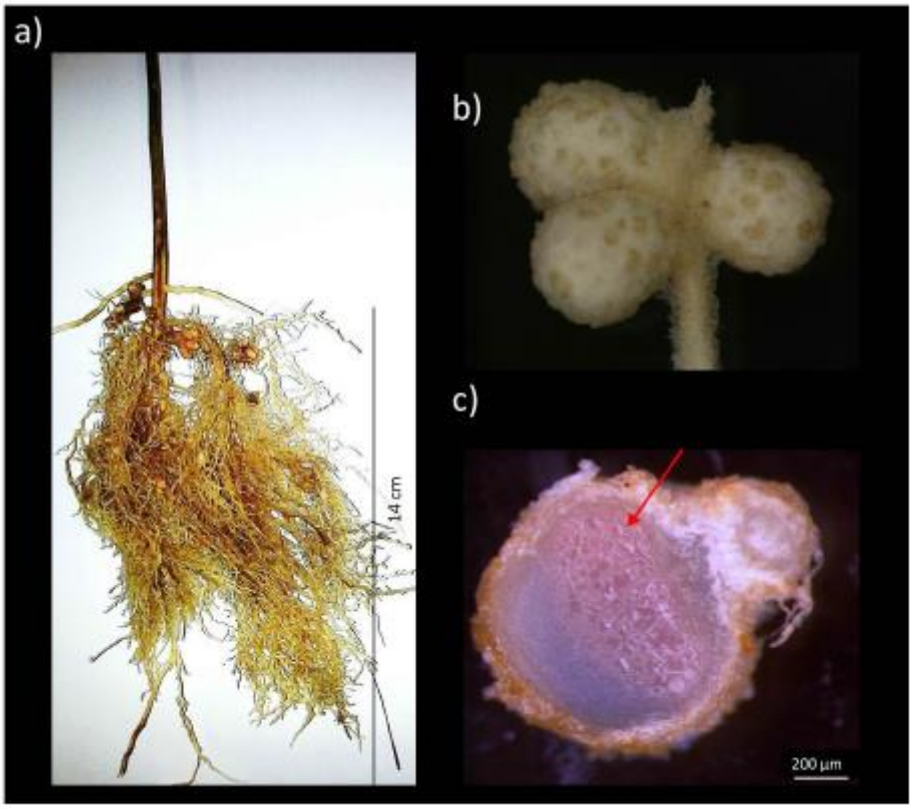

Figure 4.1 Nodules of *Virgilia divaricata* (Adamson). (a) Nodules mostly occur at the top region of the root system and are typically (b) clustered, occurring in groups of two or more. For the various analyses in this study, only active N_2 fixing nodules were selected based on the presence of the pink coloration caused by leghemoglobin (red arrow) as seen in (c).

4.4.2 Biomass and allocation

Total plant biomass accumulation was lower in P starved plants, as root, shoot, and nodule growth was lower, compared to plants of the control treatment (Table 4.1). The period of resupply (RP) to previously P starved plants resulted in a two-fold increase in root, shoot, and nodule mass compared to the LP treatment (Table 4.1). LP plants, however, maintained a higher root: shoot ratio, a morphological response typical of low P exposure, but the number of nodules produced was fewer compared to the other two treatments (Table 4.1).

Table 4.1 Biomass of *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low) and resupplied phosphorus conditions.

Parameters	Phosphate treatment		
	High (500µM,control)	Low (5µM)	Resupplied (5µM; 500 µM)
Plant dry weight (g)	1.166 ± 0.08 b	0.478 ± 0.02 a	0.953 ± 0.04 b
Root dry weight (g)	0.217 ± 0.02 b	0.105 ± 0.01 a	0.200 ± 0.02 b
Shoot dry weight (g)	0.651 ± 0.09 b	0.283 ± 0.03 a	0.568 ± 0.01 b
Nodule dry weight (g)	0.297 ± 0.05 c	0.091 ± 0.02 a	0.195 ± 0.02 b
Root:shoot	0.332 ± 0.02 a	0.371 ± 0.01 b	0.304 ± 0.06 a
Nodule number	42 ± 9 c	18 ± 3 a	31 ± 8 b

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$)

Table 4.2 Relative growth rate and allocation of new tissue of *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low) and resupplied phosphorus conditions.

Parameters	Phosphate treatment		
	High (500µM,control)	Low (5µM)	Resupplied (5µM; 500 µM)
a. Relative growth rate			
Shoot (mg g^{-1} d^{-1})	0.055 ± 0.002 b	0.041 ± 0.001 a	0.047 ± 0.002 ab
Root (mg g^{-1} d^{-1})	0.052 ± 0.002 b	0.040 ± 0.001 a	0.040 ± 0.001 a
Nodule (mg g^{-1} d^{-1})	0.057 ± 0.001 b	0.038 ± 0.001 a	0.049 ± 0.001 b
b. Allocation			
Root (mg g^{-1} d^{-1})	0.033 ± 0.002 a	0.068 ± 0.001 b	0.032 ± 0.002 a
Nodule (mg g^{-1} d^{-1})	0.033 ± 0.002 a	0.064 ± 0.003 b	0.039 ± 0.002 a

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

The RGR for low resupplied P roots were similar but significantly less compared to HP. Nodule RGR for resupplied plants was restored to levels established with the HP treatment (Table 4.2). For root and nodule allocation, the response of low P plants was greater (Table 4.2), suggesting that more biomass is apportioned towards these organs during P deprivation.

4.4.3 Concentration and localization of important N fixing elements

Quantitative micro-particle induced X-ray emissions (PIXE) distribution maps of P, K, Ca, Mg, CL, Fe, Al, S, Si, Mo, Mn, and Cu from PIXE were obtained, but only those elements essential to the process of N_2 fixation are presented. In addition to P, these include Fe and potassium (K). Fe is a component of leghemoglobin that functions in oxygen supply to bacteroids and of the nitrogenase enzyme complex involved in N_2 fixation while K is important for nodule development.

Elemental maps showed that P concentration did not differ significantly amongst treatments (Figure 4.2 and 4.3). Inorganic phosphate (Pi), the form of P used for metabolic functioning,

was also compared among treatments which indicated that Pi concentration in P deficient nodules decreased. Following P deprivation, resupplied nodules seem to recover easily (Figure 4.4), acquiring Pi at levels analogous to those under high P supply.

The concentration of Fe (Figure 4.3b) was significantly higher in P deficient nodules (up to 300 mg/kg), compared to high and resupplied P nodules (Figure 4.3b). K was distributed in high concentrations with values ranging between 20,000 and 30,000 mg/kg throughout nodules except the deficiency in the central region (Figure 4.3c) and was highest in P sufficient and resupplied nodules. This is expected since K relates to nodule development and number, and P sufficient plants produced the greatest number of nodules.

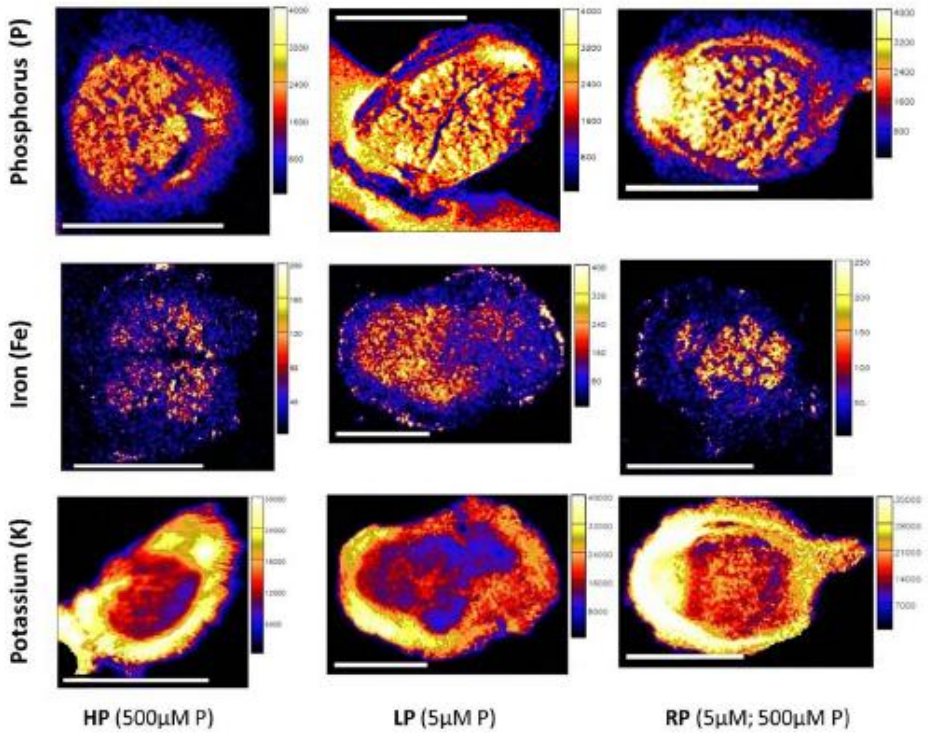

Figure 4.2 Representative maps showing the distribution of important N_2 fixing elements such as (a) phosphorus, iron, and potassium in *Virgilia divaricata* (Adamson) nodules grown under adequate (High), deficient (Low), and after the resupply of phosphorus conditions, obtained using micro-PIXE. Concentrations in mg/kg^{-1} and the scale bars represent 1000 µm.

4.4.4 Nitrogen and phosphorus nutrition and N_2 fixation

The lower level of Pi in LP nodules (Figure 4.3 and 4.4) was accompanied by a lower level of SPAR and SPUR (Table 4.3). A two-fold level in root SPUR occurred with P deprivation but SPAR was substantially lower. Resupplied roots recovered to levels found for the HP treatment for both SPUR and SPAR (Table 4.3). Furthermore, SNAR was lower in P deficient roots but when resupplied, it reached levels similar to HP roots. SNUR in nodules was also less with P deprivation (Table 4.3). HP plants obtained a greater percentage of N from the atmosphere compared to LP plants (Figure 4.5a). However, on a nodule-mass basis, P deficient plants had a higher BNF (Figure 4.5b).

Table 4.3 Nitrogen and phosphorus nutritional parameters for *Virgilia divaricata* (Adamson) nodules (a) and roots (b) grown under adequate (High), deficient (Low), and after the resupply of phosphorus conditions.

Nutrition parameter	Phosphate treatment		
	High (500µM,control)	Low (5µM)	Resupplied (5µM; 500 µM)
a. Nodule			
Specific P absorption rate (mgP g^{-1} DW d^{-1})	0.086 ± 0.002 b	0.039 ± 0.001 a	0.074 ± 0.001 b
Specific N utilization rate (g dw mg^{-1} N d^{-1})	0.018 ± 0.003 b	0.008 ± 0.001 a	0.013 ± 0.000 b
Specific P utilization rate (g dw mg^{-1} P d^{-1})	0.026 ± 0.001 b	0.010 ± 0.001 a	0.016 ± 0.000 a
b. Root			
Specific N absorption rate (mgN g^{-1} DW d^{-1})	0.091 ± 0.004 b	0.056 ± 0.006 a	0.080 ± 0.006 b
Specific P absorption rate (mgP g^{-1} DW d^{-1})	0.081 ± 0.002 b	0.027 ± 0.027 a	0.086 ± 0.001 b
Specific P utilization rate (g dw mg^{-1} P d^{-1})	0.061 ± 0.001 a	0.103 ± 0.103 b	0.053 ± 0.000 a

Values are presented as means ± SE of separate replicates (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

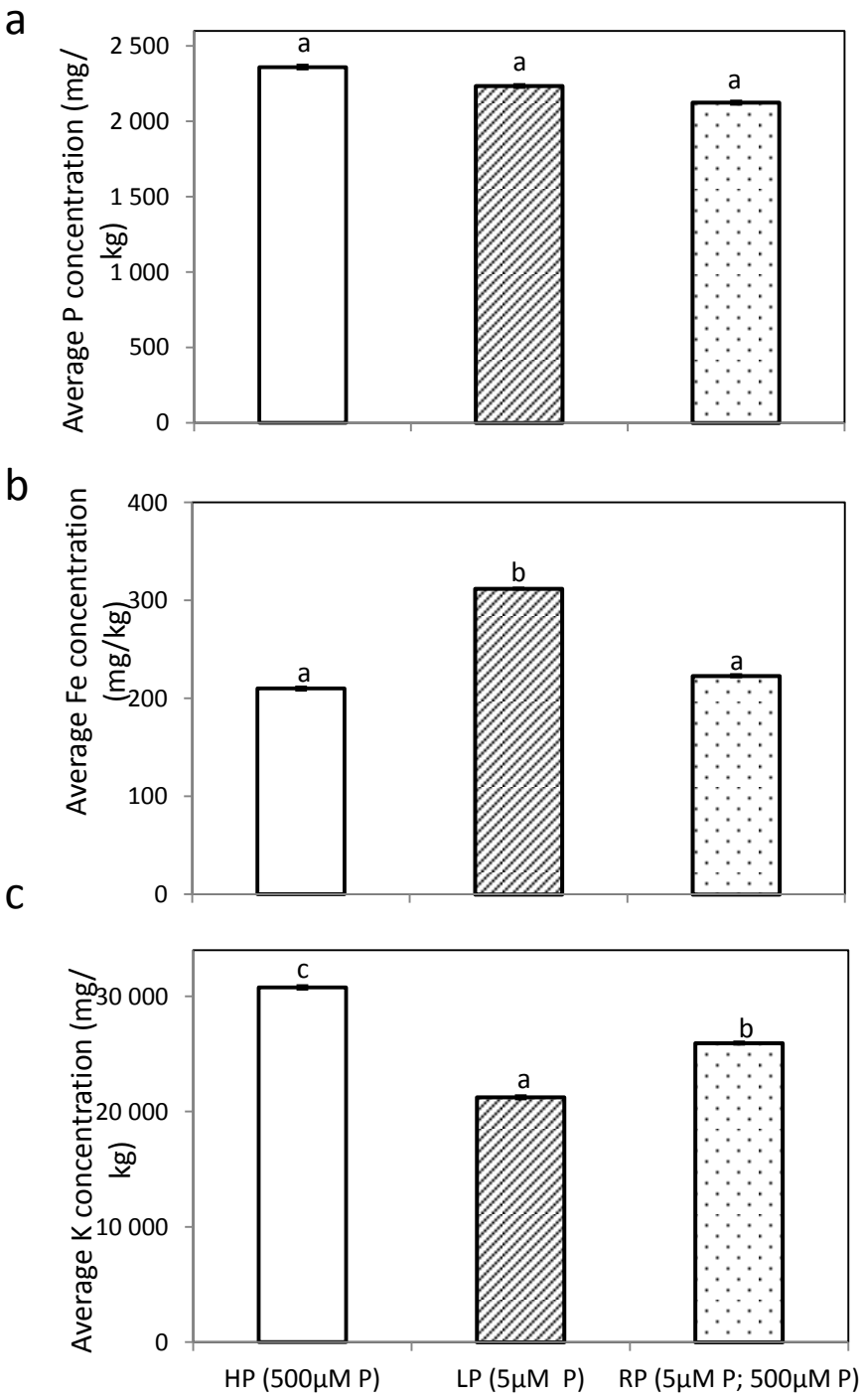

Figure 4.3 Micro-PIXE average concentrations of phosphorus, iron, and potassium in cross-sections of *Virgilia divaricata* (Adamson) nodules grown under adequate (High), deficient (Low), and after the resupply of phosphorus, conditions. Values are presented as means ± SE (minimum detection limit) of three separate replicates per treatment.

