Legume nodules from nutrient-poor soils exhibit high plasticity of cellular phosphorus recycling and conservation during variable phosphorus supply

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Abstract

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

Keywords: adenylates, nitrogen fixation, nodules, phosphohydrolases, *Virgilia divaricata*

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and a key structural constituent for nucleic acids, phospholipids, sugar phosphates and other catalytic cofactors, apart from 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 required for biological nitrogen fixation (BNF) (Schulze et al. 1999) and has been reported to affect the energy costs of BNF (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 diffusion rates in the soil 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 responses include transformed root architecture (Williamson et al. 2001), increasing root hair density and length which is common in legumes, and producing specialized roots known as proteoid roots for nutrient acquisition (Johnson et al. 1996, Neumann et al. 1999). Biochemical changes encompass increasing the abundance of Pi transport proteins and alternate enzymes to bypass Pi- or 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, and Pi is a by-product of their reactions. P 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). 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 cause the cessation of organic phosphate monoesters in the soil, while intracellular APases remobilize and scavenge Pi from internal sources (Duff et al. 1994, Marschner 1995). Many organic P compounds occur in soil, with soil phytate (inositol hexaphosphates) forming a major component (around 25%), which could be hydrolyzed by APases or phytases. The latter represent a special group of phosphatases that are able to hydrolyze phytate to myo-inositol and phosphate (Richardson et al. 2000).

The correlation between P deficiency and BNF is not consistent among legumes, and nodular P metabolism is fairly understudied. In addition, many of the legume studies examining the effect of P deficiency on BNF focuses on model legumes (Tang et al. 2001, Le Roux et al. 2006, Schulze et al. 2006; Sulieman et al. 2013, Thuynsma et al. 2014). The P poor soils of the Cape Floristic Region (CFR) in South Africa has a high legume diversity (Goldblatt and Manning 2002), yet not much is known about the functional adaptations they elicit with nutrient fluctuations.

The aim of this study was therefore to investigate the effects of P stress on BNF through response mechanisms of recycling and conservation inside the nodules of *Virgilia divaricata* (Adamson) (Figure 1). This legume 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).

Figure 1. *Virgilia divaricata* seedlings (a), plants at 22 days of growth (b) and determinate nodules attached to the root system (c).

2. Materials and methods

2.1 Seed germination, bacterial inoculation and growth

Virgilia divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were germinated as described in Vardien et al. (2014). Following the initial leaf emergence (Figure 1 a), seedlings were transferred to and inoculated with a locally sourced strain of *Burkholderia*. Inoculum was prepared as previously documented (Vardien et al. 2014). Three treatment categories, based on P concentration, were used: low P, high P (control), and resupplied P (four weeks of low P followed by three weeks of high P). All plants were supplied with 100 mL of a quarter strength Long Ashton nutrient solution twice a week. The nutrient solution was adjusted 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 until they were harvested.

2.2 Nutrient analysis

A subset of the harvested material was dried at 50 ˚C for 72 h and the dry weights (dw) recorded. The material was milled and analysed for their C, N and P concentrations according to previously established protocols in Vardien et al. 2014.

2.3 Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} dw d^{-1}) and P utilization rate (SPUR) (g dw mg^{-1} P d^{-1}) of plant organs were calculated according to Nielson et al., 2001. These equations were however modified to include nodules instead of roots for SPAR and whole plants for SPUR, in accordance with previous work by Vardien et al. (2014).

Construction costs, C_w (mmol C/ g dw) were determined according to Mortimer et al. (2005), adjusted from the equation 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 assimilated 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.

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

The $\delta^{15}N$ analyses were carried out as described in Vardien et al. (2014). The values obtained were subsequently used to determine the percentage N derived from the atmosphere (NDFA) (Vardien et al. 2014).

2.5 *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 5 mm 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 using the areas of their resonance peaks.

2.6 Extracellular acid phosphatase assay

To determine nodule surface acid phosphatase activity, whole nodules (approximately 3 g fresh weight [fw]) were washed in distilled water, blotted dry and placed into 5ml of incubation medium with substrate mixture (6 mM para-nitrophenylphosphate (pNPP) and 1 mM dithiothreitol (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 1M of NaOH to stop the reaction. Absorbance was then determined at 410 nm and enzyme activity expressed as µmol para-nitrophenyl (pNP) min^{-1} g^{-1} FW (Ciereszko et al. 2002).

2.7 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 ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM thiourea, and 1% (w/v) insoluble PVPP (Hurley et al. 2010). Homogenates were centrifuged at 4 °C at 14,000 g for 5 min, and the supernatants reserved as clarified extract. Protein extracts were quantified with the Bradford method using bovine serum albumin (BSA) as the standard.

APase activity was measured by coupling the hydrolysis of phosphoenolpyruvate (PEP) to pyruvate to the lactate dehydrogenase (LDH) reaction and monitoring nicotinamide adenine

dinucleotide (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 continuously, spectrophotometrically at 340 nm and 25 °C for a total of 5 min.

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 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 p-NP 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 phosphorous 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}.

2.8 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

variances, the means were separated using the post-hoc Tukey's LSD multiple range test (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$).

