Variation in phosphorus supply alters nitrogen metabolism in the nodules and roots of *Virgilia divaricata,* a Cape fynbos indigenous legume from the Cape Floristic Region.

By

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Declaration

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Summary

This study determined how phosphorus (P) deficiency alters the nitrogen (N) metabolism in the root nodules of *Virgilia divaricata* (Adamson). The legume is indigenous to nutrient rich forest soils, but is also known to grow across a wide range habitats these including N and P poorer soils of the mature fynbos, a nutrient-poor ecosystem in the Cape Floristic Region of South Africa. Although this implies that the legume has a wide functional tolerance for variable soil N and P levels, it is not known how the plant utilizes inorganic N under variable P supply. This was evaluated in three separate experiments.

The first experiment identified the bacterial species that nodulate *V. divaricata* and their biological N_2 fixing (BNF) efficiency during P deficiency. In the experiment, we also integrated the plant C and N metabolism to the N product exported via xylem to the shoots for plant use. Plants were grown at sufficient and low P levels, with four concentrations of inorganic N supply (NH_4NO_3). At both levels of P, soil N supply reduced the reliance of legumes on BNF. Although the bacterial composition of nodules remained unchanged by P and N supply, the nodule function was greatly altered. In this regard, plants reliant on only N_2 at both P levels had higher and more efficient BNF, which resulted in greater plant N. At high P, plants exported more amino acids relative to inorganic N and ureides in their xylem sap, whereas at low P the plants exported more ureides relative to amino acids and NH_4. The bacterial tolerance for changes in P and N determined via nodule metabolites and xylem export might be a major factor that underpins the growth of *V. divaricata* under these variable soil conditions.

The second experiment determined whether the P deficiency affects the metabolic status of roots and nodules, and the consequent impact on the routes of N assimilation. The findings show that *V. divaricata* had a reduced biomass, plant P concentration and BNF during P deficiency. 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 PPi-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.

The third experiment identified the Glutamate dehydrogenase (GDH) transcripts, their relative expressions and activity in P-stressed *V. divaricata* roots and nodules during N metabolism. GDH might contribute to the functional tolerance of *V. divaricata* to variable soil N and P levels in the mature fynbos by aminating N via aminating GDH and recycling amino acids via deaminating GDH. The analysis of the GDH cDNA sequences in *V. divaricata* revealed the presence of *GHD 1* and *GHD 2* subunits, these corresponding to the *GDH1*, *GDH-B* and *GDH3* genes of legumes and non-legume plants. The relative expression of *GDH1* and *GDH2* genes was analysed in the roots and nodules, our results indicate that two subunits were differently regulated depending on the organ type and P supply. Although both transcripts appeared to be ubiquitously expressed in the roots and nodules, the *GDH1* transcript evidently predominated over those of *GDH2*. Furthermore, the higher expression of both *GDH* transcripts in the nodules than roots in this study may play a role in the ability of nodules to regulate and conserve their internal P better than roots during P deficiency. With regards to GHD activity, both aminating and deaminating GDH activities were induced during P deficiency.

Opsomming

Hierdie studie bepaal hoe fosfor (P) tekort die stikstof (N) metabolisme verander in die wortelknoppies van *Virgilia divaricata* . Die peulplant is inheems aan ryk bosbodems voedingstof, maar is ook bekend om die N en P armer gronde van die volwasse Fynbos, 'n voedingstof -arm ekosisteem in die Kaapse Floristiese Streek van Suid-Afrika binne te val . Hoewel dit impliseer dat die peulplant 'n wye funksionele verdraagsaamheid vir N en P vlakke het, is dit nie bekend hoe die plant anorganiese N onder wisselende P aanbod gebruik nie. Dit is geëvalueer in drie afsonderlike eksperimente.

Die eerste eksperiment het die bakteriële spesies wat *V. divaricata* en hul biologiese N_2 vaststelling (BNF) doeltreffendheid tydens P-tekort nodulate, geïdentifiseer. In die eksperiment, het ons ook die C en N metabolisme aan die N produk wat uitgevoer word via xileem na die lote, geintegreerd. Plante is gegroei op voldoende en lae P-vlakke, met vier konsentrasies van anorganiese N. Op beide vlakke van P, grond N toevoer verminder die afhanklikheid van peulplante op BNF. Hoewel die bakteriële samestelling van nodules onveranderd gebly deur P en N toevoer, was die nodule funksie grootliks verander. In hierdie verband, plante afhanklik net N_2 by beide P vlakke het hoër en meer doeltreffende BNF, wat gelei het tot 'n groter aanleg N. Met hoë P, het plante meer aminosure relatief tot anorganiese N en ureides in hul xileemsap, terwyl by lae P voer die plante meer ureides relatief tot sure en NH_4 amino. Die bakteriële verdraagsaamheid vir veranderinge in P en N via nodule metaboliete en xileem uitvoer kan 'n belangrike faktor onder hierdie veranderlike grondtoestande onderlê.