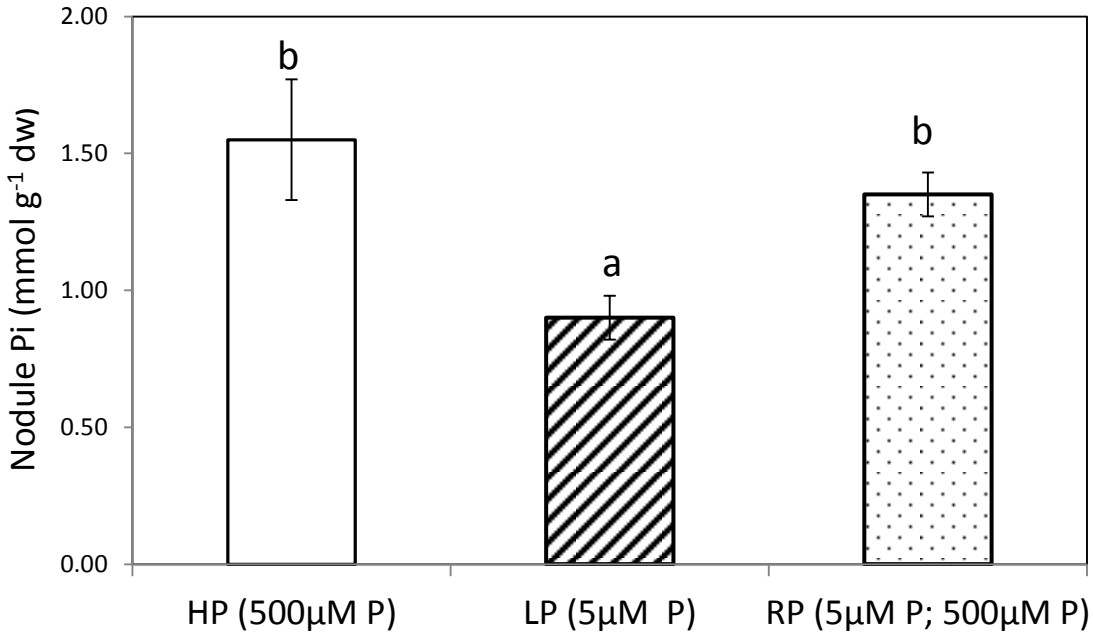

Figure 4.4 Nodule orthophosphate (Pi), the form used for metabolic functioning in *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low), and after the resupply of phosphorus conditions. Values are presented as means ± SE of separate replicates (n = 5). Different letters indicate significant differences between treatments ($P \leq 0.05$).

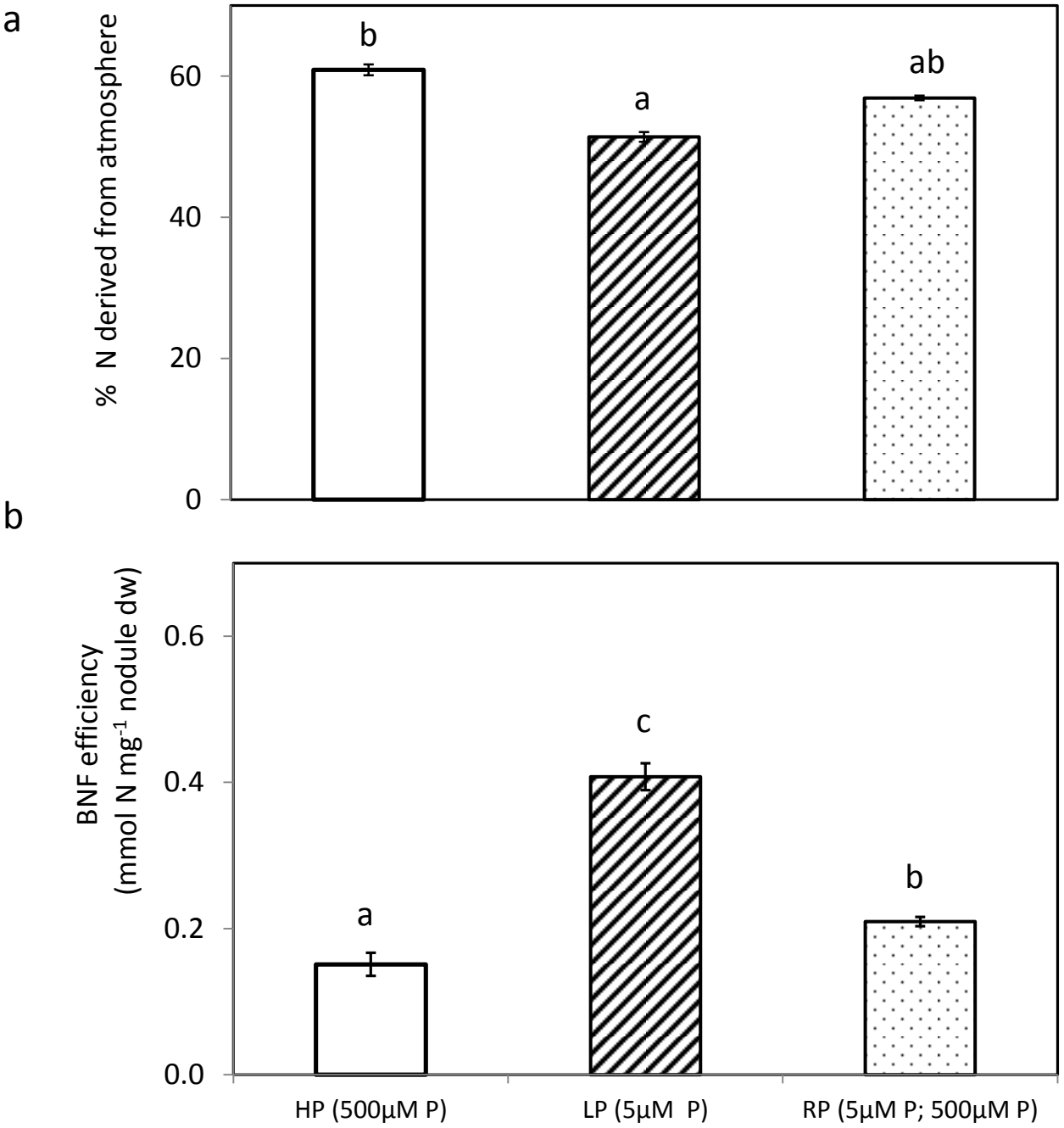

Figure 4.5 (a) Percentage nitrogen derived from the atmosphere (% NDFA) of whole plants and (b) biological nitrogen fixation on a mass basis in nodules, for *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low), and after the resupply of phosphorus conditions. Values are presented as means ± SE of separate replicates (n = 3) Different letters indicate significant differences between treatments ($P \leq 0.05$).

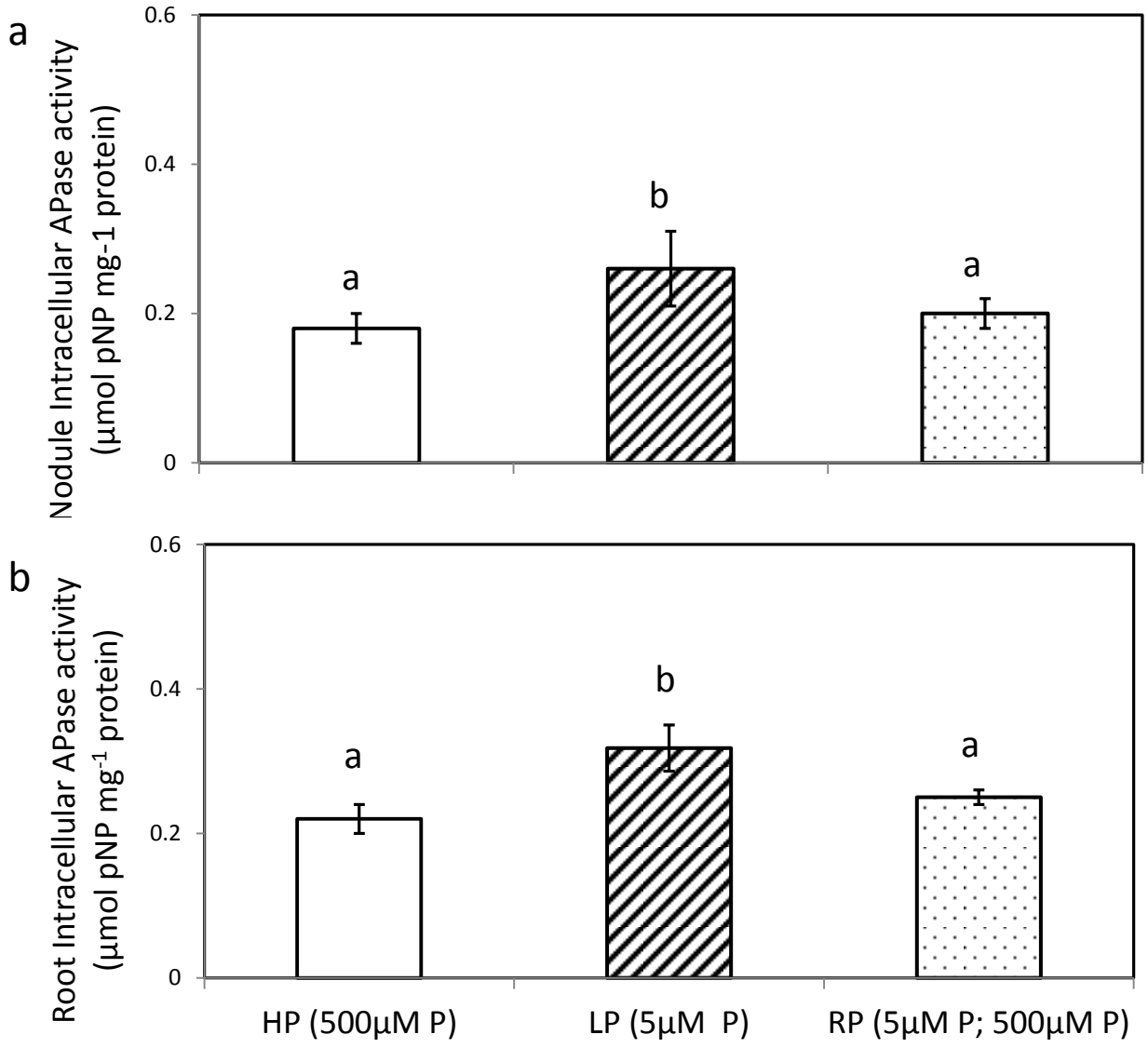

Figure 4.6 Intracellular acid phosphatase (APase) enzyme activity in *Virgilia divaricata* (Adamson) nodules (a) and roots (b) grown under adequate (High), deficient (Low), and after the resupply of phosphorus conditions. Values are presented as means ± SE of separate replicates (n = 5). Different letters indicate significant differences between treatments ($P \leq 0.05$).

4.4.5 Acid phosphatase activity

Greater APase activity was found in P deficient roots and nodules, than under P sufficient or resupplied conditions (Figure 4.6a and b). Root activity was however greater than nodule activity.

4.5 Discussion

During fluctuations in P supply, the nodules of *V. divaricata* exhibited both physiological and morphological responses to the variations in P availability. These findings indicate that the legume *V. divaricata* can acquire N during fluctuations in soil P concentrations, owing to the functional plasticity of its nodule and root physiology. The recovery responses from P deficiency observed in this study are similar to earlier reports in which P supply to previously starved plants increased plant dry weights progressively (Rao and Terry 1995) and enhanced P uptake (Drew et al. 1984, Jungk et al. 1990). Although these earlier studies focused on short term resupply, the current study on *V. divaricata* used a more extended period as it is a slow-growing legume tree and may respond more slowly to variations in P availability. The resupply of P after extended low P conditions provides insight into P uptake of indigenous legumes in CFR where soils are typically P poor within a range of concentrations (Stock and Lewis 1986, Witkowski and Mitchell 1987). Moreover, legumes growing in the CFR would have to engage P conservation and uptake strategies, in order to maintain nodule functioning and sustain N-metabolism. Under long term low P supply, *V. divaricata* was able to maintain a higher level of N_2 fixation which may be due to fewer nodules with lower biomass and increased P recycling and Fe concentration within nodules.

The increase in the allocation to both roots and nodules of *V. divaricata* under low P supply is an adaptive feature in view that altered biomass allocation to root organs is common in plants (Hermans et al. 2006), but less well known in nodules of legumes. The ability of plants to increase P uptake under low P supply is well documented (Shimogawara and Usuda 1995). The enhanced P uptake by P deficient roots suggests that these plants are adapted to cope with P deficiency. Under P deficient and P resupply conditions, nodule P utilization was analogous but P absorption differed. P deficient roots and nodules had a lower P absorption rate while the resupply treatment rates resembled that of the P sufficient treatment. P

absorption following a period of P starvation is thought to be concomitant with a higher capacity of roots for P transport, possibly by the formation of additional carriers and transporters of Pi (Drew et al. 1984, Katz et al. 1986).

Under low P conditions, *V. divaricata* had a higher N_2 fixation per nodule, despite lower levels of total P and Pi in the nodules. This can be attributed to two nutritional factors explored in this study. The first is elevated Fe concentration and localization under low P and the second is the greater P recycling by APase in both the roots and nodules under low P.

Firstly, the functional significance of the increased Fe concentration in P deficient nodules is that Fe is important for nodulation (Rai et al. 1982, Tang et al. 1990). Limited Fe availability has been associated with poor nodulation, impeded nodule development and functioning, as well as a decrease in the production of leghemoglobin in lupins (Rai et al. 1982, Tang et al. 1990). In pigeon pea nodules, a decline in leghemoglobin was associated with a decline in nitrogenase enzyme activity and lower N_2 fixing levels (Nandwal et al. 1991). Tang et al. (1990) found that Fe sufficient lupins contained more than double the amount of leghemoglobin compared to Fe deficient lupins and that nodules contained the highest levels of Fe compared to roots and shoots suggesting a high internal nodular requirement for Fe in these legumes. Because Fe is the key constituent of leghemoglobin, nodule Fe levels are generally correlated to N_2 fixation and any influence of Fe deficiency on leghemoglobin production will likely reduce N_2 fixation (Tang et al. 1990). In *Phaseolus vulgaris*, N_2 fixation levels are positively correlated with increasing Fe concentrations (Slatni et al. 2008). Furthermore, the Fe requirement by nodules with an active symbiosis is large because many other symbiotic proteins (apart from leghemoglobin and nitrogenase) such as cytochrome, ferredoxin and hydrogenase incorporate Fe (Brear et al. 2013). While leghemoglobin levels in this study was not measured, the eminent Fe levels in P deficient nodules may imply better (although fewer) nodule development, which may have led to greater production of

leghemoglobin or an enhanced symbiotic interaction between the plant and bacteria, resulting in increased N_2 fixation per nodule mass.

Secondly, the importance of the greater APase activity may be related to the role of phosphatase exudation into the rhizosphere, as an important mechanism for ensuring P acquisition from low P resources and from forms of P which are not readily available to other plants (Lambers et al. 2006). Induction of intracellular and secreted acid phosphatase activity has been correlated with de novo acid phosphatase synthesis in several Pi depleted plants, including *Brassica nigra* (black mustard), *Solanum lycopersicum* (tomato), and *Arabidopsis* suspension cells and seedlings (Duff et al. 1991, Bozzo et al. 2002, Veljanovski et al. 2006). P deficient roots and nodules exhibited greater APase activity compared to the P resupply and P sufficient treatments. The greater activity in roots, however, suggests that roots scavenge for P and transport P to nodules where nodules conserve P and typically do not exchange P with other organs. Nodules are thus strong sinks of P (Hart 1990, Schulze and Drevon 2005). Previous studies have also shown that an increase in nodule APase activity may constitute an adaptive mechanism for N_2 fixing legumes to tolerate P deficiency (Kouas et al. 2008, Bargaz et al. 2012). White lupin secretes copious amounts of APases from its roots and proteoid roots when subjected to Pi starvation (Miller et al. 2001, Wasaki et al. 2008). Similarly, common bean nodules increase APase activity under P deficiency (Kouas et al. 2009). Our findings on *V. divaricata* therefore agree with many studies reporting that P stress induces APase activity, aiding in the internal recycling of P and the increased APase activity in nodules and roots under P deficiency (Vadez et al. 1997, Al-Niemi et al. 1997).