3. Results

3.1 Plant growth and biomass

Total plant biomass accumulation was significantly reduced in P deficient plants compared to the control and previously starved plants that were supplied with sufficient P (Figure 2) owing to decreased shoot, root and nodule growth (Figure 2). Plants grown under P deficient

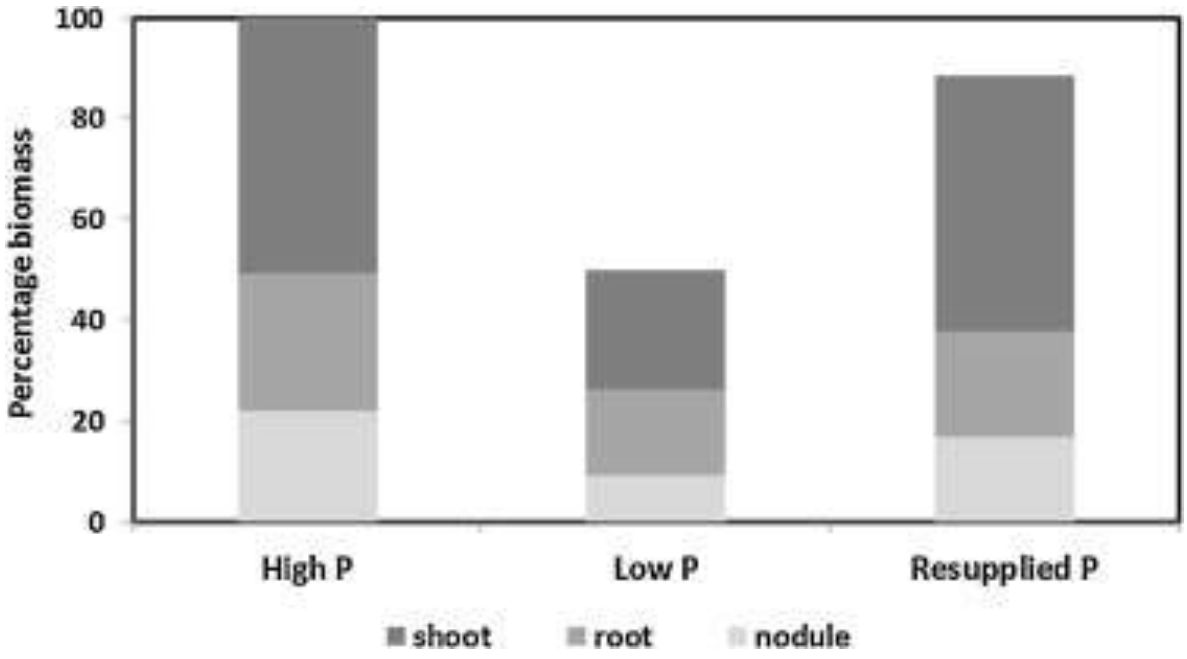

Figure 2. Percentage biomass of *Virgilia divarica*ta (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. High P was used as a standard (for optimal growth conditions) and percentages for Low P and Resupplied P indicate differences in mass on a percentage basis from the standard. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

conditions produced less than half the amount of nodules that was produced under P sufficient conditions but maintained a higher root: shoot ratio, a morphological response characteristic of low P exposure (Figure 3).

