

Variable P supply affect N metabolism in a legume tree, *Virgilia divaricata*, from nutrient-poor Mediterranean-type ecosystems

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Abstract

Virgilia divaricata is an indigenous forest margin legume growing in nutrient richer soils, but it is also known to invade the N and P poorer soils of the mature fynbos. This implies that the legume has a functional tolerance for variable soil N and P levels. It is not known how the legume utilizes inorganic N from soil and atmospheric sources under variable P supply. Moreover, very little is known about how P deficiency affects root nodule metabolic functioning of *V. divaricata* and their associated energy costs of N assimilation. Therefore the aim of this study was to determine whether the P deficiency affects the metabolic status of root and nodules and the consequent impact on the routes of N assimilation in a Fynbos legume, *V. divaricata*. Our results show that *V. divaricata* had a reduced biomass, plant P concentration and BNF during P deficiency. Based on the adenylates data, P stressed nodules maintained their P status better than P stressed roots. Furthermore *V. divaricata* was able to alter C and N metabolism in different ways in roots and nodules, in response to P stress. For both roots and nodules, this was achieved via internal cycling of P, by possible replacement of membrane phospholipids with sulpholipids and galactolipids and increased reliance on the P_i-dependant metabolism of sucrose via UDPG and to Fru-6-P. P stressed roots exported mostly ureides as organic N and recycled amino acids via deamination glutamate dehydrogenase (GDH). In contrast, P stressed nodules largely exported amino acids. Compared to roots, the nodules showed a greater degree of P conservation during low P supply, this resulted in the roots and nodules of *V. divaricata*, metabolising N differently during P stress, this meaning that these organs may contribute differently to the success of this plant in soils ranging from forest to fynbos.

Keywords

Fynbos, P deficiency, N₂ fixation, N assimilation, amino acids, ureides.

Abbreviations:

%Ndfa, nitrogen derived from atmosphere; BNF, biological nitrogen fixation; Pi, inorganic P; GlcN6P, gluconate 6-phosphate; Glc-6-P, glucose 6-phosphate; PGA, phosphoglycerate; Fru-6-P, fructose 6-phosphate; P-cho, phospho-choline; ATP, adenosine triphosphate; ADP, adenosine diphosphate; UDPG, Uridyl diphosphoglucose; NR, nitrate reductase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase/ glutamate 2-oxoglutarate aminotransferase; XDH, xanthine dehydrogenase.

Introduction

In the Cape Floristic Region (CFR) of South Africa, amongst the plant families indigenous to the Cape fynbos, Leguminosae is one of the most species-rich (Goldblatt and Manning 2000). Nevertheless it has been reported that legumes are mostly absent or rare in the mature Cape fynbos (Hoffmann *et al.* 1987). Since the Cape fynbos is adapted to regular fires to maintain both the ideal microclimate for the vegetation and to disrupt the nutrient cycle associated with tree dominated vegetation (Manders *et al.* 1992; Coetsee and Wigely 2013). Cock and Stock (2001) suggested that post-fire changes in the soil nutrient dynamics could be one of the most important factors limiting legume abundance in the mature fynbos. As Cape fynbos legumes are considered to be short lived, post fire colonizers in the ecosystem due to a temporary flush of nutrient availability, specifically P and N, which may sustain legumes until it is exhausted (Brown and Mitchell 1986). Furthermore, the Cape fynbos soils bear resemblance to the soils of the Western Australian heathlands (Groves 1983; Groom and Lamont, 2015), characterized by leached, acidic sandy soils associated with low nutrient concentrations, specifically with regards to P and N (Wisheu *et al.* 2000; Herppich *et al.* 2002).

Cape fynbos soils contain about 58%-77% organic P (Straker 1996), most is unavailable to plants due to complexation with cations (Ca, Fe), under acidic conditions P ions can easily precipitate with cations (Dakora and Phillips 2002) and is also bound to organic compounds by microbial action (Vance *et al.* 2003). Therefore P is

generally present in micro molar (μM) concentrations or less for plant use in the fynbos soils (Maseko and Dakora 2013). The low P concentrations of the fynbos soils are extremely low to drive the P-requiring metabolic processes.

Soil P availability is one of the most limiting factors for legumes that symbiotically fix atmospheric N_2 in association with rhizobia (Vance *et al.* 2003). Therefore P deficiency forms a critical constraint for plants, particularly legume plants, as P has a key role in the energy metabolism during symbiotic N_2 fixation (Dilworth 1974; Maseko and Dakora 2013; Sulieman *et al.* 2013). Rhizobial N_2 fixation takes place in the root nodules formed during the symbiotic interaction (Gordon *et al.* 2001). It can be predicted that P deficiency will have a negative impact on the energy status of legume root nodules. Nevertheless, *Virgilia divaricata* is reported to invade the mature fynbos soils even in the absence of fire and efficiently fix N_2 in these P deficient soils (Coetsee and Wigely 2013; Magadlela *et al.* 2014; Vardien *et al.* 2014). Even more, *V. divaricata* has been described as a forest precursor and it enhances fynbos soils fertility (Coetsee and Wigely 2013). *V. divaricata* (Adamson), closely related to *V. oroboides* (P.J. Bergius) Salter, indigenous to the CFR, are confined to the southwestern and southern coastal regions (Greinwald *et al.* 1989). Therefore it is important to better understand the effects of P deficiency on the root nodule function of *V. divaricata*, associated to the energy status during ammonium assimilation.

In roots and nodules, some of the N derived from N_2 fixation and assimilated from the soil generates ammonium, which is reduced via the glutamine synthetase (GS) and NADH-dependent glutamate synthase (NADH-GOGAT) cycle (Lea and Mifflin 1974; Olivera *et al.* 2004). This process and the subsequent conversion into amino acids (glutamine and asparagine) is an energy-consuming processes (Olivera *et al.* 2004).

The glutamate made during the GS-GOGAT pathway could be incorporated into aspartate amino-transferase (AAT) or into ureides by xanthine dehydrogenase (XDH) and uricase activities (Hank *et al.* 1981).