In conclusion, although prolonged low P conditions reduced *V. divaricata* growth and the costs of nutrient acquisition, these P stress responses had sufficient flexibility to revert to normal during P resupply. Long term exposure to P deficiency therefore does not compromise the ability of these plants to respond to enhanced P availability following

deficiency. These findings indicate that *V. divaricata* is able to acquire N under various conditions of P availability and contributes to our understanding of legume distribution in nutrient poor regions such as the CFR.

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Chapter 5: Legume nodules from nutrient-poor soils exhibit a high plasticity in cellular P recycling and P conservation mechanisms

5.1 Abstract

Nitrogen (N) fixing legumes rely on phosphorus (P) for nodule formation, nodule function and the energy costs of fixation. P is however very limited in soils, especially in ancient sandstone-derived soils such as those in the Cape Floristic Region (CFR) of South Africa. Plants growing in such areas have evolved the ability to tolerate P stress by eliciting an array of physiological and biochemical responses. In this study we investigated the effects of P limitation on N_2 fixation and P recycling in the nodules of *Virgilia divaricata* (Adamson), a native CFR legume distributed over a wide range of P poor soils. In particular, we focused on nutrient acquisition efficiencies, P fractions and the exudation and accumulation of phosphatases. Our findings indicate that during low P, *V. divaricata* internally recycles P and has a lower P uptake rate as well as lower levels of Pi and adenylates but greater levels of phosphohydrolase exudation suggesting it engages in recycling internal nodule P pools and making use of alternate bypass routes in order to conserve P.

5.2 Introduction

Phosphorus (P) is one of the most essential nutrients for plant growth and is an important structural constituent for nucleic acids, phospholipids, sugar phosphates and other catalytic cofactors in addition to the role it plays in metabolic regulation and energy transfer (Bosse and Köck 1998). Plants thus depend heavily on P for plant growth and development especially legume plants since P is also a significant requirement for N_2 fixation and has been reported to affect the energy costs of N_2 fixation (Schulze et al. 1999, Valentine et al. 2010), as well as nodule formation and function (Israel 1987). Soil P is however, limited and its availability is contingent on various factors such as its diffusion rates and solubilisation of P containing compounds (Vance et al. 2003).

Plants have evolved an array of morphological and biochemical mechanisms to obtain adequate P or Pi (the metabolic form of P) under P deficient conditions (Vance et al. 2003, Tran et al. 2010). Morphological strategies include altered root architecture e.g. shifting from primary to lateral root growth (Williamson et al. 2001), increasing root hair density and length, which is common in legumes and developing specialized roots known as proteoid roots for nutrient acquisition (Johnson et al. 1996, Neumann et al. 1999). Biochemical changes involve an increase in the abundance of Pi transport proteins and alternate enzymes to bypass Pi- or the adenylate dependant reactions of glycolysis and mitochondrial respiration (Theodorou and Plaxton 1993, Plaxton 2004, Sieger et al. 2005, Tran et al. 2010). These alternate enzymes promote Pi recycling, and the synthesis of organic acids, with Pi as a by-product of their reactions. Phosphorus deficiency causes a decline in cytosolic Pi and adenylates (Rychter et al. 1992) and under these conditions the increased engagement of these alternative routes, eliminate the necessity for adenylates and Pi (Duff et al. 1989, Nagano et al. 1994).

Plants also increase their efficiency of Pi use during P deficiency by inducing phosphohydrolases such as ribonucleases (RNases) and acid phosphatases (APases) which scavenge Pi from P-esters (Raghothoma 1999, Tran et al. 2010, Hurley et al. 2010) and APase activity has been used as a marker for P deficiency. APases release P (Miller et al. 2001) and have been implicated in the synthesis of glycolate and glycerate especially those associated with carbon metabolism (Duff et al. 1991, Vance et al. 2003). Extracellular APases break down organic phosphate monoesters in the soil, whereas intracellular APases remobilize and scavenge Pi from intracellular phosphate monoesters (Duff et al. 1994, Marschner 1995). Many organic P compounds occur in soil, with soil phytate (inositol hexaphosphates) forming a major component (around 25%), that can be hydrolyzed by APases or phytases, a special group of phosphatases, capable of hydrolyzing phytate to myo-inositol and phosphate (Richardson et al. 2000).

The effects of P limitation on N_2 fixation are not fully understood although legumes occur worldwide and occur in soils where P is limiting. Most studies investigating the effect of P limitation on N_2 fixation have largely been confined to model legumes such as lupins (Le Roux et al. 2006, Schulze et al. 2006, Thuynsma et al. 2014) and alfalfa (Tang et al. 2001, Sulieman et al. 2013). The P poor soils of the CFR have a high legume diversity (Goldblatt and Manning 2002), but little is known about the functional mechanisms that affects N metabolism within the nodules of these indigenous legumes. The aim of this work was therefore to investigate the effects of P limitation on BNF and P recycling in the nodules of *V. divaricata*, a native CFR legume that can occur over a range of soil P concentrations (Coetsee and Wigley 2013). In particular, the focus was on biomass and growth kinetics, nutrient acquisition efficiencies, P pools and adenylate levels as well as the exudation and accumulation of phosphatases.

5.3 Materials and methods

5.3.1 Seed germination, bacterial inoculation and growth

V. divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were placed in smoke solution (Smoke Plus, Kirstenbosch National Botanical Garden, South Africa) (Magadlela et al. 2014) and incubated in a water bath at 50 °C for 4 h. Thereafter, seeds were surface sterilized, rinsed with distilled water and germinated in 5 cm deep seed trays containing sterile sand under natural light conditions (midday irradiances between 600–800 µmol m^{-2} s^{-1}) in a temperature-controlled (15–25 °C), north-facing glasshouse at Stellenbosch University, South Africa.

After the first fully expanded leaf emergence, seedlings were transferred to pots containing sterile sand and inoculated with *Burkholderia phytofirmans* (isolated from *V. divaricata* nodules grown in soil from Stellenbosch Mountain, Stellenbosch (natural fynbos inoculum). Inoculum was prepared by growing the bacterium on yeast mannitol agar (YMA) containing 0.5 g/ L yeast extract (Biolab), 10 g/L mannitol (Saarchem), 0.5 g/L dipotassium hydrogen orthophosphate (K_2HPO_4, Biolab), 0.2 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, Biolab), 0.1 g/L sodium chloride (NaCl, Biolab), 15 g/L bacteriological agar (Biolab), and 2.5 g/L Congo red (Saarchem) (Somasegaran and Hoben, 1994). After incubation at 28 °C for 4 days, single colonies were selected and cultures prepared in tryptone–yeast medium containing 5 g/L tryptone (Biolab), 3 g/L yeast extract (Biolab), and 2 mL of a 440 g/L calcium chloride dihydrate ($CaCl_2.2H_2O$, Biolab) solution (Somasegaran and Hoben 1994). After incubation for 24 h at 28 °C, 50 mL of the bacterial culture was applied to each seedling.

Plants were separated into three phosphorus treatment groups: low P, high P (control), and resupplied P (four weeks of low P followed by three weeks of high P). The plants in these groups were supplied with 100 mL of a quarter strength Long Ashton nutrient solution twice

a week. The nutrient solution was modified to contain either 5 µM P (LP) or 500 µM P (HP) (pH 5.8), and 500 µM NH_4NO_3. Plants were grown for 55 days under the same conditions as described for germination.

5.3.2 Nutrient analysis

Upon harvesting, a subset of plants was separated into nodules, roots, and shoots. The harvested material was dried at 50 °C for 72 h and dry weights (dw) recorded. The dried material was milled and analysed for their respective C and N concentrations at the Archeometry Department (University of Cape Town, South Africa) and P concentration at the Central Analytical Facility (Stellenbosch University, South Africa) using inductively coupled mass-spectrometry (ICP-MS) with suitable standards.

5.3.3 Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} dw d^{-1}) reflects the net P absorption rate per unit root dry weight (Nielson et al., 2001) and was determined using the formula:

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

where *M* is the P content per plant, *t* is the time, and *R* is the root dry weight. This equation was however, modified to calculate the net P absorption rate for nodules. As such the nodule dry weight was used instead of root dry weight. Specific P utilization rate (SPUR) (g dw mg^{-1} P d^{-1}) is a measure of the dry weight gained for the P taken up by the plant (Nielson et al. 2001) and was estimated with the following formula:

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

where M is the P content of the plant and W is the plant dry weight. This equation was modified to calculate the dry weight gained for the P uptake by nodules, hence the nodule dry weight was used instead of plant dry weight.

The construction cost of the tissue, C_w (mmol C/ g dw) was calculated according to Mortimer et al. (2005), modified from the equation used by Peng et al. (1993):

$C_w = [(C + kN14 \times 180)/24] \times (1/0.89) \times (6000/180)$,

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

5.3.4 Calculations of $\delta^{15}N$

The $\delta^{15}N$ analyses were also carried out at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000\% [R_{sample}/R_{standard}]$, where *R* is the molar ratio of the heavier to the lighter isotope of the samples and standards as defined by Farquhar et al. (1989). Approximately 2 mg of each sample was weighed into an 8 mm x 5 mm tin capsule (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. These values were used to determine the percentage N derived from the atmosphere (NDFA) according to the calculation by Shearer and Kohl (1986):

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

The reference plant was non-nodulated *V. divaricata*, grown under the same glasshouse conditions. The *B* value is the $\delta^{15}N$ natural abundance of the N derived exclusively from

biological N fixation of nodulated *V. divaricata*, also grown under same conditions as the reference plants, but with an N-free nutrient solution.

5.3.5 In vitro NMR measurements

Perchloric acid (PCA) extracts were prepared from 8-10 g of nitrogen frozen nodules according to the method described by Gout et al. (2000) and divalent paramagnetic cations were chelated by the addition of 180 nmol CDTA. Spectra of neutralised PCA extracts were obtained on Varian INOVA 600 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5mm probe for ^{31}P detection. The deuterium resonance of D_2O was used as a lock signal/ internal reference. The conditions used for ^{31}P-NMR acquisition were as follows: 60° radio-frequency pulses (0.899-s) at 1-s intervals; spectral width 36429 Hz; 121930 repetitions; Waltz-16 1H decoupling (on during acquisition, off during delay). Free induction decays were collected and processed with a 2-Hz line broadening. H_3PO_4 was used as an external standard. Relative amounts of identified compounds were determined by the areas of their resonance peaks.

5.3.6 Extracellular acid phosphatase assay

To determine nodule surface acid phosphatase activity, whole nodules (approximately 3g fresh weight [fw]) were washed in distilled water, blotted dry and placed into 5ml of incubation medium with substrate mixture (6 mM p- nitrophenyl phosphate (pNPP) and 1 mM DTT in 50 mM Na-acetate buffer, pH 5.0), and incubated at 25°C (Zebrowska et al. 2012, Ciereszko et al. 2002). After 30min, 100µl of the reaction medium was removed and added to 100 µl 1M NaOH to stop the reaction. Absorbance was then to determine at 410 nm and enzyme activity expressed as µmol pNP min^{-1} g^{-1} fresh weight (Ciereszko et al. 2002).

5.3.7 Intracellular acid phosphatase (APase) and phytase assays

Nodules were homogenized (1:2, w/v) in ice-cold extraction buffer containing 20 mM Na-acetate (pH 5.6), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 mM thiourea, and 1% (w/v) insoluble PVPP (Hurley et al. 2010). Homogenates were centrifuged at 4 °C at 14,000g for 5 min, and the supernatants reserved as clarified extract. Protein extracts were quantified with the Bradford method using BSA as the standard.

APase activity was measured by coupling the hydrolysis of PEP to pyruvate to the LDH reaction and monitoring NADH oxidation (Hurley et al. 2010). The assay mixture contained 50 mM Na-acetate (pH 5.6), 5 mM PEP, 10 mM $MgCl_2$, 0.2 mM NADH, and 3 units of rabbit muscle LDH. The blanks consisted of reaction medium without PEP (Hurley et al. 2010). All reactions were initiated by adding 30 µl crude extract to assay mixture in a total volume of 250 µl and read at 340 nm, at 25 °C.

APase assays were also carried out in an assay mix containing 50 mM Na- acetate (pH 5.6), 5 mM pNPP, and 10 mM $MgCl_2$ by monitoring the formation of mM p- nitrophenyl (pNP). All reactions were initiated by adding 30 µl crude extract to assay mixture in a total volume of 250 µl and read at 405 nm. APase activity was defined as the amount of pNP released relative to known pNP standards derived from a standard curve. All APase assays were linear with respect to time and concentration of enzyme assayed. One unit of activity was defined as the amount of enzyme resulting in the hydrolysis of 1 µmol of substrate min^{-1} at 25 °C.

Phytase activity was assayed by measuring the Pi hydrolysed from phytate. The assay mix contained in a total volume of 0.2 ml, 0.1 M acetate buffer (pH 5), 2.5 mM phytic acid (sodium salt hydrate from rice) and 30 µl crude enzyme. The mixtures were incubated for 3 h at 30 °C and the reaction stopped by the addition of ice-cold trichloroacetic acid (TCA).

The inorganic P released was quantified by the molybdovanadate method at 460 nm (Kouas et al. 2009). One unit of activity was defined as the amount of enzyme resulting in the hydrolysis of 1 μmol of substrate min^{-1}.

5.3.8 Statistical analysis

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Kaleidagraph, Synergy Software, USA). Where the ANOVA revealed significant differences between treatments, the means were separated using the post-hoc Tukey's LSD multiple range test (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences among treatments.

5.4 Results

5.4.1 Plant growth and biomass production

Total plant biomass accumulation was significantly lower in P deficient plants compared to the control and previously starved plants that were resupplied with sufficient P (Figure 5.1). Plants grown under the latter treatment produced 100% more nodules than the P starved plants while P starved plants maintained a higher root: shoot ratio, a morphological response characteristic of low P exposure (Figure 5.2).