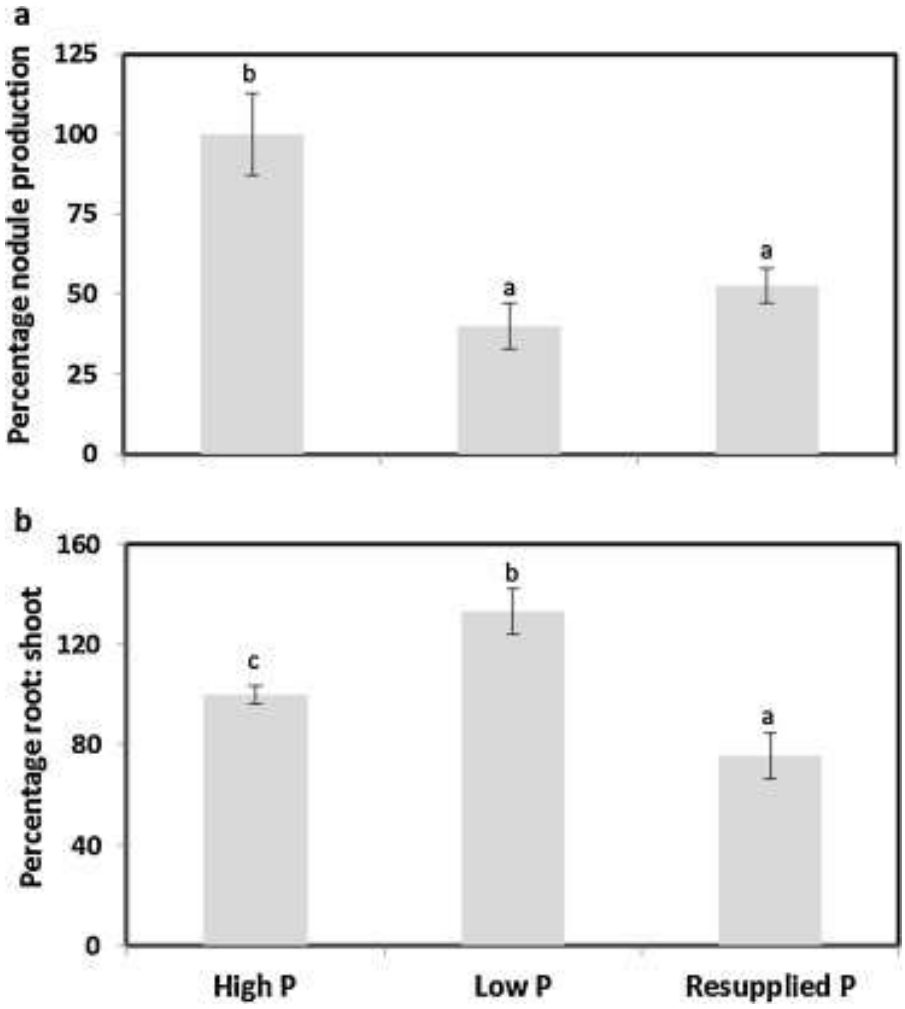

Figure 3. Percentage nodule production (a) and root: shoot ratio (b) of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. High P was used as a standard (for optimal growth conditions) and percentages for Low P and Resupplied P indicate differences from the standard. Percentages are presented as means (n = 3) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

3.2 Mineral nutrition and fixation efficiency

The concentration of N and P in P sufficient nodules was higher with values ranging between 3.6 – 4.5 mmol g^{-1} dw (Figure 4). The decline in P concentration in low P nodules was accompanied by a decrease in the net P absorption rate as well as P uptake by these nodules (Figure 4). The net P absorption rate of resupplied plants was parallel to that of P sufficient plants (Figure 4).

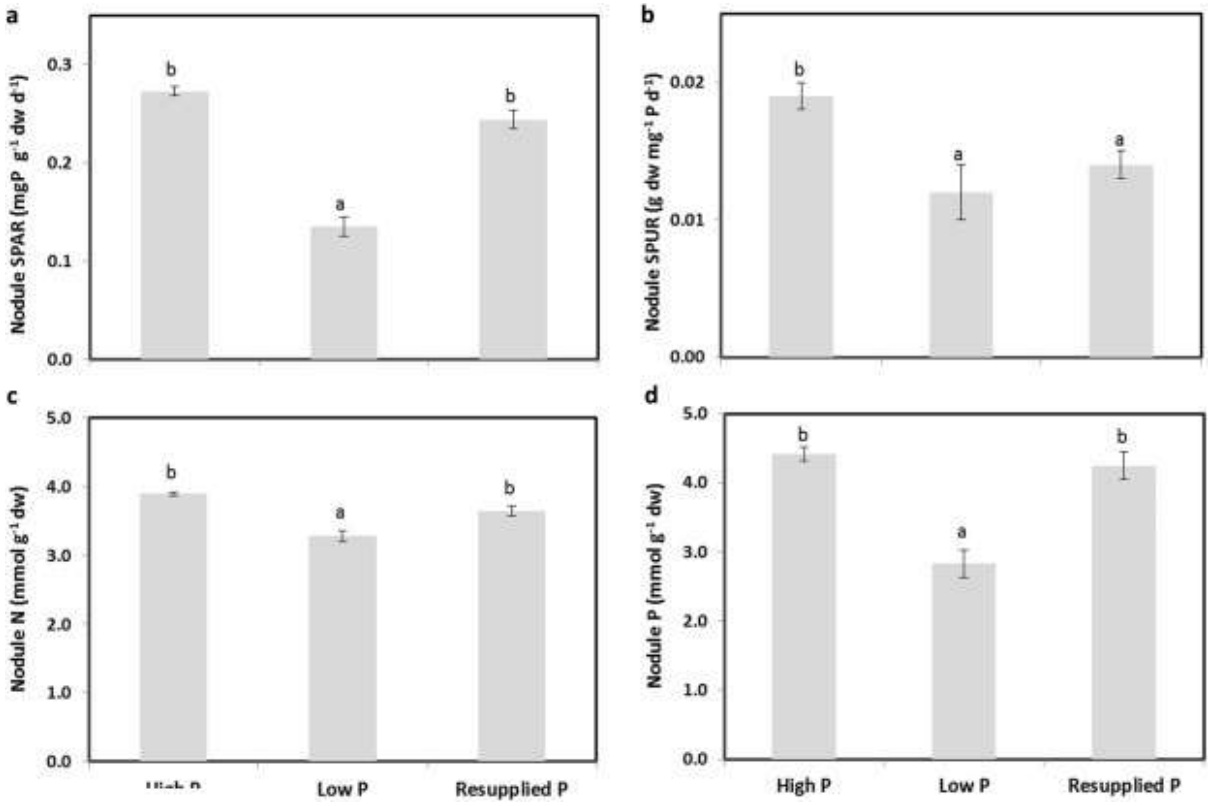

Figure 4. Specific phosphorus acquisition rate (a), specific phosphorus utilisation rate (b), nitrogen concentration (c) and phosphorus concentration (d) of *Virgilia divaricata* (Adamson, Fabaceae) nodules grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions.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

Nodules of control plants derived almost 20% more N from the atmosphere compared to low P plants. However, on a nodule-mass basis, low P plants as well as resupplied P plants were much more efficient at fixing N_2 (Figure 5). No significant difference in nodule construction costs was found between treatments (Figure 5).