Furthermore early literature also indicated that mineral nutrient deficiencies might have induced alternate pathways, stimulating large increases in asparagine concentration (Stewart and Larher 1980). In parallel, Sieciechowicz *et al.* (1988) and Lea *et al.* (2007) identified that nitrogen is often diverted from glutamine to

asparagine during periods of a wide range of stress conditions. Almeida *et al.* (2000) also found higher asparagine concentration in the roots and nodules of white clover (*Trifolium repens* L.), inducing a N feedback mechanism, affecting nodulation and proportion of N derived from the symbiotic N₂ fixation during P deficiency. This meaning that aspartate aminotransferase (AAT) plays a very important role in nitrogen and carbon metabolism. AAT catalyses the formation of 2-oxoglutarate and aspartate via reversible amino group transfer from glutamate to oxaloacetate (Givan 1980; Silvente *et al.* 2003). Several functions have been attributed to AAT, these the assimilation of fixed nitrogen into asparagine in amide exporting nodules (Farnham *et al.* 1990), the catabolism and biosynthesis of aspartate (Bryan 1980) and the conversion of tricarboxylic acid cycle intermediates to amino acids (Ryan and Fottrell 1974). This enzyme is also supposed to control the redistribution of nitrogen and carbon pools between plant cell cytoplasm and other compartments, and between microbial symbionts and host cytoplasm as proposed in a model involving a metabolic shuttle system, such as a malate-aspartate shuttle (Appels and Haaker 1991; Wallsgrove *et al.* 1983). Glutamate dehydrogenase (GDH) had been originally regarded as the main ammonium assimilatory enzyme (Skopelitis *et al.* 2006).

Lea and Miñin (1974) however demonstrated that the GS/GOGAT cycle was the major route for ammonium assimilation in plants. The enzyme GDH catalyzes the reductive amination of 2-oxoglutarate and the reverse catabolic reaction of the oxidative deamination of glutamate (Lehmann *et al.* 2011). It has been reported that ammonium assimilation could be attributed to GDH during salt stress conditions (Skopelitis *et al.* 2006). Furthermore GDH was up regulated in response to elevated ammonium levels, this suggesting that GDH maybe important in the detoxification of ammonium by assimilating some of the excess ammonium ions (Tercé-Laforgue *et al.* 2004a, b). This might suggest that these N metabolizing enzymes might play functional role in fynbos legumes during P deficiency, specifically *V. divaricata*.

Although some work has been done on how P deficiency affects plant biomass, carbon costs and symbioses of Cape fynbos legumes, very little is known about how P deficiency affects root nodule metabolic functioning of these legumes and their associated energy costs of N assimilation. Therefore the aim of this study was to determine whether the P deficiency affects the metabolic status of root and nodules

and the consequent impact on the routes of N assimilation in a Fynbos legume, *V. divaricata*.

Materials and methods

Plant material and growth conditions

Seeds of *V. divaricata* were obtained from (Silverhill Seeds, Kenilworth, Cape Town, South Africa). Seeds were scarified using an acid scarification method that entailed soaking the seeds in 95-99% Sulphuric acid (H₂SO₄) for 30 minutes and then rinsing them 10 times in distilled water (Magadlela *et al.* 2014). Hereafter seeds were treated overnight at room temperature with diluted smoke water, which was obtained from Kirstenbosch Botanical Gardens, Claremont, Cape Town, South Africa. The seeds were germinated in sterile sand. Seeds were germinated and grown under the conditions in the glasshouse of the Department of Botany and Zoology, University of Stellenbosch. The range of midday irradiances was between 600-800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and the average night/day temperatures were 15-25 °C. After seedling emergence, they were transferred to pots with sterile sand. Supplied with distilled water for a week to acclimatize then inoculated with effective *Burkholderia sp.* Thereafter seedlings were treated with quarter strength Long Ashton nutrient solution (pH 5.8), modified to 500 μM P (HP) and 5 μM P (LP) as NaH₂PO₄.2H₂O and 500 μM NH₄NO₃.

Harvesting occurred at 80 days after seedling were transplanted to pots. Upon harvesting, the plants were kept chilled and separated into nodules, roots, stems and leaves. The nodules and roots were frozen with liquid nitrogen and store at -80°C for biochemical analysis. The remaining plant material was placed in a drying oven, at 50°C for a week, and their dry weights (DW) were recorded. The dried material was ground with a tissue-lyser (Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa). The ground samples were analysed for their respective N and P concentrations by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (Central Analytical Facilities, Stellenbosch University and at the Archeometry Department, University of Cape Town, Cape Town, South Africa).

Measurements of nitrogen metabolizing enzyme activities

Crude enzyme extraction was done following the methods of El-Shora and Ali (2011), where -80°C stored fresh plant organs (roots and nodules) were ground in liquid N in a prechilled mortar and pestle in a 50mM KH₂PO₄ buffer, pH 7.5, containing 2mM EDTA, 1.5% (w/v) soluble casein, 2mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpyrrolidone (PVP). Complete Protease Inhibitor Cocktail tablet (Roche) was dissolved in the buffer. The homogenate was then centrifuged at 3,000g for 5min at 4°C, thereafter the supernatant was centrifuged at 30,000g for 20min at 4°C. The resulting supernatant was used to measure enzyme activities (nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AAT) and both aminating and deaminating glutamate dehydrogenase (GDH). The protein concentration was determined by the method of Bradford (1967), using protein assay reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard.

NR activities were measured following the methods of El-Shora and Ali (2011). NR activities were assayed in a reaction medium containing 100mM KH₂PO₄ buffer, pH 7.5, 100mM KNO₃, 2mM NADH and root or nodule extract, then incubated at 30°C for 30min. Thereafter the reaction was stopped by the addition of 1% (w/v) of sulphanilamide in 1.5M HCl and 0,02% (w/v) of n-l-naphthyl-ethylenediamine dihydrochloride solution. All samples centrifuged at 500g for 5min to remove any suspended matter and activities measured spectrophotometrically, monitoring the absorbance at 540nm.

GS activities were assayed following the methods of Kaizer and Lewis (1986), in a reaction medium containing 250µl of imidazole (pH7.2), 20mM MgSO₄, 25mM hydroxylamine, 100mM glutamate, 10mM ATP and crude enzyme extract. Thereafter the reaction was incubated and stopped. After all samples were centrifuged, glutamyl hydroxamate was determined by measuring absorbance at 500nm.

GOGAT activities were assayed spectrophotometrically, monitoring the oxidation of NADH at 340nm at 30°C for 15min according to Groat and Vance (1981). The assay medium consisted of 1mM Na₂-EDTA, 25mM 2-oxoglutarate, 10mM aminooxyacetate, 0.15mM NADH, 10mM L-glutamate and 0,1% (v/v) 2-mercaptoethanol in 50mM potassium phosphate buffer, pH 7.5.

AAT activities were determined according to González *et al.* (1995) in a reaction medium containing 4mM MgCl₂, 10mM aspartic acid, 0.2mM NADH, 1mM 2-oxoglutarate and 50mM Tris-HCl buffer, pH 8.0.