Die tweede eksperiment het bepaal of P-tekort die metaboliese status van die wortels en wortelknoppies affektuur, en die impak op die roetes van N assimilasie. Tydens P- tekort, het

V. divaricata plante 'n verminderde biomassa en P konsentrasie. P- tekort wortelknoppies handhaaf hul P status beter as P beklemtoon wortels. *V. divaricata* kan C en N metabolisme verander in verskillende maniere in reaksie op P stres. Vir beide wortels en wortelknoppies, bereik dit via interne meganisme van P, deur moontlike vervanging van membraanfosfolipiede met sulpholipids en galactolipids en verhoogde afhanklikheid van die PPI-afhanklike metabolisme van sukrose via UDPG en FRU-6-P.

Die derde eksperiment het die GDH transkripsies, hul relatiewe uitdrukkings en aktiwiteit in P-beklemtoon wortels en wortelknoppies tydens N metabolisme geïdentifiseer. Glutamaat dehidrogenase (GDH, EG 1.4.2-4) kan bydra tot die funksionele toleransie van *V. divaricata* veranderlike grond N en P-vlakke in die volwasse fynbos. Die relatiewe uitdrukking van GDH1 en GDH2 gene is ontleed in die wortels en wortelknoppies, wys dat twee subeenhede gereguleer afhangende van die tipe orrel en P aanbod. Alhoewel beide transkripsies verskyn word, is die GDH1 transkipsie oorheers oor GDH2. Verder kan die uitdrukking van beide GDH transkripsies 'n rol speel in die vermoë van wortelknoppies interne P te reguleer. Met betrekking tot GHD aktiwiteit, is albei aminating en deaminating GDH aktiwiteite veroorsaak tydens P-tekort.

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Chapter 1

Literature review

1. Introduction

1.1 Family Leguminosae (Fabaceae)

The Leguminosae (Fabaceae) began diversifying in the Palaeocene, about 60-64 million years ago. It is the third largest family of flowering plants (Cronquist, 1981; Mabberley, 1997) after the Orchidaceae and Asteraceae, and includes approximately 720 genera and more than 18 000 species worldwide (Lewis *et al*., 2005). Although the Fabaceae has a greater diversity of forms and number of habitats, the family is second to Poaceae in its agricultural and economic importance (Wojciechowski, 2003). The fabaceae includes two monophyletic subfamilies, the Mimosoideae and Papilionoideae (Polhill *et al*., 1981), both of which are nested within a paraphyletic subfamily Caesalpilionoideae (Käss & Wink, 1996; Doyle *et al*., 2002; Kajita *et al*., 2001) (Fig. 1.1). Caesalpinioids have been considered to comprise a number of unrelated lineages, which were subsequently confirmed by molecular phylogenetic analyses (Kajita *et al*., 2001).

Subfamily Caesalpinioideae, with an estimated 161 genera and about 3,000 species (Lewis *et al*., 2005), comprises of the most basal elements in these phylogenies. The relationships between these basal elements are, however, poorly resolved and not always supported in molecular studies (Tucker & Douglas, 1994). The subfamily Mimosoideae, with an estimated 76 genera and some 3 000 species (Lewis *et al*., 2005) is the smallest of the legume subfamilies. Despite its relatively small size, however, this subfamily is phylogenetically the least understood. The Papilionoideae is the largest of the 3 subfamilies, with an estimated 483 genera and 12 000 species (Lewis *et al*., 2005). It is also the most widely distributed of the three traditionally recognised subfamilies of Fabaceae. It is distinguished from the other subfamilies by

vegetative, floral and fruiting characteristics (Polhill, 1981a), including floral development (Tucker, 1987, 2002; Tucker & Douglas, 1994).

Ecologically, the family Fabaceae is important in a diversity of ecosystems, especially the members of subfamily Papilionoideae, which are present and dominant in nearly every vegetation type on earth, from tropical forests to deserts, and play an important role in global biogeochemistry (Sprent & McKey, 1994; Sprent, 2001).