3.3 Phosphorus fractions

Peak areas of compounds from NMR spectra (see Figure 6 for representative spectrum) were used to derive relative amounts of compounds. All nucleotide (adenosine triphosphate (ATP), adenosine diphosphate (ADP) and Uridyl diphosphoglucose (UDPG)) levels in P sufficient nodules were higher compared to P-deficient plants (Figure 7). This decline in ADP and ATP

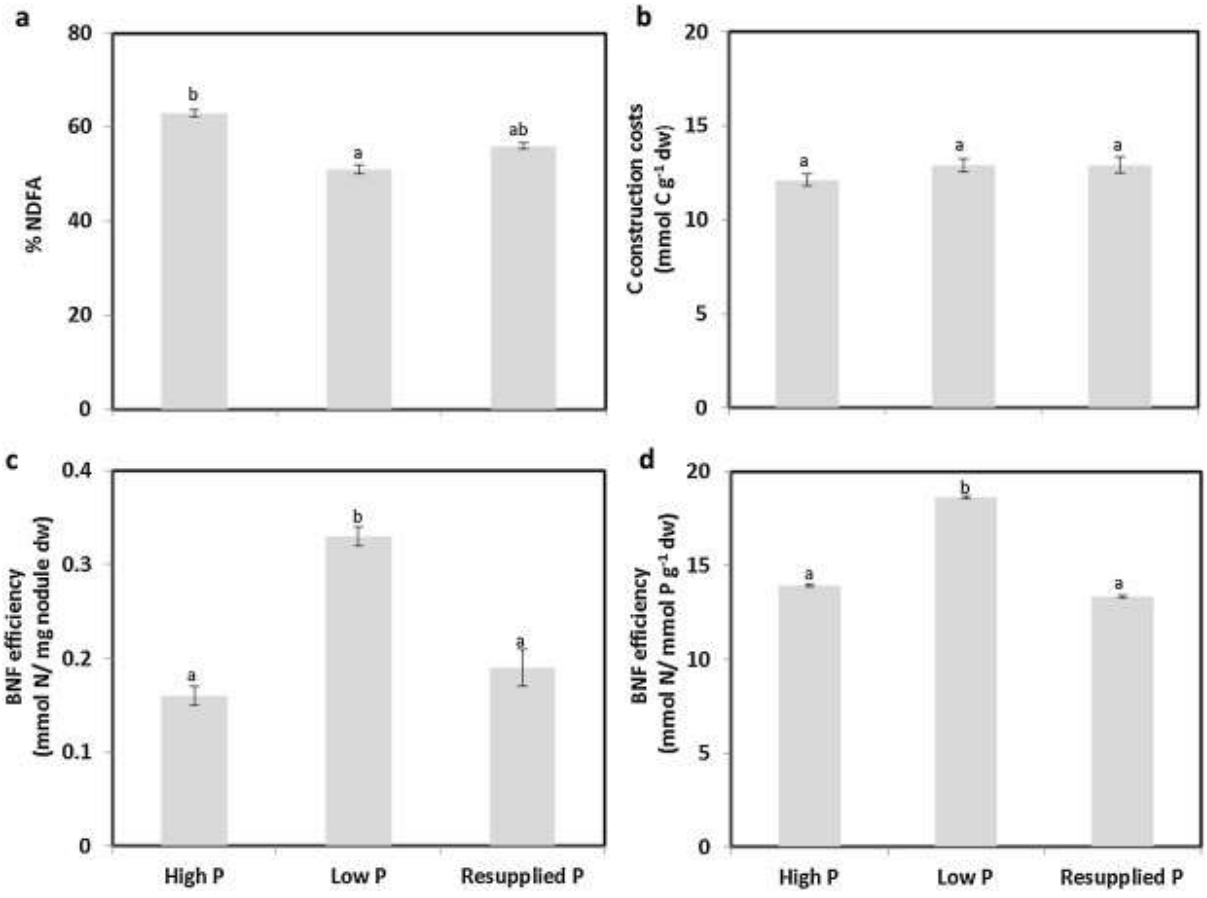

Figure 5. Percentage nitrogen derived from the atmosphere (% NDFA) for whole plants (a), nodule carbon construction costs (b) biological nitrogen fixation (BNF) efficiency on a nodule mass basis (c) and BNF efficiency on a P concentration basis (d), for *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. Values are presented as means (n = 4) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

corresponds with lower levels of Pi in LP nodules (Figure 7). The ADP: ATP ratio increased significantly in LP nodules (Table 1). Most of the sugar phosphate levels (gluconate 6-phosphate (Glcn6P), glucose 6-phosphate (Glc6P) and phosphoglycerate (PGA)) were not significantly different between treatments with the exception of fructose 6-phosphate (Fru6P) which increased in low P nodules (Table 1). An increase in the phospholipid, phospho-choline (P-cho) was observed under high P (Table 1).