Aspartate GDH activities were assayed according to Glevarec *et al.* (2004) by following the oxidation of NADH at 340nm. The reaction mixture contained Tris-HCl (100 mM, pH 8), 1mM CaCl₂, 13mM 2-oxoglutarate, 50mM (NH₄)₂SO₄ and 0.25mM NADH. Deaminating-GDH activity was assayed by following the reduction of NAD. The reaction mixture contained Tris-HCl buffer (100mM, pH 9) supplemented with 1mM CaCl₂ (pH 9), 33mM Glu and 0.25mM NAD or NADP.

For activities of Xanthine dehydrogenase (XDH) and uricase the extraction medium consisted of 25mM TES-KOH buffer, pH7.5, containing 0.15M sorbitol and the macerates were centrifuged at 3500g for 15min. XDH and uricase activities were determined following the procedure of Schubert (1981). For the XDH activity the reaction medium consisted of 3.5mM NAD⁺, and 0.5mM hypoxanthine in 50mM TES-KOH buffer, pH 8.4. For uricase assay, the reaction medium consisted of 50mM uric acid in 85mM glycine-KOH buffer pH 9.0.

Inorganic phosphate

A frozen sample (about 0.5g) was homogenized in 1ml of 10% (w/v) PCA, using an ice-cold mortar and pestle. The homogenate was then diluted 10 times with 5% (w/v) PCA and placed on ice for 30 min. After centrifugation at 10,000g for 10 min at 4°C, the supernatant was used to measure the Pi, using the molybdate-blue method: 0.4% (w/v) ammonium molybdate melted in 0.5M H₂SO₄ (solution A) was mixed with 10% ascorbic acid (solution B) (A:B = 6:1). Two milliliters of this solution was added to 1 ml of the sample solution, and incubated in a water bath at 40°C for 20 min. After being cooled in ice, the absorbance was measured at 820nm wavelength (Nanamori *et al.*, 2004).

Nuclear Magnetic Resonance (NMR) Measurements

Perchloric acid extraction and preparation was done according to Gout *et al.* (2000). For perchloric acid extraction, cells (9g wet weight) were quickly frozen in liquid nitrogen and ground to fine powder with a mortar and pestle with 1mL of 70% (v/v)

perchloric acid. The frozen powder was then placed at -10°C and thawed. The thick suspension thus obtained was centrifuged at 15,000g for 10 min to remove particulate matter, and the supernatant was neutralized with 2M KHCO_3 to approximately pH 5.0. The supernatant was then centrifuged at 10,000g for 10min to remove KClO_4 , and the resulting supernatant was lyophilized and stored in liquid nitrogen. This freeze-dried material containing non-volatile compounds was redissolved in 2.5mL of water containing 10% (v/v) $^2\text{H}_2\text{O}$, neutralized to pH 7.5 and buffered with 50mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES). Divalent cations (particularly Mn^{2+} and Mg^{2+}) were chelated by the addition of sufficient amounts of 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) ranging from 50 to 100 μmol depending on the samples. The elimination of paramagnetic cations is a pre- requisite for obtaining sharp resonance signals.

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. 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 concentrations of identified compounds were determined by the areas of their resonance peaks

Calculations of %NDFA

The $\delta^{15}\text{N}$ analyses were carried out at the Archeometry Department, University of Cape Town, South Africa. The isotopic ratio of $\delta^{15}\text{N}$ was calculated as $\delta=1000\%$ ($R_{\text{sample}}/R_{\text{standard}}$), where R is the molar ratio of the heavier to the lighter isotope of the samples and standards are as defined by Farquhar *et al.* (1989). Between 2.100 and 2.200 mg of each milled sample were weighed into 8 mm x 5 mm tin capsules (Elemental Micro-analysis Ltd., Devon, UK) 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 $\delta^{15}\text{N}$ 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 analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the

samples for machine drift: two in-house standards (Merck Gel and Nasturtium) and the IAEA (International Atomic Energy Agency) standard $(\text{NH}_4)_2\text{SO}_4$.

%Ndfa was calculated according to Shearer and Kohl (1986):

$$\%Ndfa = 100((\delta^{15}N_{\text{reference plant}} - \delta^{15}N_{\text{legume}}) / (\delta^{15}N_{\text{reference plant}} - B))$$

Where B is the $\delta^{15}\text{N}$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *Virgilia divaricata*, grown in a N-free culture. The B value of *V. divaricata* was determined as -2.58‰.

N nutrition absorption rate calculation

Specific N absorption rate (SNAR) ($\text{mg N} \cdot \text{g}^{-1} \text{ root DW} \cdot \text{d}^{-1}$) is the calculation of the net N absorption rate per unit root DW (Nielson *et al.*, 2001):

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

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

The absorption rate of the specific net N was also calculated per unit nodule DW and per unit root DW according to N sources.

Where M is the N content specific of the plant and R is the root dry weight.

Statistical analysis

The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (Super-Anova). Where the ANOVA revealed significant differences between treatments, the means (based on 6 replicates) were separated using post-hoc Student Newman Kuehl's (SNK) multiple-range test (* $P < 0.05$). Different letters indicate significant differences between treatments

Results

Plant biomass and mineral nutrition

Total biomass accumulated during growth was significantly reduced during P deficiency compared to sufficient P (Table 1). Similarly total plant P concentration was significantly reduced during P deficiency (Table 1). The P reduction was further shown by Pi , which was reduced both in the roots and nodules during P deficiency (Fig. 2A). Furthermore there was a reduction in percentage N derived from the atmosphere (%Ndfa) during P deficiency compared to P sufficient plants (Fig. 4A).

Table 1. Total plant DW, total plant P concentrations, and uricase and xanthine dehydrogenase (XDH) activities of *Virgilia divaricata* plants grown in sand culture under high P (500 mM) or low P (5 mM) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

Both the high and low P plants were either supplied with 500 pM NH_4NO_3 as a soil N source. Values are presented as means (n = 10). The different letters indicate significant differences among the treatments at $P < 0.05$

Total plant parameters		500 pM	5 pM
Plant DW (g)		0.56 ± 0.042b	0.28 ± 0.031a
Plant P concentration (pmol.g ⁻¹)		348.2 ± 58.96b	171.4 ± 11.89a
Ureide activities (pmol min ⁻¹ mg ⁻¹ protein)			
Nodules	XDH	0.096 ± 0.007b	0.005 ± 0.001a
	Uricase	0.283 ± 0.033b	0.095 ± 0.001a
Roots	XDH	0.033 ± 0.006a	0.048 ± 0.005b
	Uricase	0.291 ± 0.018a	0.330 ± 0.021a

In contrast, there was an increase in absorption rate by the roots during P deficiency, this presented by SNAR (Fig. 4B).