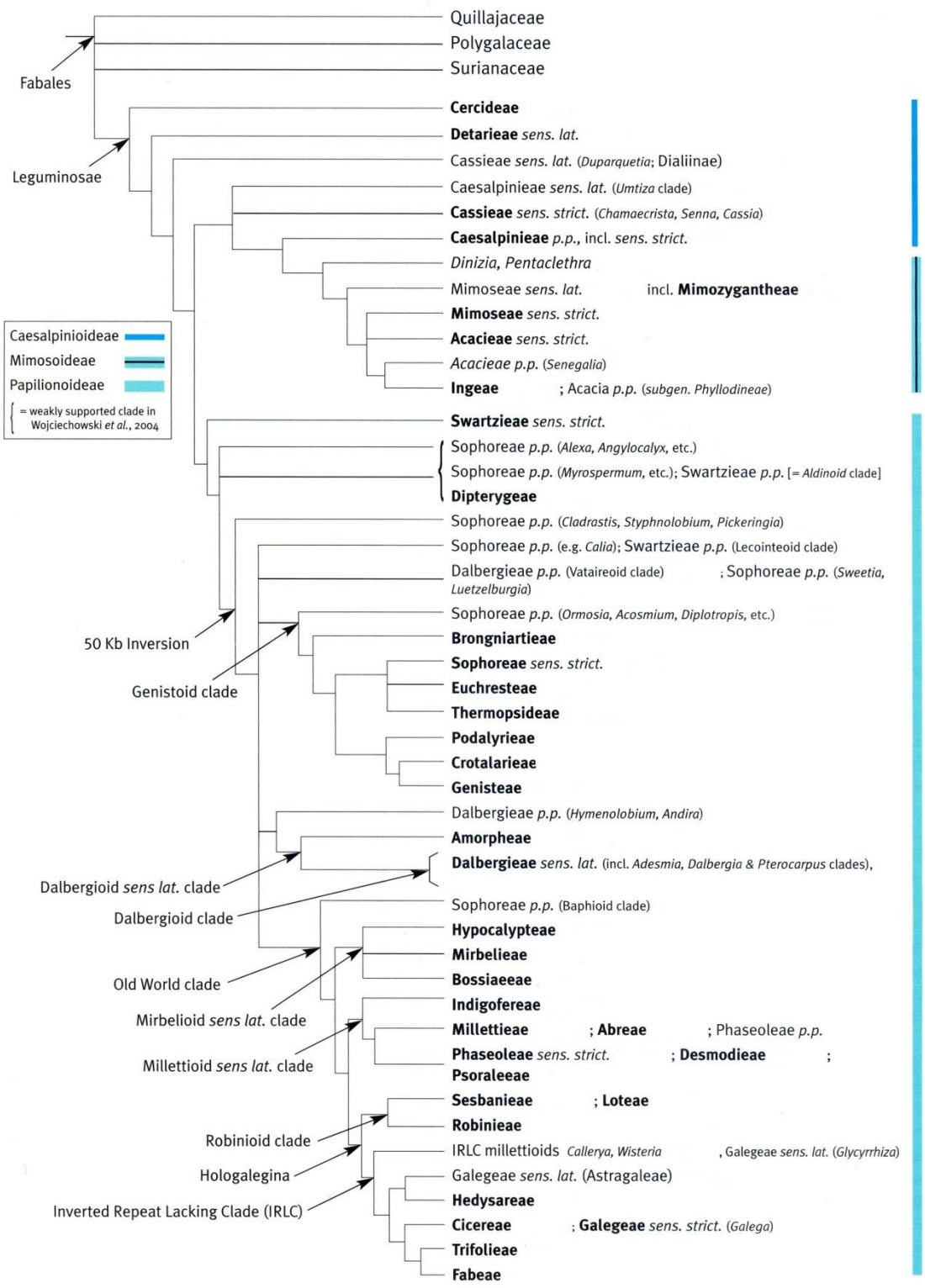


Figure 1.1 Phylogeny of Fabaceae compiled as a supertree based on genetic data derived from the matK gene, analysed by Doyle *et al*., (2000); Kajita *et al*. (2001); Wojciechowski, 2003. Redrawn from Lewis *et al*. (2005).