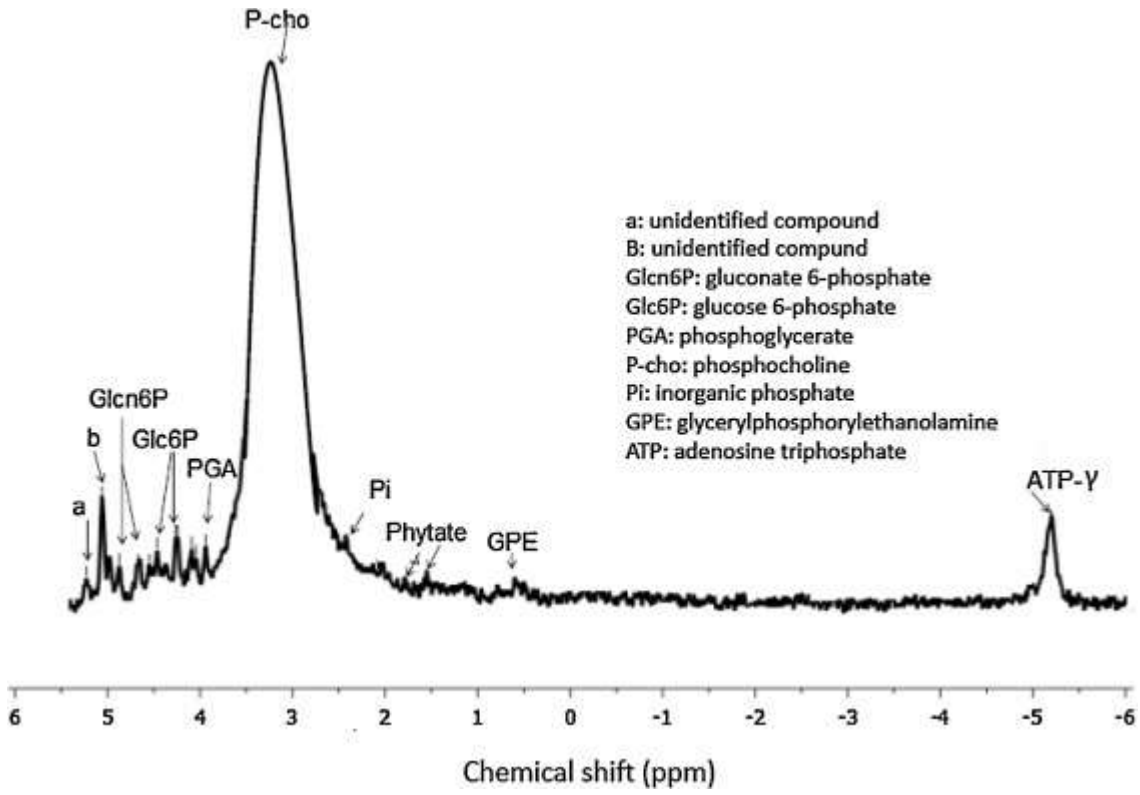

Figure 6. Representative NMR spectrum of nodules of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P) phosphorus conditions. Peak areas of spectra were used to derive relative amounts of P compounds, represented in Figure 6 and Table 1.

Table 1. Phosphorus metabolites determined from *Virgilia divaricata* (Adamson, Fabaceae) nodule extracts. Values are expressed as 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-metabolites	**Biochemical group**	**High P**	**Low P**	**Resupplied P**
(μmol × g^{-1} fw)		**500 μM**	**5 μM**	**5 μM to 500 μM**
Glcn6P	Sugar phosphate	0.300 ± 0.009a	0.29 ± 0.114a	0.33 ± 0.089a
Glc6P	Sugar phosphate	0.160 ± 0.01a	0.12 ± 0.047a	0.14 ± 0.039a
PGA	Sugar phosphate	0.110 ± 0.005a	0.08 ± 0.03a	0.15 ± 0.05a
Fru6P	Sugar phosphate	0.250 ± 0.009a	0.35 ± 0.137b	0.32 ± 0.073b
P-cho	Phospholipid	0.240 ± 0.011b	0.14 ± 0.051a	0.13 ± 0.033a
Ratios:				
ADP: ATP	Nucleotide: Nucleotide	1.400 ± 0.000a	2.50 ± 0.001b	1.60 ± 0.001a
Pi: ATP	Inorganic phosphate: Nucleotide	1.240 ± 0.001a	1.16 ± 0.000a	1.62 ± 0.001b