Phosphorus fractions

Peak areas from the NMR spectra were used to derive relative concentrations of the P compounds (Fig. 1). All nucleotides (ADP, ATP, UDP) were reduced during P deficiency in both roots and nodules compared to P sufficient plants (Fig. 2B and C and Fig 3B). This reduction corresponds to the reduction of total plant P concentration and Pi concentration. The ADP: ATP ratio increased in the nodules during P deficiency (Fig. 2D), while the roots had a slight increase during P deficiency compare to P sufficient roots but were not significantly different statistically. Sugar P, Fru-6-P showed a significant increase in the nodules and roots during P deficiency compared to P sufficient plant organs (Fig. 3C). The ratio of UDP-Glu and Fru-6-P showed a significant decline during P deficiency compared to P sufficient plant organs (Fig. 3D). Furthermore the membrane phospholipid (P-cho) was significantly reduced during P deficiency (Fig. 3A).

N metabolizing enzyme assays

In roots there was a significant increase in nitrate reductase (NR) activities during P deficiency compare to the roots of P sufficient plants. However in nodules, NR activity was reduced during P deficiency (Fig. 5A). Aminating GDH and GS activities in the roots followed the same trend as NR, as they showed increased activities during P deficiency (Fig. 5B and C). The nodules showed a reduction in aminating GDH

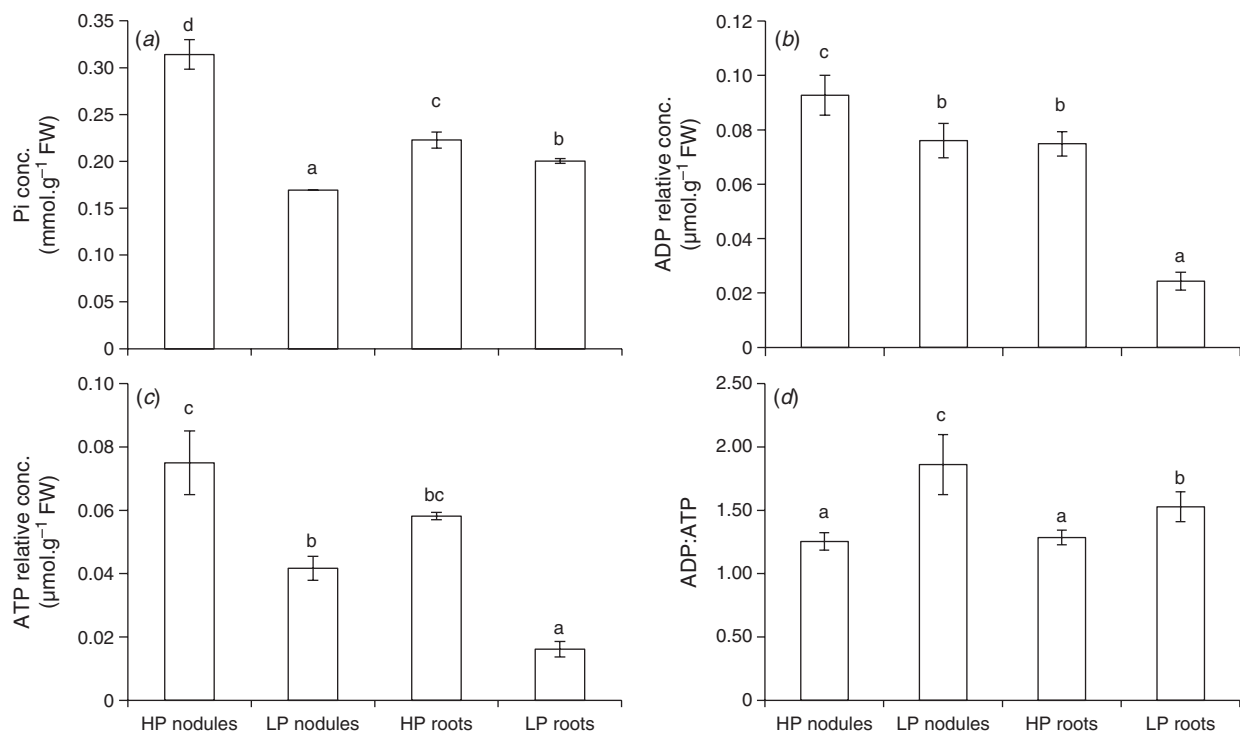


Fig. 1. (a) P_i concentration, (b) ADP and (c) ATP relative concentrations, and (d) ratio of ADP to ATP in *Virgilia divaricata* plants grown in sand culture under high P (HP, 500 μM) or low P (LP, 5 μM) as $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). Different letters indicate significant differences between the treatments at $P < 0.05$.

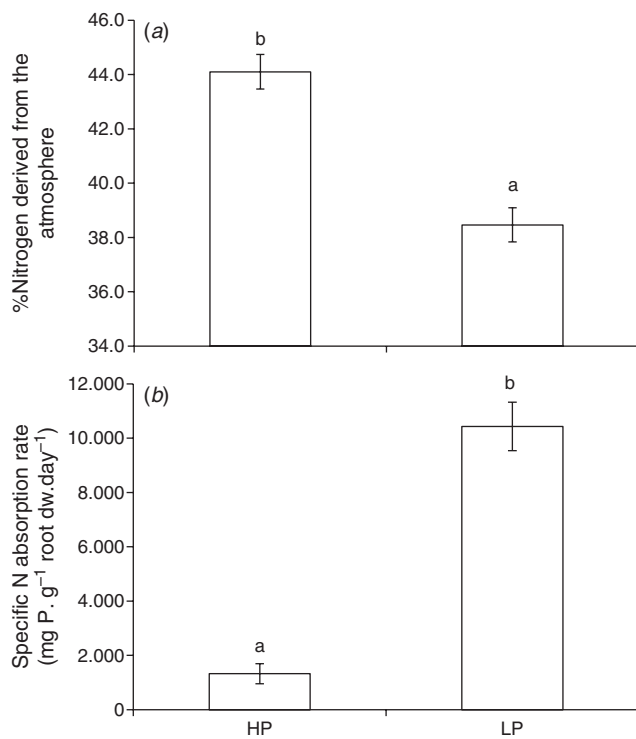


Fig. 2. (a) Percentage of N derived from the atmosphere and (b) specific N absorption rate of *Virgilia divaricata* plants grown in sand culture under high P (HP, 500 μM) or low P (LP, 5 μM) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). Different letters indicate significant differences between the treatments at $P < 0.05$.