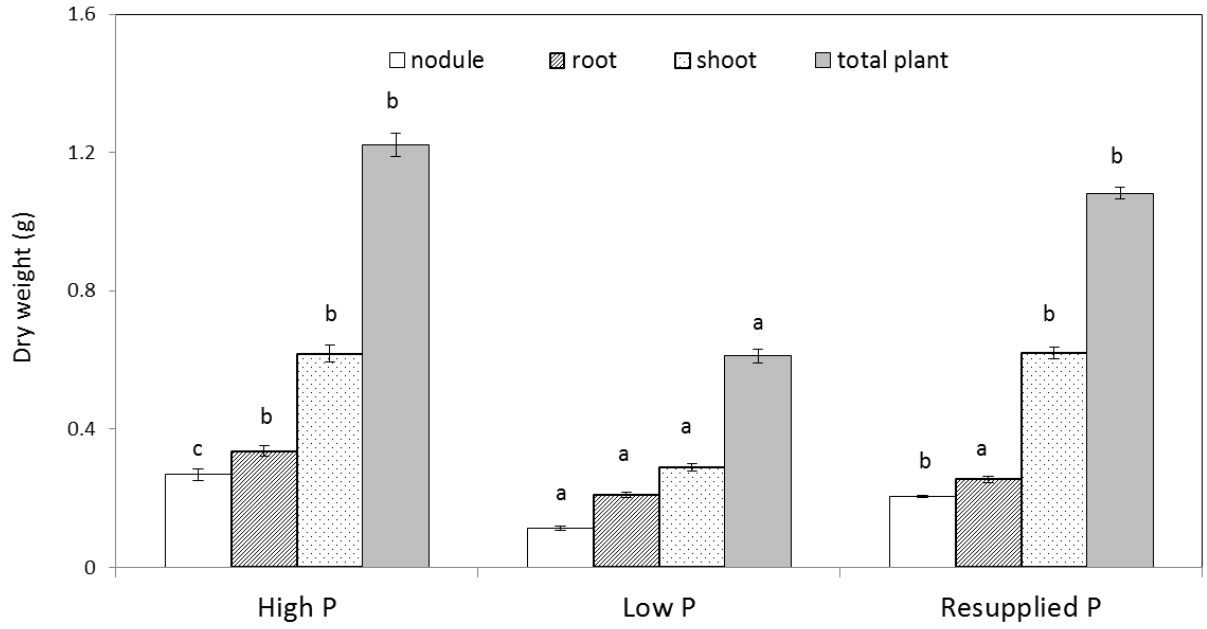

Figure 5.1 Dry weight (root, shoot, nodule and total plant) of *Virgilia divarica*ta (Adamson) grown under adequate (High), deficient (Low) and after the resupply of phosphorus conditions. Values are presented as means (n = 3) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

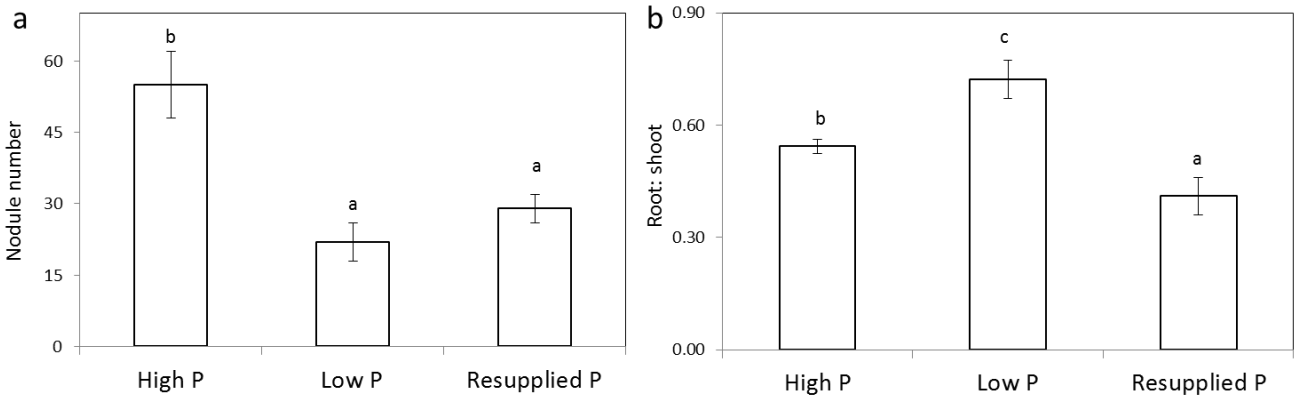

Figure 5.2 Nodule number (a) and root: shoot ratio (b) of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and after the resupply of phosphorus conditions. Values are presented as means (n =5 for nodule number and n= 3 for root: shoot) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

5.4.2 Mineral nutrition and N_2 fixation

The concentration of N and P concentration in P- sufficient and resupplied nodules was higher with values ranging between 3.6 – 4.5 mmol g^{-1} dw (Figure 5.3). The lower P concentration in P deficient nodules was complemented by a lower net P absorption rate as well as P uptake by these nodules (Figure 5.3). The net P absorption rate of resupplied plants was parallel to that of P sufficient plants (Figure 5.3).

Nodules of control plants derived almost 20% more nitrogen from the atmosphere compared to LP plants. However, on a nodule-mass basis, LP plants had a higher BNF (Figure 5.4). No significant difference in nodule construction costs was found between treatments (Figure 5.4).

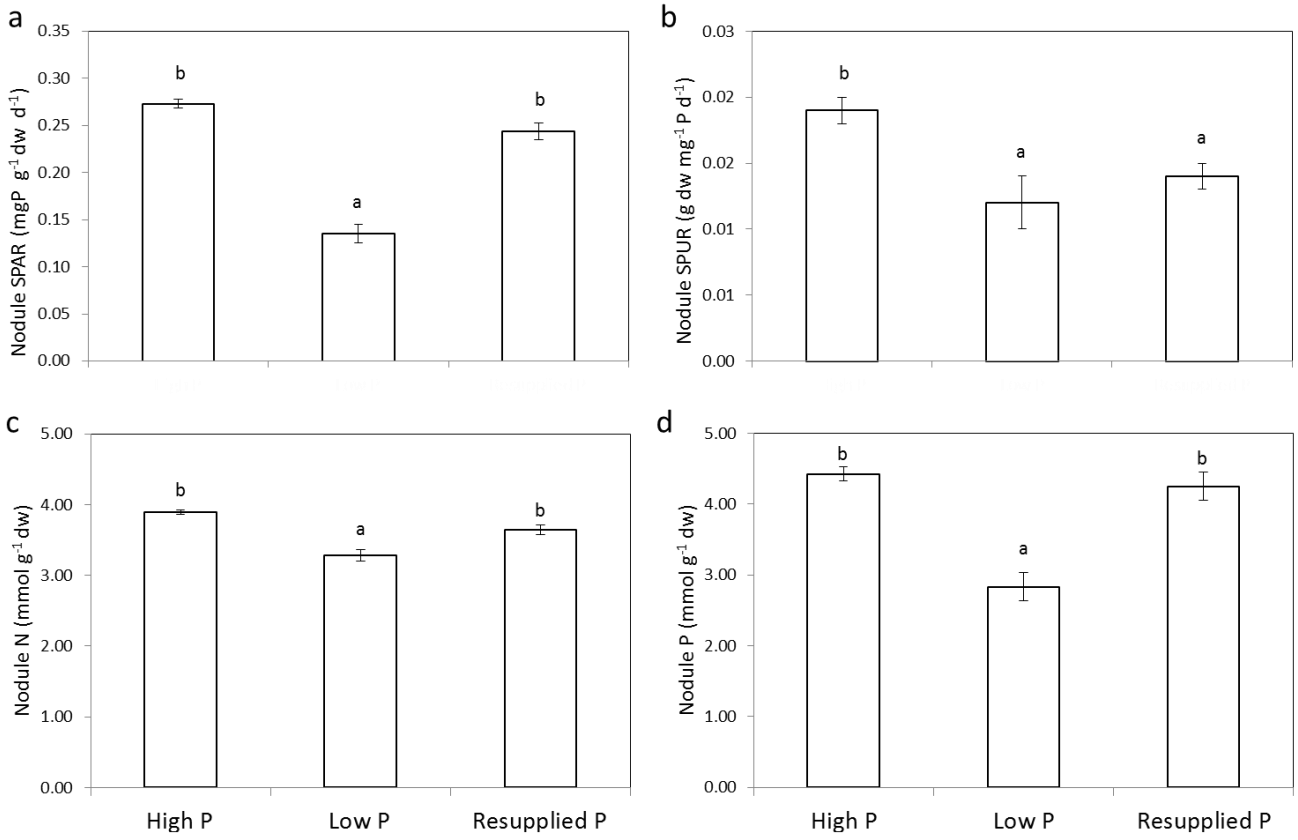

Figure 5.3 Specific phosphorus acquisition rate (a), specific phosphorus utilisation rate (b), nitrogen concentration (c) and phosphorus concentration (d) of *Virgilia divaricata* (Adamson) nodules grown under adequate (High), deficient (Low) and after the resupply of phosphorus conditions. Values are presented as means (n = 4) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

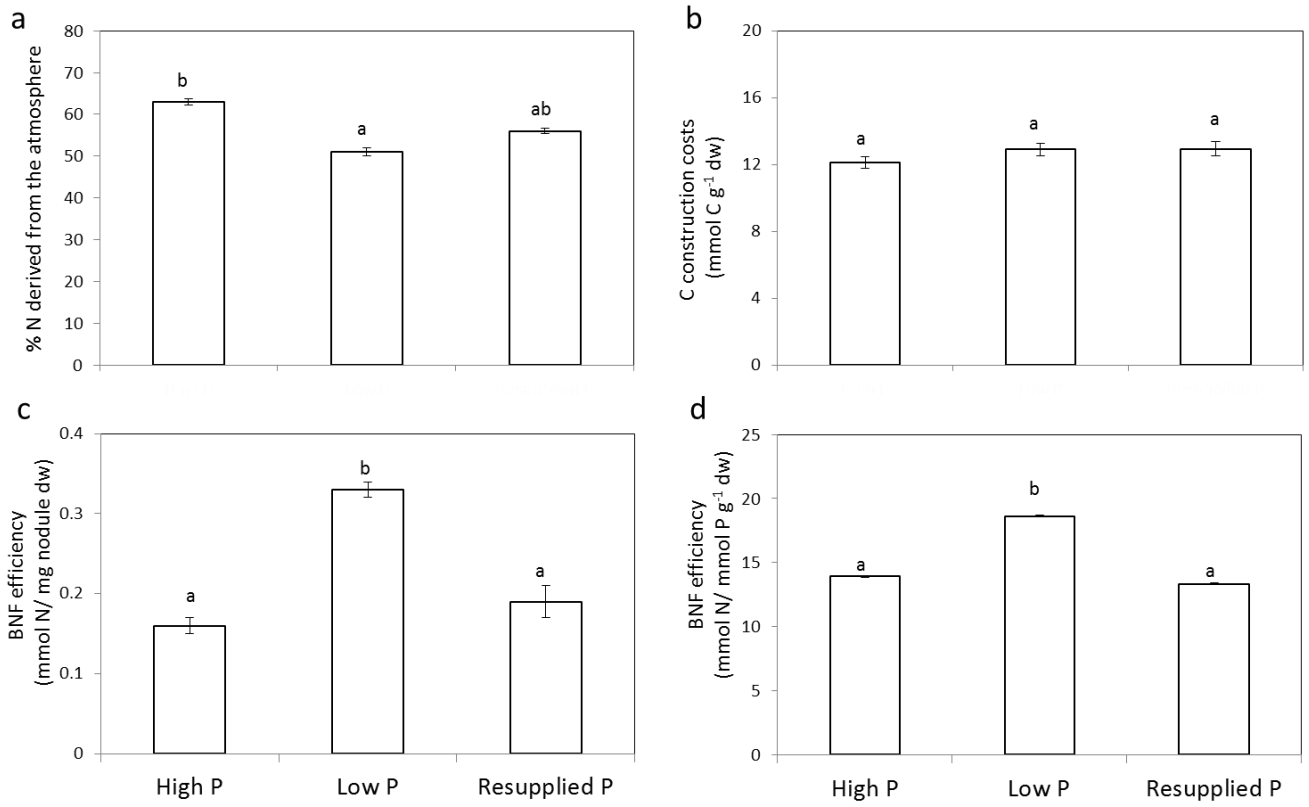

Figure 5.4 Percentage nitrogen derived from the atmosphere (% NDFA) for whole plants (a), nodule carbon construction costs (b) biological nitrogen fixation (BNF) on a nodule mass basis (c) and BNF on a P concentration basis (d), for *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low) and after the resupply of phosphorus conditions. Values are presented as means (n = 4) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

5.4.3 Phosphorus fractions

Peak areas of compounds from NMR spectra (see Figure 5.5 for representative spectrum) were used to derive relative amounts of compounds. All nucleotide (ATP, ADP and UDPG) levels in P sufficient nodules were higher compared to P deficient plants (Figure 5.6). This decline in ADP and ATP corresponds with lower levels of Pi in LP nodules (Figure 5.6). The ADP: ATP ratio increased significantly in LP nodules (Table 5.1). Most of the sugar phosphate levels (Glcn6P, Glc6P and PGA) were not significantly different between treatments with the exception of Fru6P which increased in LP nodules (Table 5.1). An increase in the phospholipid, P-cho was observed under HP (Table 5.1).

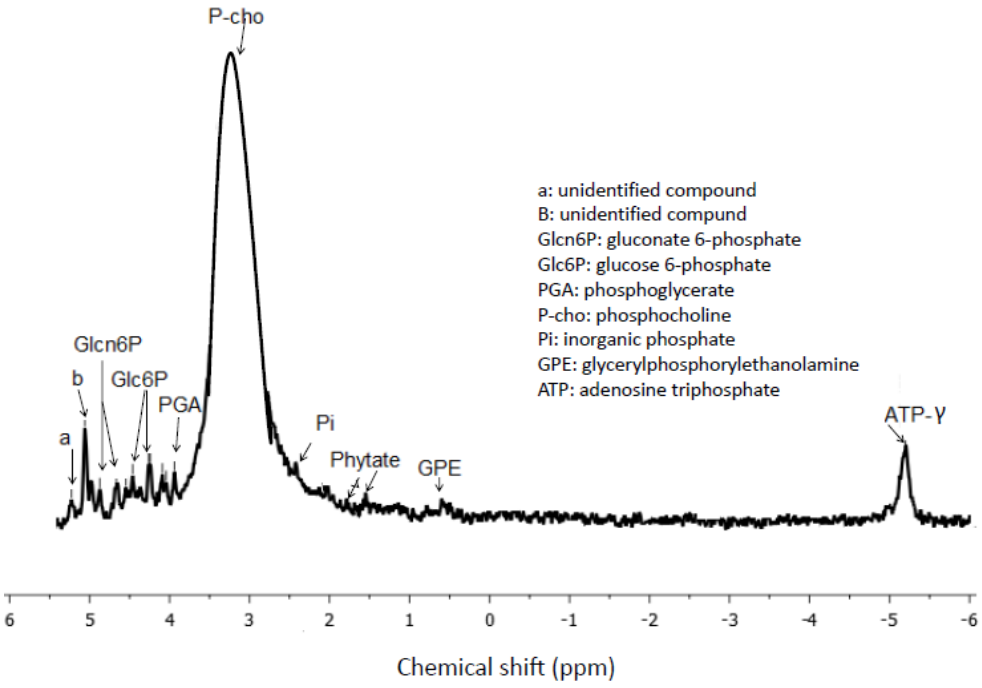

Figure 5.5 Representative NMR spectrum of nodules of *Virgilia divaricata* (Adamson) grown under adequate (High) phosphorus conditions. Peak areas of spectra were used to derive relative amounts of P compounds, represented in Figure 5.6 and Table 5.1.