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

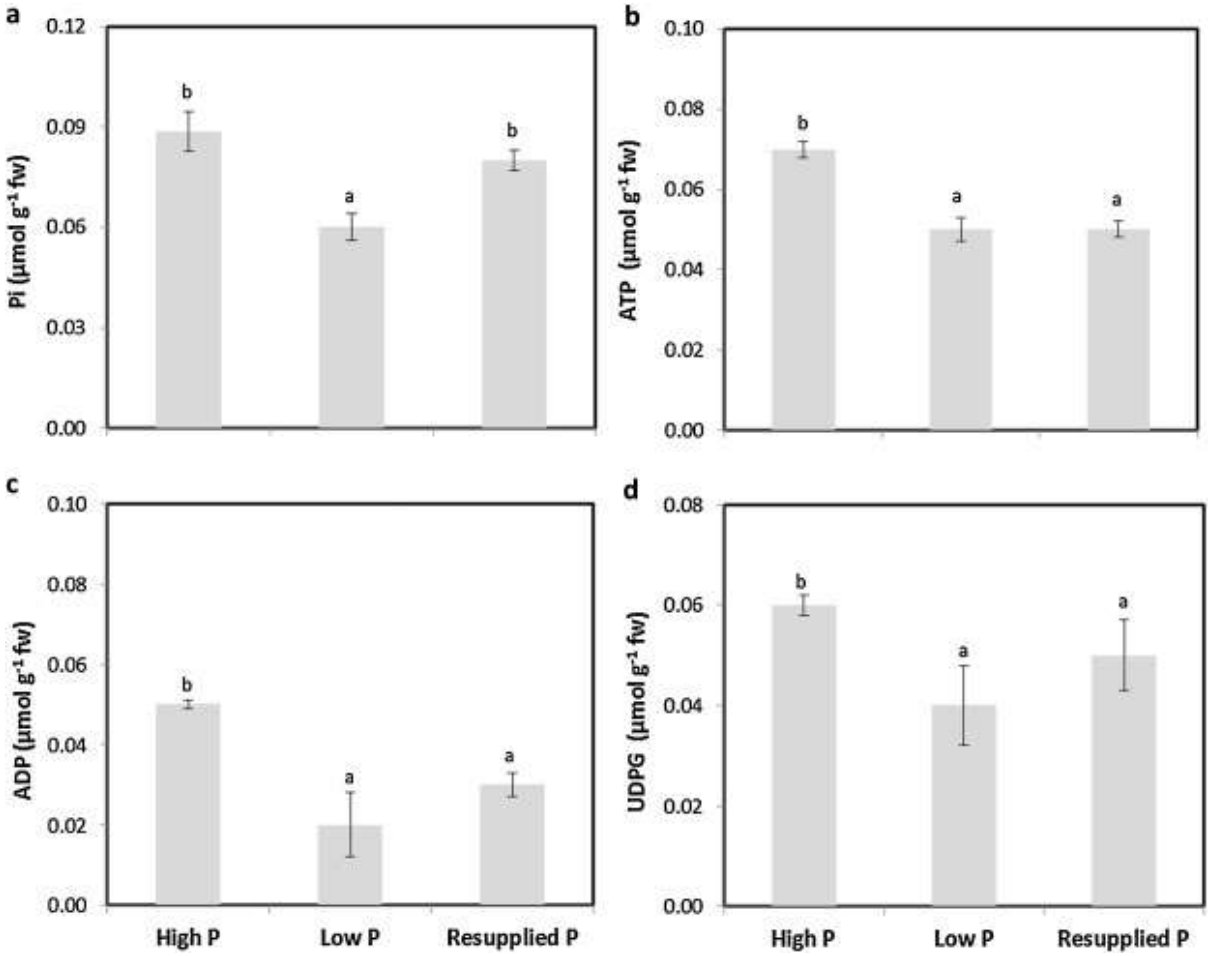

Figure 7. Pi (a), and adenylate- ATP (b), ADP (c), and UDPG (d) levels in nodules of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. Values are presented as means (n = 3) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

3.4 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 was quantified from nodule surfaces because although it is produced and secreted largely by roots, it also attaches to nodules to P acquisition. Nodule extracellular APase activity significantly increased in P deficient plants (Figure 8). The data obtained for the PEP- and pNP- based intracellular APase assays were consistent with extracellular APase assays, showing an increase in these