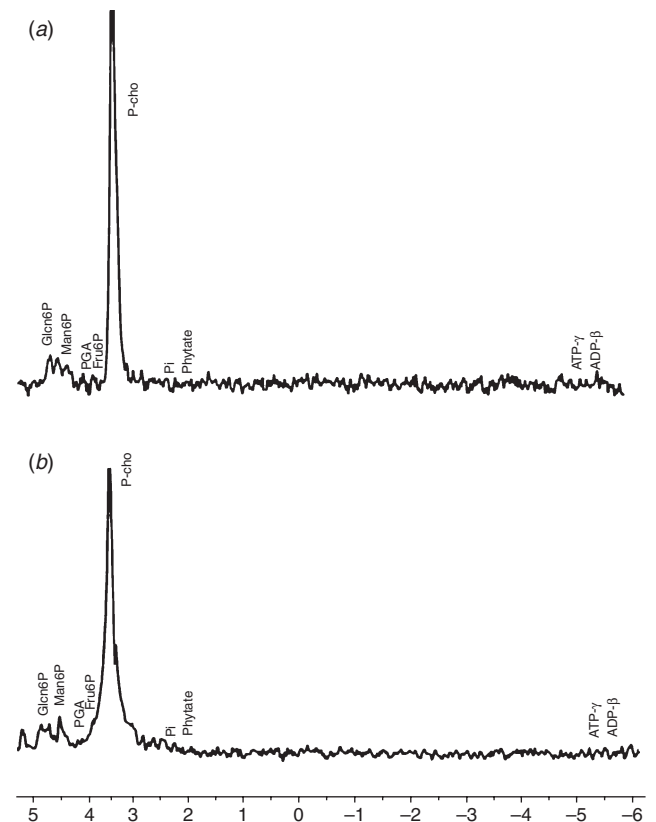


Fig. 3. Representative nuclear magnetic resonance spectra of *Virgilia divaricata* (a) nodules and (b) root tissue, grown under low P conditions. Peak areas of the spectra were used to derive the relative amounts of P compounds. Only compounds that occurred consistently in all replicates were used to derive relative amounts, and presented in the results and discussion. Glcn6P, gluconate 6-phosphate; Glc-6-P, glucose 6-phosphate; PGA, phosphoglycerate; Fru-6-P, fructose 6-phosphate; P-cho, phospho-choline; Pi, inorganic P.

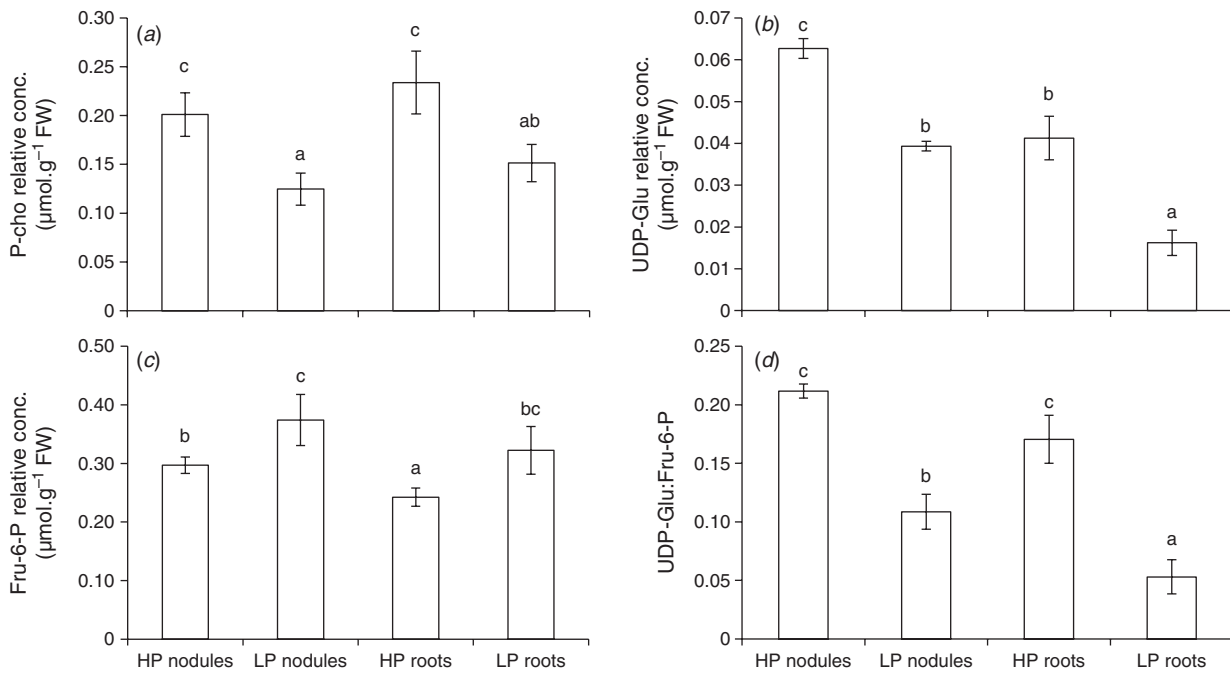


Fig. 4. (a) Membrane phospholipid (P-cho), (b) uridyl diphosphoglucose (UDPG) and (c) fructose 6-phosphate (Fru-6-P) relative concentrations, and (d) ratio of UDPG to Fru-6-P of *Virgilia divaricata* plants grown in sand culture under high P (HP, 500 μM) or low P (LP, 5 μM) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). Different letters indicate significant differences between the treatments at $P < 0.05$.