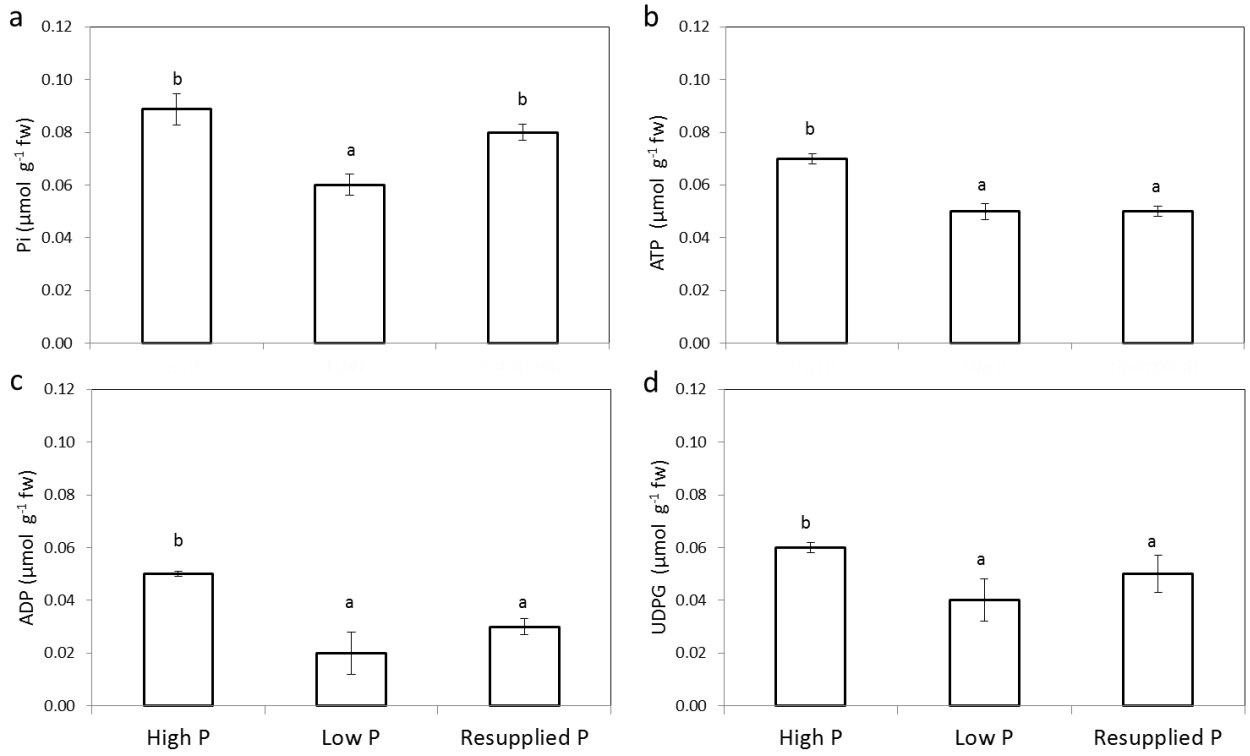

Figure 5.6 Pi (a), and adenylate- ATP (b), ADP (c), and UDPG (d) levels in nodules of *Virgilia divaricata* (Adamson) grown under adequate (High), deficient (Low) and after the resupply of phosphorus conditions. Values are presented as means (n = 3) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

Table 5.1 Phosphorus metabolites determined from *Virgilia divaricata* (Adamson) nodule extracts. Values are expressed in µmol g^{-1} FW derived from areas of resonance peaks and are presented as means (n = 3) with standard error. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

P- metabolite	Group	High P	Low P	Resupplied P
Glcn6P	sugar phosphate	0.30 ± 0.009 a	0.29 ± 0.114 a	0.33 ± 0.089 a
Glc6P	sugar phosphate	0.16 ± 0.010 a	0.12 ± 0.047 a	0.14 ± 0.039 a
PGA	sugar phosphate	0.11 ± 0.005 a	0.08 ± 0.030 a	0.15 ± 0.050 a
Fru6P	sugar phosphate	0.25 ± 0.009 a	0.35 ± 0.137 b	0.32 ± 0.073 b
P-cho	phospholipid	0.24 ± 0.011 b	0.14 ± 0.051 a	0.13 ± 0.033 a
Ratios:				
ADP: ATP	nucleotide: nucleotide	1.40 ± 0.000 a	2.50 ± 0.001 b	1.60 ± 0.001 a
Pi: ATP	inorganic phosphate: nucleotide	1.24 ± 0.001 a	1.16 ± 0.000 a	1.62 ± 0.001 b

Abbreviations for the different compounds are: GlcN6P, gluconate 6-phosphate; Glc6P, glucose 6-phosphate; PGA, phosphoglycerate; Fru6P, fructose 6-phosphate; P-cho, phospho-choline; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

5.4.4 Phosphohydrolase activities

Measurements of APase and phytase activities were carried out to establish their role in enhancing P availability through recycling of P. Extracellular APases were quantified from nodule surfaces because although it is produced and secreted largely by roots, it also attaches to nodules to enhance P uptake. Nodule extracellular APase activity was significantly greater in P deficient plants (Figure 5.7). The data obtained for the PEP- and pNP- based intracellular APase assays was consistent with the data for extracellular APase assays, showing an increase in these enzymes under P stressed conditions and recovery with resupplied P (Figure 5.7). Intracellular APase levels were however far greater than extracellular APase levels. There was a two-fold increase in phytase activity in LP nodules, compared to HP nodules (Figure 5.7).

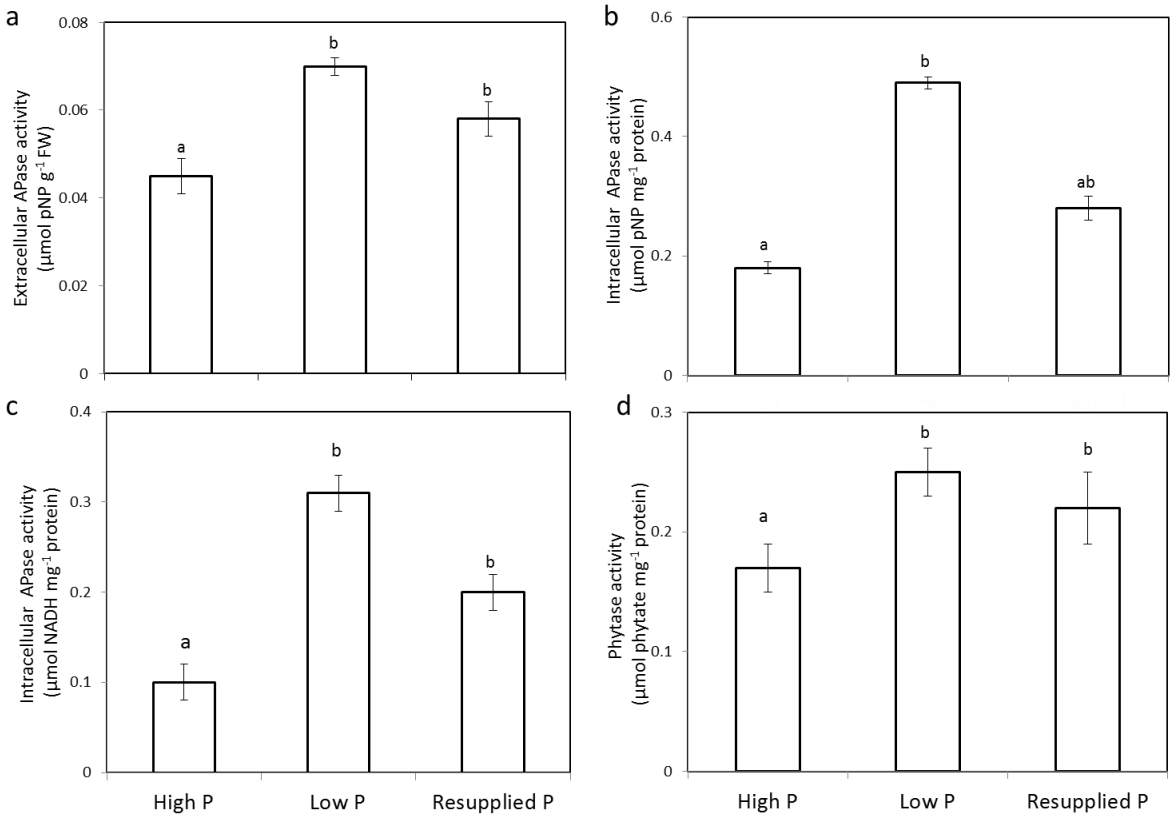

Figure 5.7 Extracellular acid phosphatase (APase) (a), intracellular APase (b, c), and phytase enzyme activity in *Virgilia divaricata* (Adamson) nodules grown under adequate (High), deficient (Low) and after the resupply of phosphorus conditions. Values are presented as means (n = 7) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

5.5 Discussion

V. divaricata nodules show responses of internal P recycling and P metabolite profiles. This is the first report on the plasticity of the biochemical and physiological mechanism of P recycling in legume nodules. The implications of these modifications are discussed in relation to N acquisition during fluctuations in soil P concentration.

A lower nodule biomass but an increased root: shoot ratio was observed in *V. divaricata* in response to low P supply. This is a typical response to P deficiency as has been documented in other legumes (Vadez et al. 1997, Le Roux et al. 2006). The recovery response from P deficiency is consistent with earlier reports in which P supply to previously P deficient plants increased plant dry weights progressively and enhanced P uptake (Drew et al. 1984, Jungk et al. 1990) and is important as it demonstrates that low P exposure has no permanent impact on these plants.

Nodule P utilization under P deficient and resupplied conditions were analogous but acquisition rates differed. Furthermore, *V. divaricata* had a higher BNF per nodule, despite a decline in metabolic Pi and adenylate levels within the nodules following P deficiency. A reduction in nucleotide-P levels has been reported in response to P deprivation in *Glycine max* (Duff et al. 1989) and *Lupinus angustifolius* (Le Roux et al. 2006). According to Theodorou and Plaxton (1993), these reductions in Pi and adenylate pools that accompany Pi starvation are important. Reduced levels of ADP and Pi, and increased ADP: ATP ratios have been reported to be associated with restricted electron flow in the Cytochrome pathway (Bryce et al. 1990). Consequently, plants then utilize alternate means of mitochondrial respiration (Vance et al. 2003). This phenomenon known as adenylate control was observed in *Phaseolus vulgaris* (Rychter et al. 1992) as well as *Lupinus albus* (Johnson et al. 1996). Conservation of internal pools of acquired Pi is considered an important adaptation for

growth on low P (Vance et al. 2003) and although several enzymes of the glycolytic pathway depend on Pi and/ or adenylates as co-substrates, metabolic functions are not compromised by P deprivation due to the generation of energy and the production of carbon skeletons as manifested in the excretion of significant amounts of organic acids (Vance et al. 2003).

Many plants show remarkable flexibility in modifying metabolic rates and utilizing alternative metabolic pathways under low P (Vance et al. 2003, Tran et al. 2010) e.g. alternative glycolytic reactions can bypass Pi- or ATP-requiring steps of glycolysis under Pi starvation (Theodorou and Plaxton 1996). While ATP and ADP-levels often decline under P deficiency as observed in this study and others (Duff et al. 1989, Le Roux et al. 2006) pyrophosphate (PPi) concentrations appear to be elevated during Pi stress (Duff et al. 1989), thereby suggesting that PPi can act as an energy donor and aid in conserving limited ATP. An alternate glycolytic pathway catalysed by PPi-dependent phosphofructokinase (PFP) under P deficiency has been documented, in which PPi- dependant PFP bypasses the ATP-dependent phosphofructokinase (PFK), generating fructose 1,6-biphosphate (Theodorou and Plaxton 1996; Plaxton and Carswell 1999). Although PPi was not determined in this study, the elevated levels of Fru6P under low P may be indicative of the engagement of this pathway. Alternatively, the accumulation of Fru6P, may also be due to the enhanced sucrose breakdown, to fuel the organic acid requirement of the low P adaptations in nodules (Le Roux et al. 2006). Apart from the potential of engagement of PPi dependant bypass reactions during P stress, internal organic P recycling may also be important.

The enhanced BNF per nodule dry weight in LP nodules in this study may have been supported by increased internal P recycling in which the nodules regulate P influx, thereby increasing amounts of P entering the nodule and minimizing the effects of P deficiency when supply is low (Tang et al. 2001). This internal recycling can be achieved via the possible mobilization of membrane phospholipids and activities of phosphohydrolases. The

mobilization of membrane phospholipids, in order to release Pi to the cell, has been found for P stressed leaves (Lambers *et al*. 2012), where the phospholipids have been replaced by sulfolipids. Although we do not have any evidence for the increased presence of sulpholipids, the decline in membrane phospholipids in P stressed nodules may support to this possibility.

Greater phosphohydrolase (APase and phytase) activity under P deficient conditions enables P from nucleotides to be remobilized to support low P concentrations in the cell. The importance of increased APase activity for P metabolism has been extensively reported (Gilbert et al. 1998, Miller et al. 2001, Hurley et al. 2010). Intracellular APases break down P nucleotides, sugar-P and P-monoesters and recycle P through the supply of P for amino acid biosynthesis and nodule metabolism during P deprivation (Penheiter et al. 1997). Previous studies have also shown that an increase in nodule APase activity may constitute an adaptive mechanism for N_2 fixing legumes to tolerate P deficiency (Kouas et al. 2008, Bargaz et al. 2012). This current work, therefore explains how the internal recycling of P via greater APase activity in nodules and roots under P deficiency, sustains nodules under P deficiency to engage in BNF (Vadez et al. 1997, Al-Niemi et al. 1997). The functional benefit of such physiological plasticity is that these plants would possibly be able to respond to a changing soil environment, where the P is known to be heterogeneously distributed.

The findings of this study suggest that *V. divaricata* plants exhibit a great degree of flexibility in functional response with variations in P levels. With the resupply of P, plants were able to regress to growth levels as expected under optimal conditions, even though they were previously P starved. Lower levels of P- nucleotides and increased levels of phosphatases were observed, which imply that alternate strategies were at play to recycle P more efficiently enabling *V. divaricata* to still fix N_2 under P deficient conditions.

5.6 References

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Chapter 6: General discussion

Phosphorus availability and accessibility strongly determines plant growth, metabolism, development, reproduction and crop yield (Marschner 1995). Phosphorus is essential for photosynthesis, respiration, regulation of enzymes activity and gene expression or signal transduction (Raghothama 1999, Ciereszko and Kleczkowski 2005, Ciereszko and Balwicka 2005, Rychter and Rao 2005) but it is very limited in the soil solution and concentrations typically do not exceed 10 µM (Lamont et al. 2003). Availability of this element is even lower in certain soils, notably acid weathered soils in Mediterranean-type ecosystems, where soils are highly leached.

The fynbos region in South Africa (Power et al. 2010), the chaparral of California (Westman 1981) and the Jarrah forests of South West Australia (Bell and Koch 1980) are all Mediterranean-type ecosystems characterised by nutrient poor (N and P) soils. Legumes are relatively well represented in these soils despite their high demand for P to engage in N_2 fixation with bacteria implying that they might have evolved modifications to cope with nutrient limitation. However, most of our knowledge on legume adaptations during P deficiency stems from the work on model legume species for obvious reasons such as agricultural importance (see Table 6.1). These model legumes have been extensively researched in terms of their mechanisms to overcome P deficiency and are the subject of recent bio-technologies to study the regulation of genes and proteins involved during P deficiency (Wang et al. 2014, Secco et al. 2014). For example, Secco et al. (2014) assessed cluster root development in white lupin under phosphate limitation by RNA-seq. The data generated from the study include sequencing data, as well as pathways and transcription factors involved in the regulation of the development of cluster roots during P limitation and may serve as a library to compare other plant P stress mechanisms against.

Evidently a need for information on the dynamics of N and P uptake and utilisation in legume plants in a wider context is however needed as patterns and mechanism cannot always be extrapolated from model legumes. A better understanding of metabolic and physiological costs associated with P acquisition strategies is needed (Lynch and Ho 2005), which may be important for the management and future conservation of local legume species. As such, an understanding of the mechanisms of legume adaptations to fix N_2 especially in nutrient impoverished environments is important. Currently, very little information about strategies that local fynbos legumes use to tolerate P deficiency is available (Table 6.2). This research therefore addresses a very important issue in P nutrition of legumes, which has been neglected in almost all the studies to date. This aspect is the functional flexibility of P recycling and localisation within roots and nodules, during exposure to low P as well as after the resupply of P to the plant. With the exception of recent work (Thuynsma et al. 2014), the physiological plasticity of P recycling during P deficiency response in nodules, has not been documented. The work by Thuynsma et al. (2014) has shown that during P resupply, lupins have remarkable flexibility for belowground allocation and organic acid metabolism within cluster roots and nodules. However, less is known about the other P deficiency strategies, such as the distribution and recycling of P in roots and nodules. Given the heterogenous distribution of mineral nutrients in natural soils, (Hodge 2004), the flexibility of the P deficiency response, involving P distribution and recycling, becomes very important for plants in nutrient poor ecosystems. This thesis has generated a body of new knowledge regarding the physiological impact of the flexibility of mechanisms involving belowground P recycling and tissue localisation in legumes from nutrient poor ecosystems.