The Fabaceae is an extremely diverse family with a worldwide distribution in the fynbos, rain forest, temperate and grassland biomes (Boerma & Curtis, 2004). It includes herbaceous plants (such as the temperate to subtropical crops species pea, vetch, soybean and common bean) to large woody lianas (such as *Wisteria*) and even tall trees (e.g. 100 meter tall trees of tropical forests) (Boerma & Curtis, 2004; Wilson *et al*., 2004). The fabaceae represents one of only a few plant families known to be able to undertake biological nitrogen fixation (BNF). By doing so, legumes form an important component of the nitrogen (N) cycle on land, and for this reason agricultural systems have traditionally relied heavily on legumes for N input.

Both legumes and grasses (cereals) are still essential to modern day agriculture. Legumes are agronomically and economically important in many cropping systems, because of their ability to assimilate atmospheric N. This importance is expected to increase with the need to develop sustainable agricultural practices (Serraj *et al*., 1999). Through their mutualistic relationship with bacteria (*Rhizobium*), they can fix a significant amount of 40 to 60 million tons of atmospheric N annually (Boerma & Curtis, 2004). This unique ability of legumes reduces the dependence of farmers on expensive chemical fertilizers, reduces our dependence on petroleum products and improves soil and water quality (Boerma & Curtis, 2004). One of the driving forces behind sustainable agriculture and the protection of the environment is effective management of N in farming systems. Increased cultivation and productivity of legumes would thus do much to reduce environmental degradation, reduce the exhaustion of non-renewable resources and provide adequate N for the population, especially in low-nutrient ecosystems where legumes provide N_2 to these ecosystems (Boerma & Curtis, 2004).

Nitrogen contribution by legumes to other crops in the system depends on the legume species, BNF and the growth of the legumes, as determined by climate and soil, and the management of residues. Grain legumes contribute less N_2 than herbaceous legumes to subsequent crops in rotation, because most of the N_2 fixed biologically by grain legumes is translocated to the grain and both the grain and the residues are constantly removed by humans and livestock (Rao & Mathuva, 1999). As legumes access atmospheric N_2 through the symbiotic relationship with rhizobia, they require minimal N fertilizer inputs (Van Kessel & Hartely, 2000). Even though housing and feeding these bacteria are costly to the legumes in terms of energy, they benefit considerably from this association when confined to soils that lack N compounds (Raven *et al*., 2008; Taiz & Zeiger, 2010).

1.2 Symbiotic Nitrogen Fixation

Nitrogen fixation is the natural process, either biological or abiotic, by which N_2 in the atmosphere is converted into ammonia (NH_3). In nature, plants acquire N by assimilation of nitrate (NO_3) and ammonium (NH_4) or from N_2 through association with bacteria. Where the plant supplies carbon source to the bacteria to undertake the energy dependent of dinitrogen and protect the oxygen sensitive nitrogenase enzyme (Stougaard, 2000). Most of the N available to plants is in the form of gaseous N (N_2). Unfortunately plants lack both the biochemical pathways and the enzyme nitrogenase that are necessary to reduce gaseous nitrogen to ammonia (Schulze, 2004; Raven & Johnson, 2008; Taiz & Zeiger, 2010). The following equation summarizes the process of N_2 fixation, the energy and other components used during N_2 fixation:

$$N_2 + 16ATP + 16H_2O + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i + 8H^+$$

Nitrogenase is an oxygen sensitive enzyme and requires micro aerobic conditions for expression and function (Mylona, *et al*., 1995; Schulze, 2004). The soil borne, Gram-negative *Rhizobia* bacteria have this reduction capacity including the nitrogenase that plants lack (Van Kessel & Hartley, 2000; Raven & Johnson, 2008; Taiz & Zeiger, 2010; Valentine *et al*., 2011).

Nitrogen fixation is utilised by bacteria, these bacteria belong to either the genus *Bradyrhizobium* or the genus *Rhizobium.* The latter genus is paraphyletic, comprising of two groups that resolve in two different classes of the proteobacteria, the alpha- and beta-proteobacteria (Raven & Johnson, 2008).

There is a symbiotic relationship between some plant groups and these bacteria, which has evolved over millions of years (Raven & Johnson, 2008). These bacteria live in close association with the roots of plants. They are contained within symbiotic sacks called symbiosomes in the plant roots that develop to provide optimal conditions for the nitrogen fixing bacteria (Stougaard, 2000). The special plant organs that enclose these bacterial symbiosomes are called nodules. One of the most important sites of BNF is inside nodules that form on these plant species as a result of symbiosis between host plant and bacteria (Serraj *et al*., 1999). The development pattern of nodules can be either indeterminate, determinate (desmodioid nodules), aeschynomenoid nodules and lupinoid nodules (Corby, 1988; Sprent, 2000, 2008; Guinel, 2009). The different types of development pattern of nodules will be described in section 1.2.1.