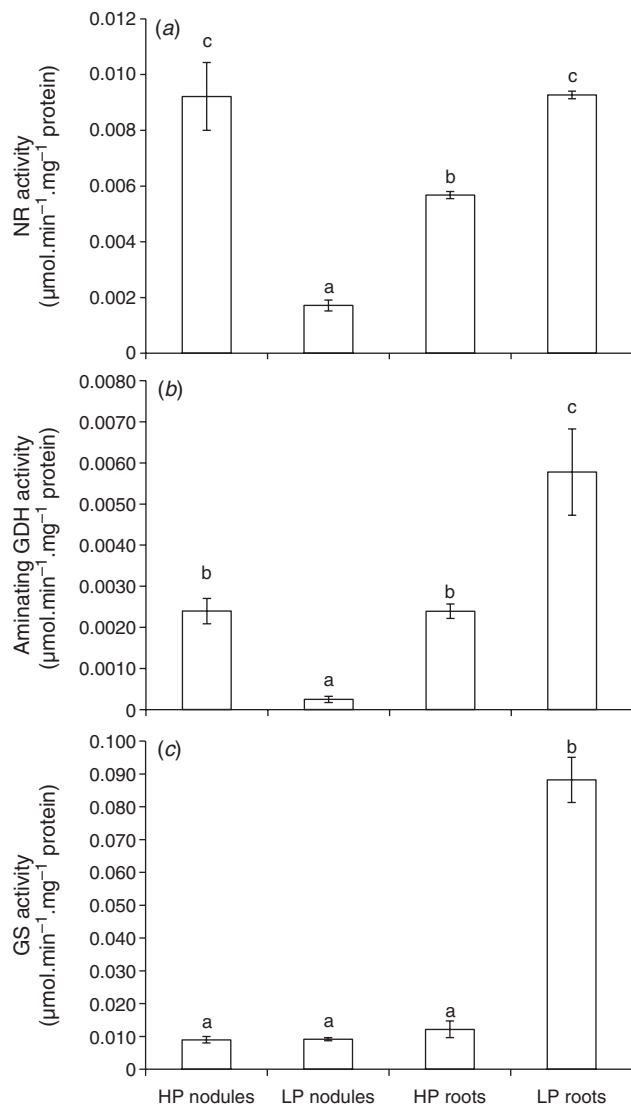


Fig. 5. (a) Nitrate reductase (NR), (b) aminating glutamate dehydrogenase (GDH), (c) glutamine synthetase (GS) activities of *Virgilia divaricata* plants grown in sand culture under high P (HP, 500 μM) or low P (LP, 5 μM) as $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). Different letters indicate significant differences among the treatments at $P < 0.05$.

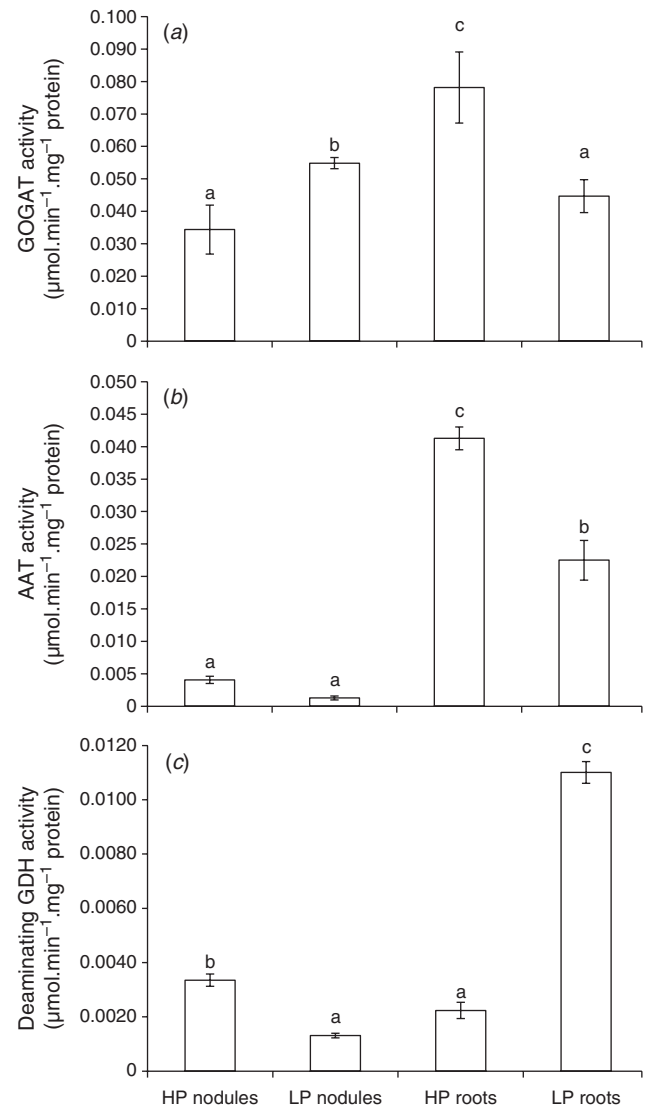


Fig. 6. (a) Glutamate 2-oxoglutarate aminotransferase (GOGAT), (b) aspartate aminotransferase (AAT) and (c) deaminating glutamate dehydrogenase (GDH) activities of *Virgilia divaricata* plants grown in sand culture under high P (HP, 500 μM) or low P (LP, 5 μM) as $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). Different letters indicate significant differences among the treatments at $P < 0.05$.

Table 2. Ratios of N-metabolising enzymes in *Virgilia divaricata* plants grown in sand culture under high P (500 μ M) or low P (5 μ M) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

Both the high and low P plants were either supplied with 500 μ M NH_4NO_3 as a soil N source. Values are presented as means ($n = 10$). The different letters indicate significant differences among the treatments at $P < 0.05$. GOGAT, glutamate synthase or glutamate 2-oxoglutarate aminotransferase; XDH, xanthine dehydrogenase GDH-A, aminating glutamate dehydrogenase; GDH-D, deaminating glutamate dehydrogenase; AAT, aspartate aminotransferase

Organ	N-metabolising enzyme ratios	500 μ M P	5 μ M P
Nodules	GOGAT : uricase	0.12 \pm 0.02a	0.58 \pm 0.02b
	GOGAT : XDH	0.374 \pm 0.09a	10.6 \pm 1.56b
	GDH-A : GDH-D	0.73 \pm 0.13b	0.19 \pm 0.05a
	AAT : uricase	0.014 \pm 0.00a	0.014 \pm 0.00a
	AAT : XDH	0.043 \pm 0.01a	0.23 \pm 0.03b
Roots	GOGAT : uricase	0.27 \pm 0.05b	0.14 \pm 0.02a
	GOGAT : XDH	2.50 \pm 0.38b	0.94 \pm 0.09a
	GDH-A : GDH-D	1.12 \pm 0.22b	0.52 \pm 0.08a
	AAT : uricase	0.14 \pm 0.01b	0.07 \pm 0.01a
	AAT : XDH	1.38 \pm 0.30b	0.48 \pm 0.07a