This study was undertaken to enhance our understanding of the nodule physiology, P fractions, N and P requirements as well as P recycling mechanisms during P deficient conditions. This was accomplished through three experimental chapters (Chapter 3, 4 and 5).

The experiments in Chapter 3 aimed to determine how the differences in nodule anatomy, P recycling and acquisition, affect the efficacy of BNF during P deficiency. In these experiments, plants were subjected to low P supply for 10 weeks. Although other studies (Le Roux et al. 2006, Kleinert et al. 2014, Thuynsma et al. 2014) use a much shorter period of time to mimic prolonged low P exposure, *V. divaricata* is slow growing (Magadlela et al. 2014), hence the decision to increase P deficiency for longer. This is important because even with a longer period of low P exposure, compared to the studies cited above, low P symptoms did not take long to be expressed.

The findings of these experiments indicate that although fewer nodules and infected cells form during P stress in *V. divaricata*, the ratio of bacteroid-filled cells relative to nodule number was very high. It is this high ratio that may contribute to the higher BNF per unit nodule dry weight. In addition, APases and RNases which is known to increase P turnover (Nanamori et al. 2004), contributed to nodule P sustenance during low P exposure. The increased secretion of APases found in this study is consistent with what has been found in other fynbos legumes such as *Aspalathus* and *Cyclopia* (see Table 6.2) (Maistry et al. 2014, Spriggs 2004). Although the findings of Chapter 3, determined the levels of P deficiency that can induce functional and anatomical changes to the roots and nodules, the flexibility of these responses remained elusive. Therefore this was addressed in the following chapter.

In Chapter 4, P distribution and recycling within the nodule and its effects on N nutrition was examined. To test if the response to P stress is flexible, an additional treatment was added in which plants are stressed and then resupplied with P. It was found that P distribution in the nodules is homogenous and not localized to specific regions. The period of P deficiency was shorter for these experiments as compared to those in Chapter 3 (ten versus eight weeks). The relative growth rates of shoots, roots and nodules were lower with P stress while biomass allocation was higher. It has previously been shown that an increase in the proportion of

allocation to roots is an adaptive feature (Hermans et al. 2006) but less is known with regard to nodules.

A higher concentration of APases and Fe was documented in nodules under P deficient conditions. Iron plays a pivotal role in the nitrogenase enzyme complex involved in N_2 fixation and is an important component of leghaemoglobin. In pigeon pea nodules lower Fe levels corresponded to lower leghaemoglobin and in turn declined N_2 fixing levels (Nandwal et al. 1991). Iron deficiency has also been correlated with the development of nodule initials but reduced nodule functioning (Tang et al. 1990). Increased activity of intracellular APase activity constitutes an adaptive mechanism to tolerate P deficiency for legumes that fix N_2 (see Table 6.1, Kous et al. 2008, Araujo et al. 2008, Bargaz et al. 2012). The distribution and recycling of P during deficiency conditions further necessitated the investigation of the metabolic changes that may underpin these observed effects. The following Chapter 5, aimed at profiling the chemical species of P involved in these responses.

In Chapter 5, the effect of P limitation on N_2 fixation was assessed with regard to adenylate levels, P partitioning as well as the accumulation and secretion of phosphatases (APases and phytase). Plants were grown under P deficient and sufficient conditions for seven weeks (short term deprivation). Both physiological and biochemical strategies to variations in P availability was observed in this study. *V. divaricata* had a higher N_2 BNF per nodule regardless of a decline in metabolic Pi, adenylate levels, as well as phospholipids within the nodules following nutritional P deficiency. Reduced nucleotide-P levels have been observed in response to P deprivation in soybean (Duff et al. 1989) and blue lupin (Le Roux et al. 2006) and have been correlated with the conservation of internal pools of P/ Pi during low P (Vance et al. 2003). When adenylate levels are low, it also suggests the plant may be using alternate pathways to conserve P (Theodorou and Plaxton 1996). It therefore seems that an internal recycling mechanism is at play in the nodules of *V. divaricata* as increased BNF per

nodule dry weight was concomitant with elevated levels of internal recycling of organic P and phospholipid mobilization. Internal activities of APase and phytase enzymes exceeded external levels of these enzymes under P stress. Phytases and APases regulate P influx, and also help minimize the effects of P deficiency when supply is low. Roots of *V. divaricata* contained higher levels of these enzymes, compared to the nodules, suggesting they scavenge P and transport them to nodules.

Phytases and APases have both been implicated in the breakdown of P from nucleotides, sugar-P and P-monoesters and recycle P through the supply of P for amino acid biosynthesis and nodule metabolism during P deprivation (Penheiter et al. 1997). Another method of P recycling is the replacement of membrane phospholipids, in order to release Pi to the cell. This has been found for P stressed leaves (Lambers et al. 2012), and this is the first report of this possibility in P stressed nodules.

Overall, the findings of these experiments show the functional flexibility in *V. divaricata* to acquire nutrients during short and long term P deficiency and the plasticity in growth when P is resupplied following P deprivation. The study highlights that the species can respond to P limitation due to the interplay of physiological and biochemical mechanisms. This study was the first to examine adenylate levels, the exudation of intracellular and extracellular phosphohydrolases, and P distribution during P deficiency in an indigenous legume species from a nutrient impoverished ecosystem. Furthermore, it serves as a better representation of how local CFR legumes may function and cope with variable P levels.

This study was restricted however, in that it focussed largely on physiological responses in relation to metabolic functioning. To gain an improved understanding of P stress and N_2 metabolism in legumes, an integrated approach to look at the interplay of mechanisms would be necessary.

At the biochemical level, several other plant adaptations to P stress exists that warrant further investigation e.g. alternate glycolytic bypasses and the role of organic acids. It would also be invaluable to investigate P stress at the molecular level is terms of transcriptional regulation and signalling during P deficiency using RNA-seq tools and gene regulatory network analyses. High-throughput sequencing technologies have facilitated the exploration of such functional genomics in model legumes (Verdier et al. 2013, Secco et al. 2014). In addition it would also be important to identify the different peptides associated with P deficiency, using a proteomic approach. This would provide insight on which proteins are involved in P deficient signaling and may also shed light whether the size of certain proteins such as APase vary, and if more than one isoform of the protein exists. It will also show whether certain proteins are nodule and/ or root specific.

Table 6.1 Mechanisms of P acquisition and utilization, documented in model legumes under P-limited conditions.

Species	Mechanism induced under limited P-supply	Effect	Reference
Medicago truncatula alfalfa, barrel medic	increased extracelluar root APase activity	mobilization of organic P	*In* Vance et al. 2003
Medicago falcata yellow lucerne	increased extracelluar root APase activity	mobilization of organic P	Li et al. 2011
Phaseolus vulgaris common bean	increased adventitious root formation, increased root hair density	facilitates acquisition of P from topsoil	Lynch and Brown 2001
	increased nodule intracellular APase activity	liberates P for utilization	Bargaz et al. 2012
	increased nodule and roots intracellular APase activity	liberates P for utilization	Kouas et al. 2009
	increased nodule intracellular APase and phytase activity	liberates P for utilization	Araujo et al. 2008
Glycine max soybean	increased nodule and roots intracellular APase activity	liberates P for utilization	Penheiter et al. 1997
Lupinus albus white lupin	formation of cluster roots	facilitates acquisition of P	Johnson et al. 1996
	increased secretion of APases from cluster roots	mobilization of organic P	Miller et al. 2001
	increased nodule intracellular APase activity	liberates P for utilization	Thuynsma et al. 2014
Lupinus angustifolius blue lupin	reduced adenylate levels, increased ADP to ATP ratio in nodules	regulates P influx	Le Roux et al. 2006

Table 6.2 Mechanisms of P acquisition and utilization, documented in legumes from the nutrient-poor fynbos ecosystem in the Cape Floristic Region of South Africa. Mechanisms documented in *Virgilia divaricata* (Adamson) in this study have been included.

Species	Mechanism induced under limited P-supply	Effect	Reference
Aspalathus linearis	increased extracellular root APase activity	mobilization of organic P	Maistry et al. 2014
rooibos	formation of cluster roots	facilitates acquisition of P	Maistry et al. 2014
Aspalathus nivea, A. subtingens	formation of cluster roots	facilitates acquisition of P	Power et al. 2010
Cyclopia	formation of cluster roots	facilitates acquisition of P	Spriggs 2004
honeybush	increased extracellular root APase activity	mobilization of organic P	Maseko and Dakora 2013
Virgilia divaricata	incrased root to shoot ratio	combined adaptations to acquire P and effciently use P internally	*chapter 3,4,5*
keurboom	increased nodular Fe concentration		chapter 3
	increased biomass allocation to roots and nodules		chapter 4
	increased extracellular root and nodule APase activity		chapter 3, 5
	increased phytase and RNase activity		chapter 3, 5
	increased intracellular root and nodule APase activity		chapter 4, 5
	reduced adenylate levels, increased ADP to ATP ratio in nodules		chapter 5

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APPENDIX

Vardien W, Mesjasz-Przybylowicz J, Przybylowicz WJ, Wang Y, Steenkamp ET, Valentine AJ. (2014) Nodules from Fynbos legume *Virgilia divaricata* have high functional plasticity under variable P supply levels. Journal of Plant Physiology 171: 1731-1739.

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Nodules from Fynbos legume *Virgilia divaricata* have high functional plasticity under variable P supply levels

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ABSTRACT

Legumes have the unique ability to fix atmospheric nitrogen (N_2) via symbiotic bacteria in their nodules but depend heavily on phosphorus (P), which affects nodulation, and the carbon costs and energy costs of N_2 fixation. Consequently, legumes growing in nutrient-poor ecosystems (e.g., sandstone-derived soils) have to enhance P recycling and/or acquisition in order to maintain N_2 fixation. In this study, we investigated the flexibility of P recycling and distribution within the nodules and their effect on N nutrition in *Virgilia divaricata* Adamson, Fabaceae, an indigenous legume in the Cape Floristic Region of South Africa. Specifically, we assessed tissue elemental localization using micro-particle-induced X-ray emission (PIXE), measured N fixation using nutrient concentrations derived from inductively coupled mass-spectrometry (ICP-MS), calculated nutrient costs, and determined P recycling from enzyme activity assays. Morphological and physiological features characteristic of adaptation to P deprivation were observed for *V. divaricata*. Decreased plant growth and nodule production with parallel increased root:shoot ratios are some of the plastic features exhibited in response to P deficiency. Plants resupplied with P resembled those supplied with optimal P levels in terms of growth and nutrient acquisition. Under low P conditions, plants maintained an increase in N_2-fixing efficiency despite lower levels of orthophosphate (Pi) in the nodules. This can be attributed to two factors: (i) an increase in Fe concentration under low P, and (ii) greater APase activity in both the roots and nodules under low P. These findings suggest that *V. divaricata* is well adapted to acquire N under P deficiency, owing to the plasticity of its nodule physiology

Introduction

Soil phosphorus (P) availability is the most limiting factor for legumes that symbiotically fix atmospheric nitrogen (N_2) with rhizobia (Vance et al., 2003). N_2-fixing legumes are autonomous at acquiring N, but depend profoundly on and require more P than legumes growing on mineral N (Drevon and Hartwig, 1997). P not only affects the energy costs of N_2 fixation (Schulze et al., 1999, Valentine et al., 2010), it is also important for nodule formation and function (Israel, 1987). After N, however, P is often the most limited element in soils, especially in ancient sandstone-derived soils which are coarse grained and highly leached such as soils encountered in the Cape Floristic Region (CFR) of South Africa (Witkowski and Mitchell 1987). The legume tree, *Virgilia divaricata* (Adamson) is native to the CFR, and is distributed over a wide range of variably P-poor soils, from relatively richer forest margins to poorer Fynbos soils (Coetsee and Wigley, 2013).

P is taken up as orthophosphate (Pi), but the chemistry of Pi results in low Pi concentrations in the soil solution, thus limiting Pi diffusion to the root system (Morgan et al., 2005). Many studies have investigated P in rhizobium–legume symbiosis (Israel,

Abbreviations: BNF, biological nitrogen fixation; %NDFA, nitrogen derived from atmosphere; RGR, relative growth rates; SNAR, specific nitrogen acquisition rate; SNUR, specific nitrogen utilization rate; SPAR, specific phosphate absorption rate; SPUR, specific phosphate utilization rate; APase, acid phosphatase; PIXE, particle-induced X-ray emission; BS, proton backscattering; ICP-MS, inductively coupled mass-spectrometry.

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1993; Høgh-Jensen et al., 2002; Olivera et al., 2004; Bucciarelli et al., 2006), and have demonstrated that improving P nutrition in legumes under P-deficient conditions is generally based on two broad mechanisms (Raghothama, 1999; Hammond et al., 2003). These are (i) increasing P acquisition that can be accomplished by increasing carbohydrate allocation to the roots, which increases the root:shoot ratio or causes a shift from primary to lateral root growth (Vance et al., 2003) and (ii) enhancing P utilization by increasing the abundance of Pi transport proteins and the exudation of organic acids, as well as phosphatases to mobilize P from organic or insoluble compounds (Plaxton, 2004). An alternative way to attain P from the soil for use in plant growth is through the use of acid phosphatases (APases) to hydrolyze organic P (Duff et al., 1994). In plant tissues, APases are mainly found in the cell wall and intracellular spaces (Yadav and Tarafdar, 2001; Olczak et al., 2003). Extracellular APases are involved in breakdown of organic phosphate monoesters in the soil, whereas intracellular APases are thought to be pivotal in the remobilization and scavenging of Pi from intracellular phosphate monoesters (Duff et al., 1994; Marschner, 1995). In common bean, the activities of APases increase in the nodules under low P conditions, indicating that N_2-fixing legumes can enhance P utilization within the nodules to tolerate P deficiency (Araújo et al., 2008).

The mechanistic effects of P limitation on N_2 fixation are not fully understood and not much is known of P metabolism in nodules. This is despite the fact that legumes occur worldwide where they thrive under a diversity of ecological, including limited P and N availability. It is therefore reasonable to expect that even in nutrient-poor ecosystems, legume species adapt to low P conditions or make use of alternate strategies to obtain and recycle P (He et al., 2011). Most studies on the effect of P limitation on N_2 fixation have largely been confined to model legumes such as *Lupinus albus* (Schulze et al., 2006; Thuynsma et al., 2014) and *Medicago truncatula* (Tang et al., 2001; Sulieman et al., 2013). Despite the high legume diversity found on the P-poor soils of the CFR (Goldblatt and Manning, 2002), little is known about the functional mechanisms which affect N nutrition within the nodules of indigenous legumes.

Our aim was therefore to investigate how P recycling and distribution in nodules affects the N nutrition of the indigenous legume *V. divaricata* during variable P supply.

Materials and methods

Seed germination, bacterial inoculation, and growth

Virgilia divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were placed in smoke solution (Smoke Plus, Kirstenbosch National Botanical Garden, South Africa) (Magadlela et al., 2014) and incubated in a water bath at 50 °C for 4 h. Thereafter, the seeds were surface sterilized, rinsed with distilled water and germinated in 5 cm deep seed trays containing sterile sand under natural light conditions (midday irradiances between 600–800 $\mu mol\ m^{-2}\ s^{-1}$) in a temperature-controlled (15–25 °C), north-facing greenhouse at Stellenbosch University, South Africa.