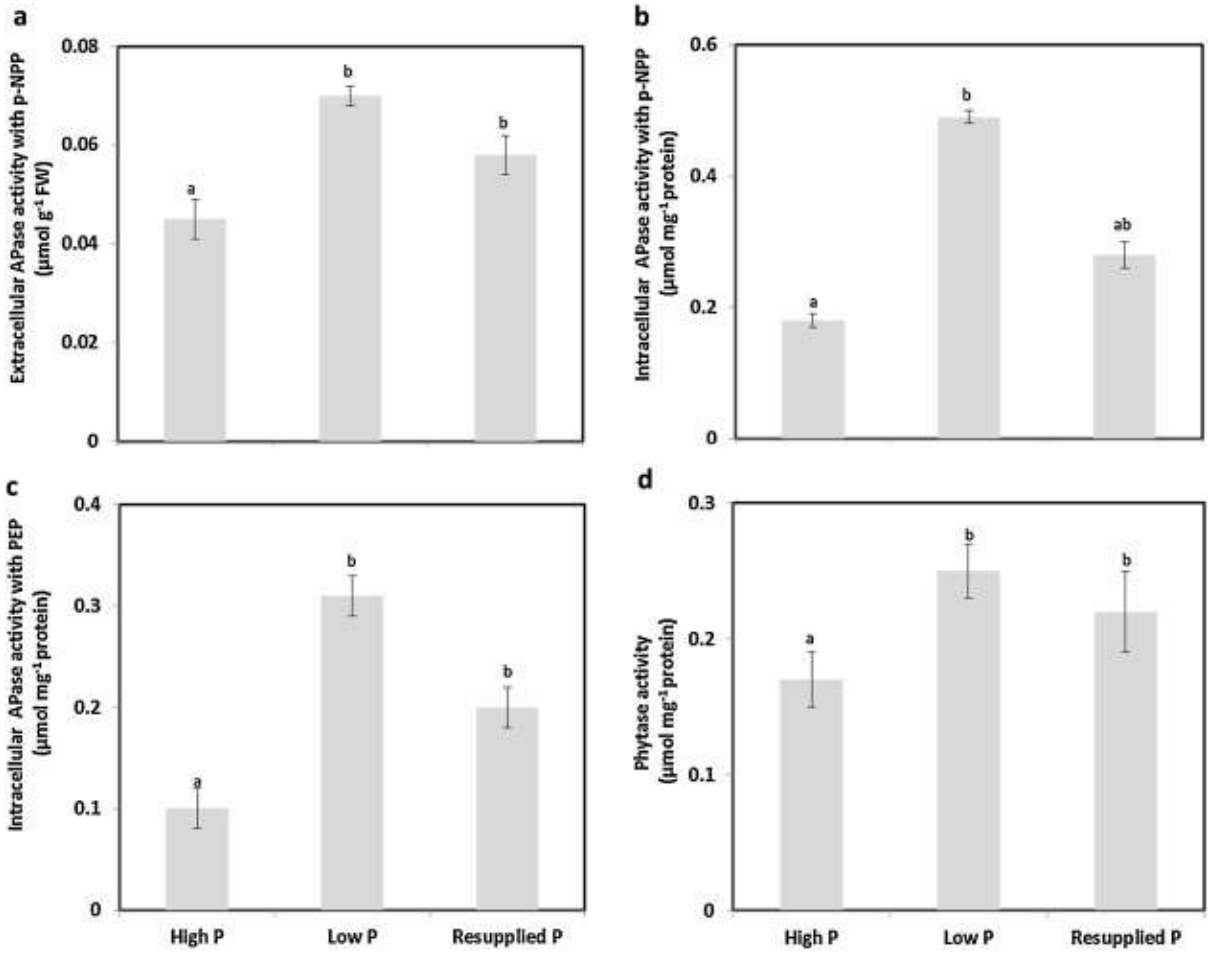

Figure 8. Extracellular acid phosphatase (APase) (a), intracellular APase (b, c), and phytase enzyme activity in *Virgilia divaricata* (Adamson, Fabaceae) nodules grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. Values are presented as means (n = 7) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

enzymes under P stressed conditions and recovery with resupplied P (Figure 8). Phytase activity was also shown to be important during internal P recycling, where low P and resupplied P nodules had higher activities (Figure 8). Although the intracellular APase levels were far greater than extracellular APase levels, the ratios of extracellular to intracellular APase activities (expressed on fresh weight basis) showed a distinct pattern (Figure 9). In this regard the ratios of extracellular to intracellular APase activity was the highest for HP plants,

then declined in LP plants and subsequently showed a recovery towards HP levels in P resupplied nodules (Figure 9).

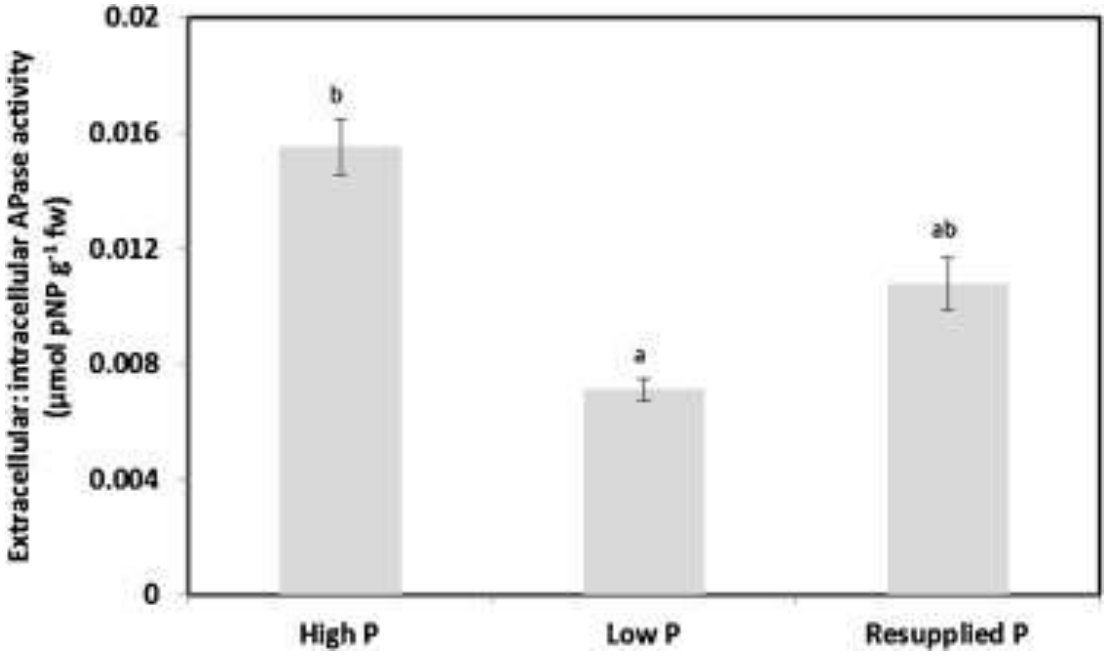

Figure 9. The ratio of extracellular to intracellular acid phosphatase enzyme activity in *Virgilia divaricata* (Adamson, Fabaceae) nodules grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. Values are presented as means (n = 7) with standard error bars. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

4. Discussion

During variable P supply, *V. divaricata* nodules display flexible internal P recycling and metabolism, which may prevent excessive changes in BNF during P deficiency and P resupply. This is the first report on the plasticity of the biochemical and physiological mechanisms of P recycling in legume nodules. The implications of these adaptations are discussed in relation to N acquisition during fluctuations in soil P concentration.