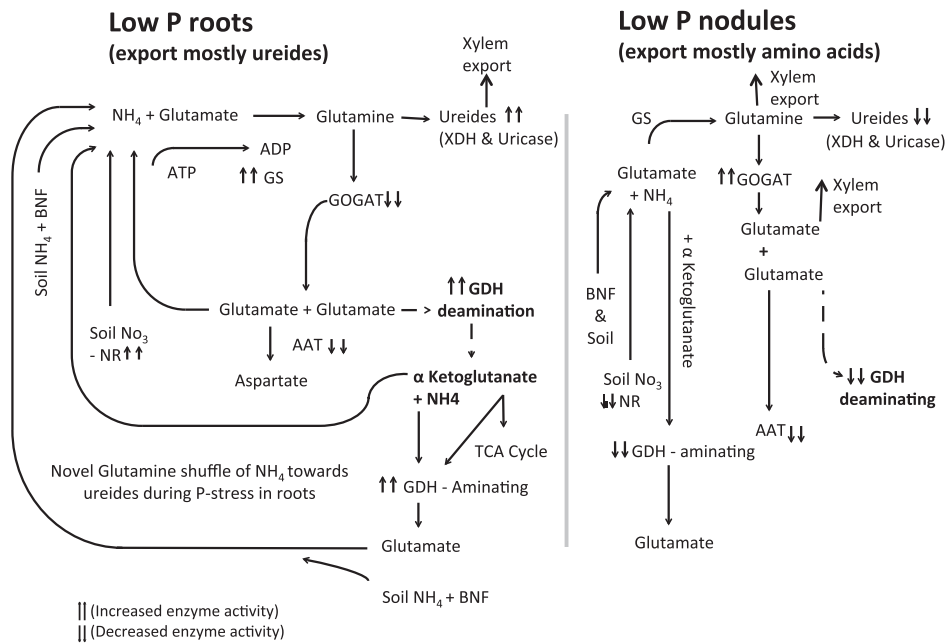


Fig. 7. Proposed routes of N metabolism in (a) roots and (b) nodules of *Virgilia divaricata* plants grown in sand culture under high P (500 μ M) or low P (5 μ M) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and low P plants were supplied with 500 μ M NH_4NO_3 as a soil N source. XDH, xanthine dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate 2-oxoglutarate aminotransferase; NR, nitrate reductase; BNF, biological nitrogen fixation; AAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; TCA, tricarboxylic acid cycle.

activity, while GS activity was maintained during P deficiency compared to P sufficient plants (Fig. 5B and C).

Furthermore, GOGAT activity was reduced in the roots during P deficiency compared to P sufficient plants, while there was an increase in GOGAT activity in nodules during of P deficiency (Fig. 6A). In the roots, the decrease in activity during P deficiency is also observed in AAT activity compared to P sufficient plants, while the nodules maintain their AAT activity (Fig. 6B). Deaminating GDH activity show a significant increase in the roots during P deficiency (Fig. 6C), while the inverse was observed in the nodules. XDH show increased activity in the roots during P deficiency compared to P sufficient plants, while uricase activity is maintained in the same organ (Fig. 6A and B). Inversely in the nodules both uricase and XDH showed decreased activities during P deficiency (Table 1).

The variation of the N metabolizing enzymes is presented in table 2 by ratios. There is a higher ratio of GOGAT: uricase and XDH in the nodules during P deficiency, this is also observed in the ratio of AAT and XDH during P deficiency (Table 2). The inverse is observed in the roots, showing a lower ratio during P deficiency (Table 2). The ratio of aminating GDH and deaminating GDH shows the same trend in both the roots and nodules, showing a decreased ratio during P deficiency compared to P sufficient plants (Table 2).

Discussion

V. divaricata under P stress showed different responses in roots and nodules. Varying metabolic changes in nodules and roots, caused alteration in the assimilation and export of N in each organ. These alternate routes of N metabolism suggest that different energy-saving mechanisms may contribute to the growth of *V. divaricata* in low P soils.

The limited P supply reduced plant growth resulting in decreased biomass, and though *V. divaricata* were supplied with a very low concentration of N as NH_4NO_3 , they formed nodules and initiated BNF however BNF was decreased by limited P supply. This is consistent with results of other studies on model and fynbos endemic legumes, where limited P reduced both plant growth and N_2 fixation (Rufty JR *et al.* 1993; Muofhe and Dakora 1999; Nielsen *et al.* 2001; Olivera *et al.* 2004; Dakora and

Phillips 2002; Power *et al.* 2010; Magadlela *et al.* 2014; Vardien *et al.* 2014). Furthermore the decline in plant P concentration and Pi during P deficiency concurs with previous studies in legumes, also grown under P-deficient conditions (Hernandez *et al.* 2007; Le Roux *et al.* 2006). However, the observation at the whole plant level may not reflect the organ-specific responses of roots and nodules to P deficiency.

Metabolic changes in the ability of roots and nodules to cope with P deficiency were observed in the decreases of metabolically active phosphate (Pi) and adenylate-P concentrations. This concurs with previous work where declines in Pi concentration and adenylates were reported during P deficiency (Le Roux *et al.* 2006; Theodorou *et al.* 1991). The lower percentage decline (below 35%) in nodule ADP and ATP, compared to a 60% decline in roots, and the lower nodule ADP: ATP ratios, all suggests that during P deficiency the nodules maintained their P status better than roots. Although these findings are for a legume from a nutrient-poor habitat, they concur with findings by Le Roux *et al.* 2006 for a model legume. It therefore shows that nodules generally have a strategy to regulate their P status, which allows them to minimize effects of P deficiency (Tang *et al.* 2001; Le Roux *et al.* 2006). This regulation may include mechanisms of P recycling and metabolic bypass reactions.

Internal recycling of P can be achieved via the possible replacement of membrane phospholipids as evidenced by the P-cho decline during P deficiency. The replacement of membrane phospholipids, in order to release Pi to the cell, has been found during P stress (Zavaleta-Pastor *et al.* 2010; Lambers *et al.* 2012), where the phospholipids have been replaced by non-phosphorus containing galactolipids or sulpholipids. Although we do not have direct evidence for the increased presence of galactolipids and sulpholipids, the decline in membrane phospholipids in P stressed nodules and roots does suggest this possibility.

Furthermore, it is known that during P deficiency, the metabolic bypasses in glycolysis would be engaged (Theodorou and Plaxton 1993; Plaxton 2010). The PPi fueled process maybe a fundamental facet of the metabolic adaptations of plants to environmental stress, where these glycolytic bypasses favour PPi-dependent reactions instead of ATP-requiring reactions, in order to conserve diminishing cellular ATP pools (Plaxton 2010). There is evidence that *V divaricata*, a legume from a nutrient-

poor ecosystem, showed an increased reliance on the P_{Pi}-dependant metabolism of sucrose via UDP-Glu to Fru-6-P in its roots and nodules. Since these organs are involved in N acquisition, the consequence of these bypasses is expected to influence N nutrition of these organs. This may involve the synthesis of alternative N containing products and recycling of N compounds.