After the first fully expanded leaf emergence, seedlings were transferred to pots containing sterile sand and inoculated with *Burkholderia*. Inoculum was prepared by growing the bacterium on yeast mannitol agar (YMA) containing 0.5 g/L yeast extract (Biolab), 10 g/L mannitol (Saarchem), 0.5 g/L dipotassium hydrogen orthophosphate (K_2HPO_4, Biolab), 0.2 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$, Biolab), 0.1 g/L sodium chloride (NaCl, Biolab), 15 g/L bacteriological agar (Biolab), and 2.5 g/L Congo red (Saarchem) (Somasegaran and Hoben, 1994). After incubation at 28 °C for 4 days, single colonies were selected and cultures prepared in tryptone–yeast medium containing 5 g/L tryptone (Biolab), 3 g/L yeast extract (Biolab), and 2 mL of a 440 g/L calcium chloride dihydrate ($CaCl_2.2H_2O$, Biolab) solution (Somasegaran and Hoben, 1994). After incubation for 24 h at 28 °C, 50 mL of the bacterial culture was applied to each seedling.

Plants were separated into three treatment groups: group I (low P, LP), group II (high P, HP, control), and group III (resupplied phosphate, RP: 4 weeks of low P followed by 4 weeks of high P). The plants in these groups were supplied with 100 mL of a quarter strength Long Ashton nutrient solution (Hewitt, 1966) twice a week. The nutrient solution was modified to contain either 500 μM P (HP) or 5 μM P (LP) (pH 5.8), and 500 μM NH_4NO_3. Plants were grown for 8 weeks under the same conditions as described for germination after which they were harvested.

Specimen preparation for elemental analysis

Nodule samples were immediately frozen by immersion in liquid propane cooled by liquid nitrogen, using a Leica EM CPC cryoworkstation (Leica Microsystems AG, Vienna, Austria). Samples were subsequently freeze dried in a Leica EM CFD cryosorption freeze dryer, following a 208 h programed cycle starting at −80 °C, and ending at ambient temperature. Transverse sections of the freeze-dried nodule samples were obtained by hand-sectioning under a stereomicroscope using a double-edge stainless steel razor blade, and mounted between two layers of 0.5% (w/v) Formvar film. To prevent charge buildup during measurements, the Formvar membrane facing the proton beam was coated with a thin layer of carbon. Light micrographs of each specimen were taken before and after proton irradiation.

Elemental analysis and data evaluation

Analyses were performed using the nuclear microprobe at the Materials Research Department, iThemba LABS, South Africa. A proton beam of 3 MeV energy from the 6 MV single-ended Van de Graaff accelerator was focused onto a $3 \times 3\ \mu m^2$ spot and scanned over specimens using square or rectangular scan patterns with a variable number of pixels (up to 128×128). Scan sizes varied according to the sizes of nodules. Particle-induced X-ray emission (PIXE) and proton backscattering (BS) were used simultaneously. PIXE spectra were registered in the energy dispersive mode, using a Si (Li) detector. BS spectra were recorded with an annular Si surface barrier detector (100 mm thick) positioned at an average angle of 176°. Data were acquired in the event-by-event mode. The normalization of results was done using the integrated beam charge, collected simultaneously from a Faraday cup located behind the specimen and from the insulated specimen holder. A more detailed description of the nuclear microprobe set-up at iThemba LABS can be found in Prozesky et al. (1995) and Przybylowicz et al. (1999, 2001, 2005).

Data evaluation was performed using GeoPIXE II software (Ryan, 2000). Quantitative elemental images were generated using the *Dynamic Analysis* method. The matrix composition and areal density were obtained from the analysis of corresponding BS spectra using a RUMP simulation package (Doolittle, 1986) with non-Rutherford cross sections for C, O, and N. In addition to elemental images, average concentrations from nodules were also obtained. For this purpose, PIXE and BS spectra extracted from the nodule cross sections were used.

Biomass parameters and nutrient concentrations

Upon harvesting, a subset of plants was separated into nodules, roots, and shoots. The harvested material was dried at 50 °C for 72 h and dry weights (DW) recorded. The latter were used to calculate growth parameters such as allocation and relative growth

rate (RGR). The dried material was milled and analyzed for their respective C and N concentrations at the Archeometry Department (University of Cape Town, South Africa) and P concentration at the Central Analytical Facility (Stellenbosch University, South Africa) using inductively coupled mass-spectrometry (ICP-MS).

Calculations of $\delta^{15}N$

The $\delta^{15}N$ analyses were also carried out at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope (^{15}N:^{14}N) of the sample and standards as defined by Farquhar et al. (1989). Approximately 2 mg of each dried organ sample was put into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. %NDFA was calculated according to Shearer and Kohl (1986):

$$\%\text{NDFA} = 100((\delta^{15}\text{Nreference plant} - \delta^{15}\text{N}_{\text{legume}})/(\delta^{15}\text{N}_{\text{reference plant}} - \text{B})).$$

The reference plant was non-nodulated *V. divaricata*, grown under the same glasshouse conditions. The B value is the $\delta^{15}N$ natural abundance of the N derived exclusively from biological N fixation of nodulated *V. divaricata*, also grown under same conditions as the reference plants, but with a N-free nutrient solution.

Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} DW d^{-1}) reflects the net P absorption rate per unit root DW (Nielson et al., 2001) and was determined using the formula:

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

where M is the P content per plant, t is the time, and R is the root DW. This equation was modified to calculate the net P absorption rate for nodules, where the nodule DW was used instead of root DW.

Specific P utilization rate (SPUR) (g DW mg^{-1} P d^{-1}) is a measure of the DW gained for the P taken up by the plant (Nielson et al., 2001) and was estimated with the following formula:

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

where M is the P content of the plant and W is the plant DW. This equation was modified to calculate the DW gained for the P uptake by roots and nodules, where the nodule and root DW was used instead of plant DW. The specific N absorption and utilization rates (SNAR and SNUR, respectively) were adapted from this equation as well, to include N instead of P.

Enzyme activity assay: Intracellular acid phosphatase

To assess P recycling, fresh nodule and root samples detached upon harvesting were frozen at −80 °C. Nodule and root samples (approximately 40 mg fresh mass) were ground with an extraction buffer according to Araújo et al. (2008) consisting of 0.1 M Na-acetate and 1% β-mercaptoethanol. The material was centrifuged at 13,000 × *g* at 4 °C during 30 min, and the supernatant was taken for enzyme assays. For APase activity, 200 μL of nodule or root crude protein extract was incubated for 30 min at 28 °C with a mixture of 50 mM Na-acetate buffer containing 5 mM *p*-nitrophenyl phosphate (p-NPP). The reaction was stopped by the addition of 1.0 mL 0.5 M NaOH, and activity was measured spectrophotometrically at 410 nm. APase activity was defined as the amount of *p*-nitrophenyl (p-NP) released relative to known p-NP standards (derived from a standard curve) and expressed per unit protein.

Table 1
Biomass of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (HP), deficient (LP), and resupplied (RP) phosphorus conditions.

Parameters	Phosphate treatment		
	High (500 μM, control)	Low (5 μM)	Resupplied (5 μM; 500 μM)
Plant dry weight (g)	1.166 ± 0.08 b	0.478 ± 0.02 a	0.953 ± 0.04 b
Root dry weight (g)	0.217 ± 0.02 b	0.105 ± 0.01 a	0.200 ± 0.02 b
Shoot dry weight (g)	0.651 ± 0.09 b	0.283 ± 0.03 a	0.568 ± 0.01 b
Nodule dry weight (g)	0.297 ± 0.05 c	0.091 ± 0.02 a	0.195 ± 0.02 b
Root:shoot	0.332 ± 0.02 a	0.371 ± 0.01 b	0.304 ± 0.06 a
Nodule number	42 ± 9 c	18 ± 3 a	31 ± 8 b

Values are presented as means ± SE of separate replicates ($n = 4$).
Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Statistical analysis

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Kaleidagraph, Synergy Software, USA). Where the ANOVA revealed significant differences between treatments, the means (4–5) were separated using the *post-hoc* Tukey's LSD multiple range test (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences among treatments.

Results

Nodule induction

All 50 *V. divaricata* plants grown, formed nodules and all subsequent results are based on nodules that were functionally fixing N_2 (Fig. 1a and c). Nodules were round-spherical in shape and often produced in clusters (Fig. 1a and b). Most nodules formed at the top portion of the root system (Fig. 1a).

Biomass and allocation

Total plant biomass accumulation decreased in P-starved plants as a result of root, shoot, and nodule growth (Table 1). The period of resupply (RP) to previously P-starved plants resulted in a two-fold increase in root, shoot, and nodule mass compared to the LP treatment (Table 1). LP plants, however, maintained a higher root:shoot ratio, a morphological response typical of low P exposure, but the number of nodules produced was fewer compared to the other two treatments (Table 1).

The RGR for LP and RP roots were similar but significantly less compared to HP. Nodule RGR for RP was restored to levels established with the control (HP) treatment (Table 2). For root and nodule allocation, the response of LP was greater (Table 2), suggesting that more biomass is apportioned towards these organs during P deprivation.

Concentration and localization of important N-fixing elements

Quantitative micro-particle induced X-ray emissions (PIXE) distribution maps of P, K, Ca, Mg, CL, Fe, Al, S, Si, Mo, Mn, and Cu from PIXE were obtained, but only those elements essential to the process of N_2 fixation are presented. In addition to P, these include iron (Fe) and potassium (K). Fe is a component of leghemoglobin that functions in oxygen supply to bacteroids and of the nitrogenase

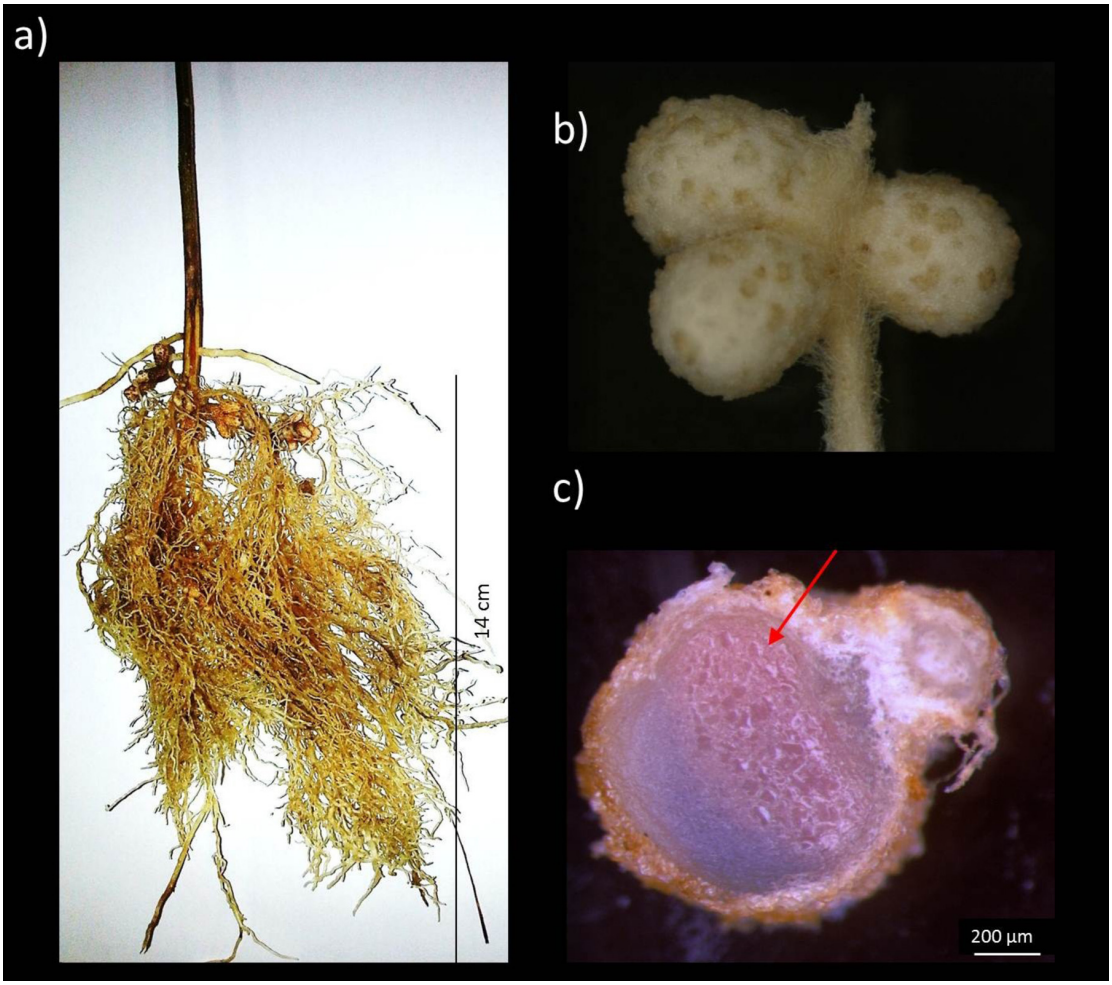

Fig. 1. Nodules of *Virgilia divaricata* (Adamson, Fabaceae). (a) Nodules mostly occur at the top region of the root system and are typically (b) clustered, occurring in groups of two or more. For the various analyses in this study, only active N_2-fixing nodules were selected based on the presence of the pink coloration caused by leghemoglobin (red arrow) as seen in (c).

enzyme complex involved in N_2 fixation while K is important for nodule development.

Elemental maps showed that P concentration did not differ significantly amongst treatments (Figs. 2 and 3). Inorganic phosphate (Pi), the form of P used for metabolic functioning, was also compared among treatments which indicated that Pi concentration in P-deficient nodules decreased. Following P deprivation, resupplied nodules seem to recover easily (Fig. 4), acquiring Pi at levels analogous to those under HP supply.

The concentration of Fe (Fig. 3b) concentration was significantly higher in P-deficient nodules (up to 300 mg/kg), compared to HP and RP (Fig. 3b). K was distributed in high concentrations with values ranging between 20,000 and 30,000 mg/kg throughout nodules except the deficiency in the central region (Fig. 3c) and was highest in P-sufficient and resupplied nodules. This is expected since K relates to nodule development and number, and P-sufficient plants produced the greatest number of nodules.

Table 2
Relative growth rate and allocation of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (HP), deficient (LP), and resupplied (RP) phosphorus conditions.

Growth parameter	Phosphate treatment		
	High (500 µM, control)	Low (5 µM)	Resupplied (5 µM; 500 µM)
(a) Relative growth rate			
Shoot (mg g^{-1} d^{-1})	0.055 ± 0.002 b	0.041 ± 0.001 a	0.047 ± 0.002 ab
Root (mg g^{-1} d^{-1})	0.052 ± 0.002 b	0.040 ± 0.001 a	0.040 ± 0.001 a
Nodule (mg g^{-1} d^{-1})	0.057 ± 0.001 b	0.038 ± 0.001 a	0.049 ± 0.001 b
(b) Allocation			
Root (mg g^{-1} d^{-1})	0.033 ± 0.002 a	0.068 ± 0.001 b	0.032 ± 0.002 a
Nodule (mg g^{-1} d^{-1})	0.033 ± 0.002 a	0.064 ± 0.003 b	0.039 ± 0.002 a

Values are presented as means ± SE of separate replicates ($n = 4$).
Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Nitrogen and phosphorus nutrition and N_2 fixation efficiency

The decline of Pi levels in LP nodules (Figs. 3 and 4) was accompanied by a decrease in SPAR and SPUR (Table 3). A two-fold increase in root SPUR occurred with P deprivation but SPAR declined substantially. Resupplied roots recovered to levels found for the control (HP) treatment for both SPUR and SPAR (Table 3). Furthermore, SNAR declined in LP roots but when resupplied, it reached levels similar to HP roots. SNUR in nodules also declined with P deprivation (Table 3).