The decline in *V. divaricata* biomass during P deficiency and its subsequent recovery upon short-term P resupply is a flexible response of allocation, as previously found for this species (Vardien et al. 2014). The decreased nodule biomass, in of the spite higher below ground

investment in relative root biomass during P stress, appears to be a typical response to P-deficiency in legumes (Vadez et al. 1997, Le Roux et al. 2006). This may be attributed to the lower P utilisation efficiency for biomass production, which was evident in the P deficient and P resupply nodules. It would appear that the growth resources are preferentially allocated increasing the root: shoot ratio, rather than nodule growth. Regardless of the smaller biomass under low P supply, the nodules maintained a high efficiency of function for acquiring N via BNF.

This high N_2 fixing efficiency during P stress was even maintained during decreases in the levels of adenylates and metabolic P*i* inside nodules. Following nutritional P starvation, *Virgilia divaricata* nodules had decline in metabolic P*i* and adenylate levels within the nodules. A reduction in nodule 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 P*i* and adenylate pools, which accompany P*i*- starvation are functionally important. Reduced levels of ADP and P*i*, and increased ADP: ATP ratios have been reported to result in restricted electron flow in the Cytochrome pathway (Bryce et al. 1990). In order to minimize the effects of P deficiency on BNF during low P supply (Tang et al. 2001), the nodules require very flexible mechanisms maintaining the functional P homeostasis during variable P supply. Although the mechanisms that underlie this high efficiency of function are not currently known in the nodules of this Fynbos legume, they may include strategies which increase the uptake, conservation and recycling of P, as found in other non-nodule plant organs (Vance *et al*. 2003).

In this study the activities of mechanisms such as phosphohydrolase enzymes, recycling of membrane phospholipids and the use of alternate biochemical pathways, are integrated to enable the internal recycling and conservation of P. These mechanisms also appear to be

highly flexible during the variations in P supply. The enhanced efficiency of N_2 fixation in low P nodules appears to have been underpinned by very flexible P recycling and internal P conservation mechanisms, rather than enhanced mechanisms aimed at acquiring external P. The nodule's internal recycling of organic P was evident in the increase in intracellular phosphohydrolases and changes in the membrane lipid composition.

Firstly, the increase in the internal recycling and alternative metabolic bypass mechanisms of the nodule, concur with decline of external mechanisms of P uptake and extracellular recycling. This shift is quite flexible and may contribute to the success of the species' nodules to continue to fix N_2 in variable P soils. During P deficiency the decline in the nodule's specific acquisition rate of P (SPAR), corresponds with the relative decreased reliance on extracellular APase activities compared to intracellular APase. Although the extracellular APase activities in nodules are known to be higher during P deficiency (Maistry *et al*. 2015 a,b, Tang et al. 2013), the relative contribution is much less compared to intracellular APase under P stress. Furthermore, the increased intracellular and extracellular phosphohydrolase (APase and phytase) activity, would serve as an additional route of P-acquisition from organically-bound sources of P, during conditions of limiting soil P. 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 intracellular 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).

Secondly, the internal recycling of P in the nodule can also be mediated via phospholipid utilisation of the cell membranes and intracellular phosphohydrolases. The replacement of

membrane phospholipids, in order to release P*i* to the cell, has been found for P stressed leaves (Lambers *et al*. 2012), where the decline in membrane phospholipids were associated with an increase in sulpholipids. Although we do not have any evidence for the increased presence of sulpholipids, the decline in membrane phospholipids in P stressed nodules does allude to this possibility.

Thirdly, P conservation mechanisms can then utilize alternate means of mitochondrial respiration to conserve the internal pool of acquired P*i* (Johnson et al. 1996, Rychter et al. 1992, 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 P*i*- or ATP- requiring steps of glycolysis under P*i* 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 (PP*i*) concentrations appear to be elevated during P*i* stress (Duff et al. 1989), thereby suggesting that PP*i* can act as an energy donor and aid in conserving limited ATP. An alternate glycolytic pathway catalysed by PP*i*-dependent phosphofructokinase (PFP) under P deficiency has been documented, in which PP*i*-dependant PFP bypasses the ATP-dependent phosphofructokinase (PFK), generating fructose 1,6-biphosphate (Theodorou and Plaxton 1996; Plaxton and Carswell 1999). Although PP*i* was not determined in this study, the elevated levels of Fru6P and UDP-Glucose under low P, may be indicative of the potential of this pathway being engaged.

This study shows the functional plasticity of internal recycling and conservation of P inside the nodules of a legume from a nutrient-poor ecosystem, to support the great requirement for P for BNF (Vadez et al. 1997, Al-Niemi et al. 1998). The functional benefits of such physiological plasticity, is that these plants would be able to respond to a changing soil environment, where the P is known to be heterogeneously distributed. In conclusion, these

findings indicate that *V. divaricata* is well adapted to acquire N under various conditions of P availability and contributes to our understanding of legumes in nutrient poor regions.

5. Acknowledgements

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