During P stress, *Virgilia divaricata* nodules have a reduced participation in the uptake of atmospheric N in favour of soil N uptake via the roots. This reduced participation is evidenced by reduced BNF and increased specific N absorption rate by roots during P deficiency as well as NR activities in P deficient roots. The switch of N sources due to limited P was reported in earlier studies on *V. divaricata* (Magadlela *et al.* 2014). During N assimilation in roots there is a difference in glutamine metabolism, relative to nodule during P stress, in order to favour ureide synthesis (Fig 7). This is evidenced by the increased GS activities, but not GOGAT and AAT activities. Furthermore, under P stress in roots, the higher capacity for ureide synthesis via unchanged uricase activities and enhanced XDH activity, suggest that glutamine may be used for ureide synthesis. This ureide synthesizing pathway may be less C expensive, than the amide pathway, as it is reported in the current study that the roots were more P stressed than the nodules (Todd *et al.* 2006). This concurs with experimental determinations of C and N budgets of ureide-forming and amide forming legumes by Atkins (1991), indicating that those based on ureides are generally more economical of C, with a C input of 1.4 g C g⁻¹ fixed N in cowpea compared to minimum of 3.9 g C g⁻¹ fixed N in lupin (Schubert 1986). Furthermore it is estimated that the ATP costs per N assimilated to produce ureide transport molecules is half that of producing glutamine and asparagine (Schubert 1986). The values deduced were 5 ATP per N for allantoin or allantoate compared with 12 for asparagine, calculated from known biochemical pathways and assuming that the starting compounds are ammonium and phosphoglycerate (Schubert 1986). Even though there are other associated costs with the ureide synthesis pathway (Winkers *et al.* 1987), it can be concluded that ureide synthesis for N assimilation might be more carbon and energy cost effective than the amine pathway. Therefore the lower ratio of the amino acid synthesizing enzyme activity of AAT and GOGAT relative to the activities of the ureide synthesizing uricase and XDH, supports the contention that the GS product, glutamine is being metabolized increasingly into ureides, rather than amino acids in roots of P stressed

legumes. The glutamate and the NH_4^+ required for glutamine synthesis via GS may come from various sources.

The NH_4^+ may be derived either from NO_3^- reduction, NH_4^+ uptake from sand medium or deamination of glutamate via deaminating GDH. In addition, the glutamate may be produced via aminating GDH in the view of the decline in GOGAT activity in these P stressed roots. Since these roots were supplied with $\text{NH}_4^+\text{NO}_3^-$ as an inorganic N source, the increases in NR activity, deaminating GDH activity and the enhanced N acquisition rates of roots suggest that NH_4^+ may have been supplied via these routes, since the BNF was lower in the P stressed plants. Furthermore, the supply of glutamate for glutamine synthesis was most likely from the increase in aminating GDH activity. It is unlikely that GOGAT could have provided this glutamate for the enhanced ureide synthesis, because GOGAT activity was lower in these low P roots. Moreover the increased activity of deaminating GDH, may serve an additional purpose in P stressed roots.

In addition to the alternate export of N as ureides, roots of P stressed plants may also engage in the recycling of N compounds during P limitation. The increased deaminating GDH activity in the P-stressed roots suggests that glutamate is being broken down to form 2-ketoglutarate and NH_4 . Although this has not been demonstrated in P stressed tissues before, the deaminating role of GDH may break down glutamate to supply the C skeletons as 2-ketoglutarate to the TCA cycle during times of C limitation (Robinson *et al.* 1991; Melo-Oliveira *et al.* 1996; Ireland and Lea 1999; Aubert *et al.* 2001; Masclaux-Daubresse *et al.* 2002; Restivo 2004; Miyashita and Good 2008). In contrast to the P stressed roots, the nodules were better able function during P deficiency based on their adenylate levels.

During P deficiency, nodules maintained a lower percentage decline in ATP and ADP, than roots under similar stress. This implies that nodules have a strategy to regulate P influx and conservation, allowing nodules to minimize the effects of P deficiency when the supply is low (Jakobsen 1985; Tang *et al.* 2001; Le Roux *et al.* 2006; Vardien *et al.* 2014). With the decline in BNF during P deficiency, the nodules also relied less on amino acid recycling via deaminating GDH, compared to roots during P deficiency. Instead, the nodules favoured the synthesis of amino acids, in

contrast to roots, which favoured ureide synthesis (Fig 7). The source of N in the P-stressed nodules was most likely NH_4^+ from soil sources, rather than NO_3^- because NO_3^- assimilation via NR was reduced, possibly owing to its requirement of ATP. Furthermore the soil-acquired NH_4^+ assimilation is energetically less expensive (Minchin and Witty 2005) than BNF and this would be more beneficial during P deficiency. This NH_4^+ assimilation is evident in the low P nodules as the constant GS activities, but the increase in GOGAT activities. Similarly, the ureide synthesis via XDH and uricase activities, also declined. Furthermore, the ratio of GOGAT to ureide synthesizing enzyme activities was higher under P deficiency in the nodules, suggesting that amino acid synthesis was favoured over ureide synthesis. The subsequent export of organic N, may have been in the form of either glutamate or glutamine, since our experimental evidence suggest that aspartate and asparagine synthesis may have been reduced under P limitation in nodules. In this regard, the decline in AAT activities during P stress concurs with previous reports, since the AAT-AS reactions to produce asparagine is an ATP requiring step (Ryan and Fottrell 1974; Bryan 1980; Schubert 1986; Farnham *et al.* 1990). This bypass of ATP-requiring steps may be part of the nodule's strategy of P conservation. Therefore, in contrast to LP roots, the nodules relied heavily on the GS-GOGAT route for N assimilation during P deficiency.

Conclusion

P deficiency had different impacts on the N metabolism of *V. divaricata* roots and nodules. Compared to roots, the nodules showed a greater degree of P conservation during low P supply, which resulted in these organs having less amino acid recycling and less ureide production in favour of amino acid synthesis. This indicates that the roots and nodules of *V. divaricata*, metabolise N very differently (Fig 7) during P stress and these organs may contribute differently to the success of this plant in soils ranging from forest to fynbos.

Acknowledgements

This work was funded by the DST/NRF-Center of Excellence for Tree Health Biotechnology, based at the University of Pretoria, South Africa. We acknowledge the Department of Botany and Zoology at the University of Stellenbosch for their research facilities, Waafeka Vardien for assisting with the NMR data analysis and

Richard Thompson for assisting with the designing of the nitrogen metabolising pathway.

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