Control (HP) plants obtained a greater percentage of N from the atmosphere compared to LP plants (Fig. 5a). However, on

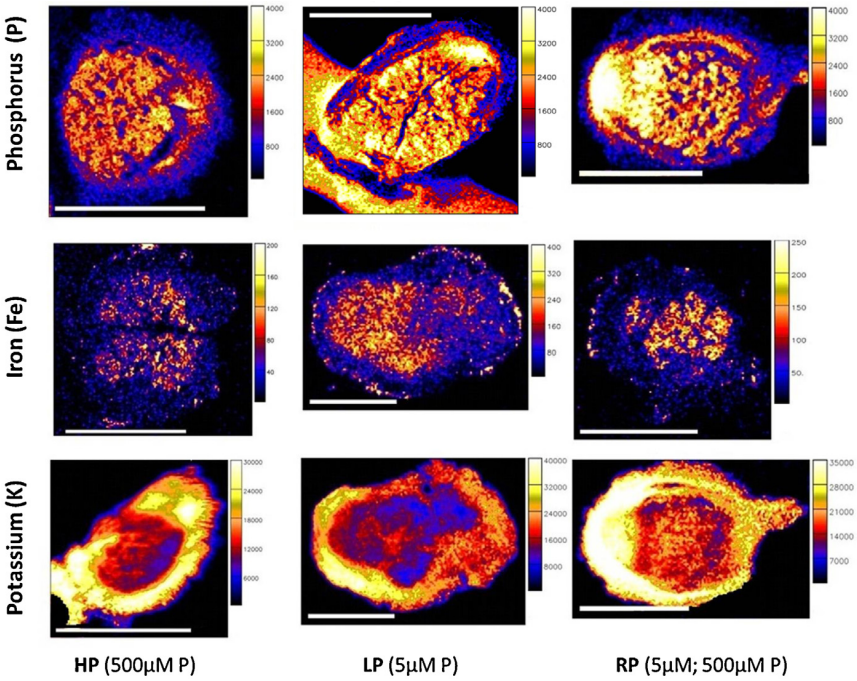

Fig. 2. Representative maps showing the distribution of important N_2-fixing elements such as (a) phosphorus, iron, and potassium in *Virgilia divaricata* (Adamson, Fabaceae) nodules grown under adequate (HP), deficient (LP), and resupplied (RP) conditions, obtained using micro-PIXE. Concentrations in mg kg^{-1} and the scale bars represent 1000 μm.

a nodule-mass basis, P-deficient plants (and to a lesser extent resupplied plants) were much more efficient at fixing N_2 (Fig. 5b).

Acid phosphatase activity

Greater APase activity was found in P-deficient roots and nodules, than under P-sufficient or resupplied conditions (Fig. 6a and b). Root's activity was however greater than nodule activity.

Table 3
Nitrogen and phosphorus nutritional parameters for *Virgilia divaricata* (Adamson, Fabaceae) nodules (a) and roots (b) grown under adequate (HP), deficient (LP), and resupplied (RP) conditions.

Nutrition parameter	Phosphate treatment High (500 μM, control)	Low (5 μM)	Resupplied (5 μM; 500 μM)
(a) Nodule			
Specific P absorption rate (mgP g^{-1} DW d^{-1})	0.086 ± 0.002 b	0.039 ± 0.001 a	0.074 ± 0.001 b
Specific N utilization rate (g DW mg^{-1} N d^{-1})	0.018 ± 0.003 b	0.008 ± 0.001 a	0.013 ± 0.000 b
Specific P utilization rate (g DW mg^{-1} P d^{-1})	0.026 ± 0.001 b	0.010 ± 0.001 a	0.016 ± 0.000 a
(b) Root			
Specific N absorption rate (mgN g^{-1} DW d^{-1})	0.091 ± 0.004 b	0.056 ± 0.006 a	0.080 ± 0.006 b
Specific P absorption rate (mgP g^{-1} DW d^{-1})	0.081 ± 0.002 b	0.027 ± 0.027 a	0.086 ± 0.001 b
Specific P utilization rate (g DW mg^{-1} P d^{-1})	0.061 ± 0.001 a	0.103 ± 0.103 b	0.053 ± 0.000 a

Values are presented as means ± SE of separate replicates ($n = 4$).
Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Discussion

During fluctuations in long-term P supply, the nodules of *V. divaricata* exhibited both physiological and morphological adaptation to the variations in P availability. These findings indicate that the legume *V. divaricata* is well adapted to acquire N during fluctuations in soil P concentrations, owing to the functional plasticity of its nodule and root physiology.

The recovery responses from P deficiency observed in this study are similar to earlier reports in which P supply to previously starved plants increased plant dry weights progressively (Rao and Terry, 1995) and enhanced P uptake (Drew et al., 1984; Jungk et al., 1990). Although these earlier focused on short-term resupply, the current study on *V. divaricata* used a more extended period as it is a slow-growing legume tree. P resupply in this legume species after extended low P conditions provides insight into P uptake of indigenous legumes in CFR where soils are typically P poor within a range of concentrations (Stock and Lewis, 1986; Witkowski and Mitchell 1987). Moreover, legumes growing in the CFR would have to engage P conservation and uptake strategies, in order to maintain nodule functioning and sustain N-metabolism. Under long-term low P supply, *V. divaricata* was able to maintain a high N_2-fixing efficiency despite a decline in nodule biomass and metabolic Pi fractions within the nodules. This may have been underpinned by observed adaptations such as the altered nodule biomass allocation, and increased P recycling and Fe concentration within nodules.

The increase in the proportion of allocation to both roots and nodules of *V. divaricata* under low P supply is an adaptive feature in view that altered biomass allocation to root organs is common in plants (Hermans et al., 2006), but less well known in nodules of legumes. The ability of plants to increase P uptake under low P supply is well documented (Shimogawara and Usuda, 1995). The enhanced P uptake by P-deficient roots suggests that these plants are adapted to cope with P deficiency. Under P-deficient and P-resupply conditions, nodule P utilization was analogous but P absorption differed. P-deficient roots and nodules had a declined P

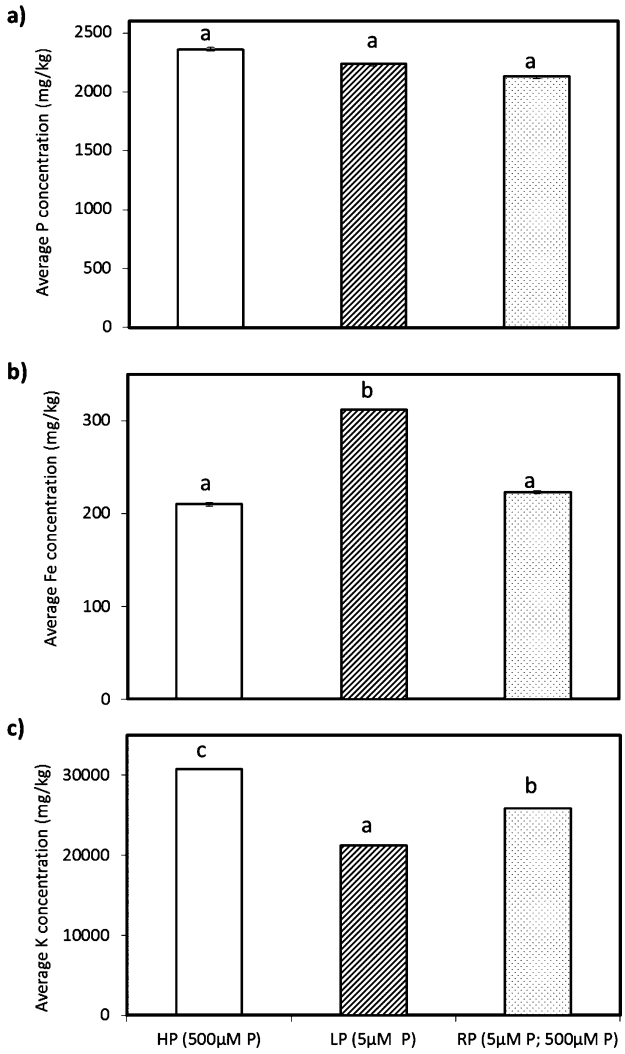

Fig. 3. Micro-PIXE average concentrations of phosphorus, iron, and potassium in cross-sections of *Virgilia divaricata* (Adamson, Fabaceae) nodules grown under adequate (HP), deficient (LP), and resupplied (RP) conditions. Values are presented as means ± SE (minimum detection limit) of three separate replicates per treatment.

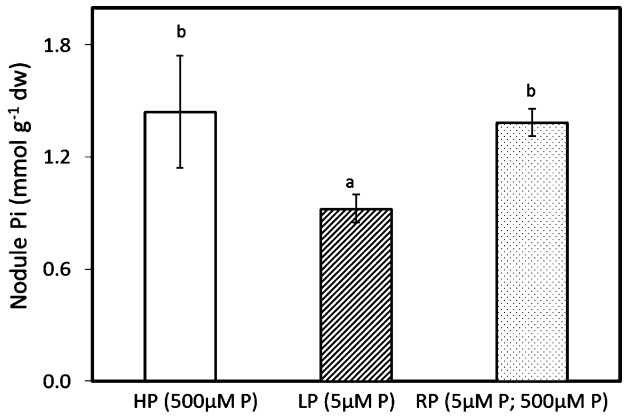

Fig. 4. Nodule orthophosphate (Pi), the form used for metabolic functioning in *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (HP), deficient (LP), and resupplied (RP) conditions. Values are presented as means ± SE of separate replicates ($n = 5$). Different letters indicate significant differences between treatments ($P \leq 0.05$).

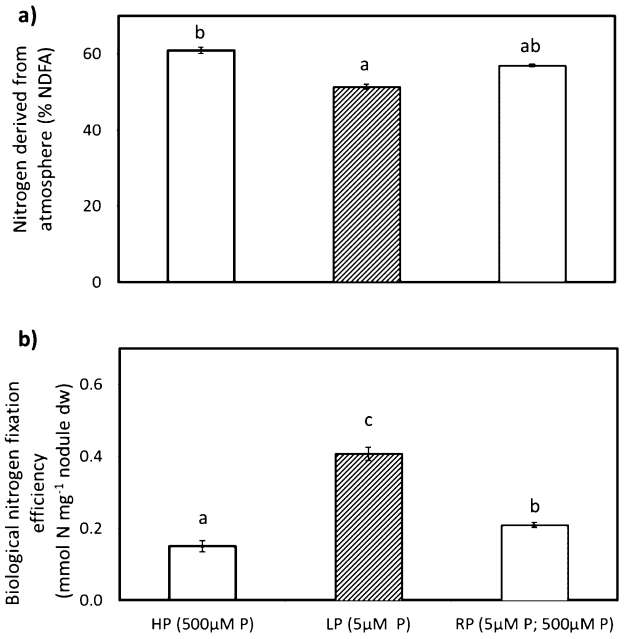

Fig. 5. (a) Percentage nitrogen derived from the atmosphere (% NDFA) of whole plants and (b) biological nitrogen fixation efficiency on a mass basis in nodules, for *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (HP), deficient (LP), and resupplied (RP) conditions. Values are presented as means ± SE of separate replicates. Different letters indicate significant differences between treatments ($P \leq 0.05$).

absorption rate while the resupply treatment rates resembled that of the P-sufficient treatment. P absorption following a period of P starvation is thought to be concomitant with a higher capacity of roots for P transport, possibly by the formation of additional carriers and transporters of Pi (Drew et al., 1984; Katz et al., 1986). Under low P conditions, *V. divaricata* maintained an increase in N_2-fixing efficiency despite lower levels of total P and Pi in the nodules.

The increased efficiency by nodules under low P conditions can be attributed to two nutritional factors explored in this study. The first is an increase in Fe concentration and localization under low P, and the second is the greater P recycling by APase in both the roots and nodules under low P.

Firstly, the functional significance of the increased Fe concentration in P-deficient nodules is that Fe is important for the nitrogenase enzyme complex involved in N_2 fixation and is a component of leghemoglobin. In pigeon pea nodules, a decline in leghemoglobin was associated with a decline in nitrogenase enzyme activity and lower N_2-fixing levels (Nandwal et al., 1991). Legumes exposed to Fe-deficiency develop many nodule initials but few functioning nodules (Tang et al., 1990). Secondly, the importance of the increased APase activity may be related to the role of phosphatase exudation into the rhizosphere, as an important mechanism for ensuring P acquisition from low P resources and from forms of P which are not readily available to other plants (Lambers et al., 2006). Induction of intracellular and secreted acid phosphatase activity has been correlated with de novo acid phosphatase synthesis in several Pi-depleted plants, including *Brassica nigra* (black mustard), *Solanum lycopersicum* (tomato), and *Arabidopsis* suspension cells and seedlings (Duff et al., 1991; Bozzo et al., 2002; Veljanovski et al., 2006). P-deficient roots and nodules exhibited greater APase activity compared to the P-resupply and P-sufficient treatments. The greater activity in roots, however, suggests that roots scavenge for P and transport P to nodules where nodules conserve P and typically do not exchange P with other organs.

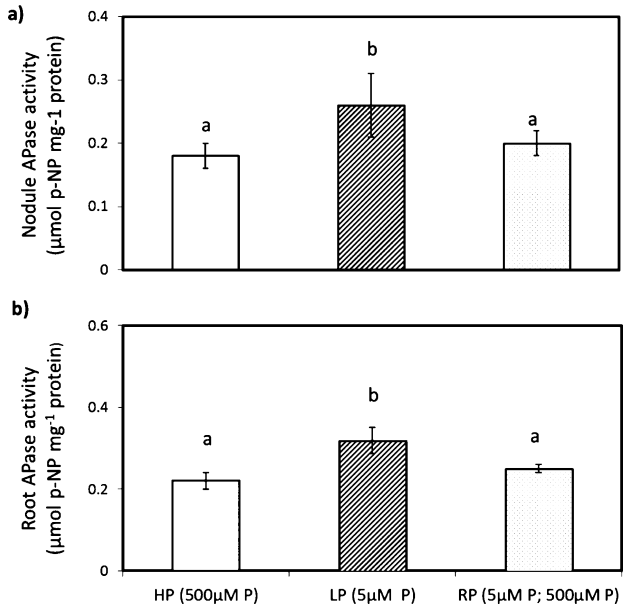

Fig. 6. Intracellular acid phosphatase (APase) enzyme activity in *Virgilia divaricata* (Adamson, Fabaceae) nodules (a) and roots (b) grown under adequate (HP), deficient (LP), and resupplied (RP) conditions. Values are presented as means ± SE of separate replicates ($n = 5$). Different letters indicate significant differences between treatments ($P \leq 0.05$).

Nodules are thus strong sinks of P (Hart, 1990; Schulze and Drevon, 2005).

Previous studies have also shown that an increase in nodule APase activity may constitute an adaptive mechanism for N_2-fixing legumes to tolerate P deficiency (Kouas et al., 2008; Bargaz et al., 2012). White lupin secretes copious amounts of APases from its roots and proteoid roots when subjected to Pi starvation (Miller et al., 2001; Wasaki et al., 2008). Similarly, common bean nodules increase APase activity under P deficiency (Kouas et al., 2009). Our findings on *V. divaricata* therefore agree with many studies reporting that P stress induces APase activity, aiding in the internal recycling of P and the increased APase activity in nodules and roots under P deficiency, explains the great requirement for P for BNF (Vadez et al., 1997; Al-Niemi et al., 1998).

In conclusion, although prolonged low P conditions reduced *V. divaricata* growth and the costs of nutrient acquisition, these P-stress responses had sufficient plasticity to revert to normal during P resupply. Specifically, the decline in N_2 fixation of low P nodules was compensated for by an increase in BNF efficiency. This can be attributed to increased Fe concentration, as well as P recycling by APases. These findings indicate that *V. divaricata* is well adapted to acquire N under various conditions of P availability and contributes to our understanding of legume distribution in nutrient poor regions such as the CFR.

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