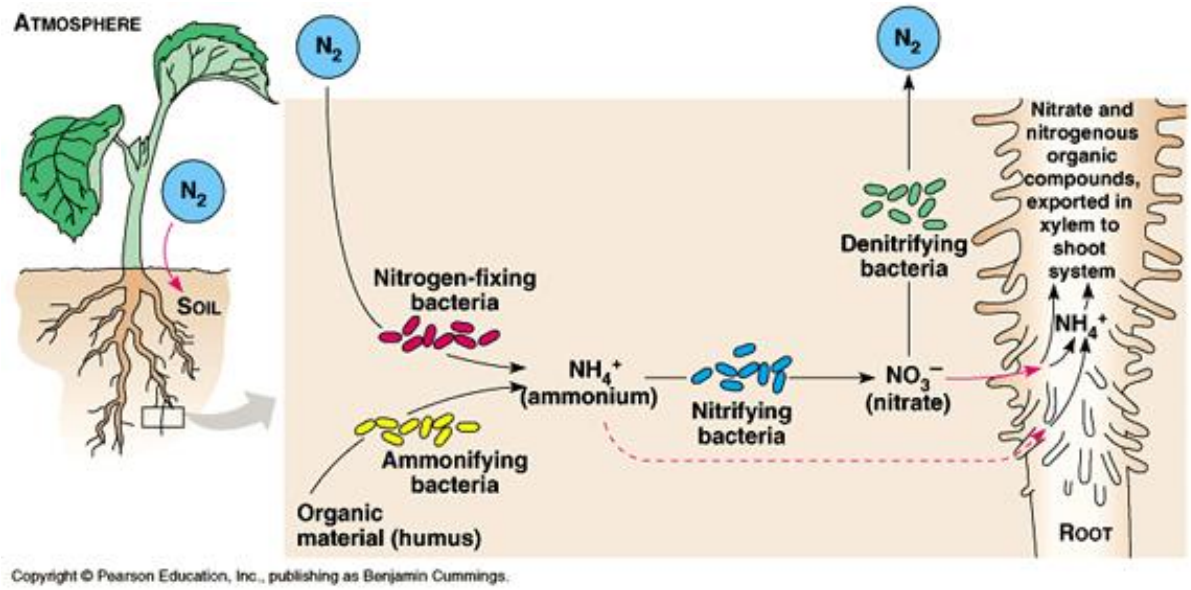


Figure 1.2 A schematic diagram of the nitrogen fixation process, by which nitrogen (N_2) in the atmosphere is converted into ammonium and assimilated by plants. http://www.bio.miami.edu/dana/226/226F08_22print.html retrieved on 14-05-2013

1.2.1 Types of nodules and nodulation

Plants of the Papilionoideae subfamilies are important, specifically in agriculture (Guinel, 2009). In this subfamily, species of Trifolieae and Fabeae tribes form indeterminate nodules, while plants belonging to the Phaseoleae and Loteae tribes develop desmodioid (determinate) nodules. Nodules of the former are bilaterally symmetrical and have a long-lived meristem arising from the root inner cortex. Nodules of the latter are more or less spherical, have radial symmetry and lack a persistent meristem and initiate from the outer cortex of the host root (Hirsch, 1992). Following the above mentioned nodule types, other forms of nodules include the aeschynomenoid type that develops from *Arachis hypogaea* L. (peanut) and *Lupinus sp*. L. develops lupinoid type nodules (Sprent, 2008). Neither of these types of nodules develop from root-hair infection. The aeschynomenoid type is characterised

by uniform infected tissue and the latter girdles the subtending root, owing its presence to the lateral meristems (Sprent, 2008).

Definite structural differences are obvious between the four nodule types, previously mentioned, but causes behind these are unknown and may the result of endogenous hormonal differences (Guinel, 2009). However amongst the nodule types, constant structural features exist, where there is presence of vascular traces enclosed in a vascular endodermis and located within a narrow band of uninfected parenchyma cells surrounding the infected tissue (Guinel, 2009). To establish symbiosis, the bacterial microsymbionts gain access to single plant cells and install themselves in compartments surrounded by a plant membrane (Stougaard, 2000).

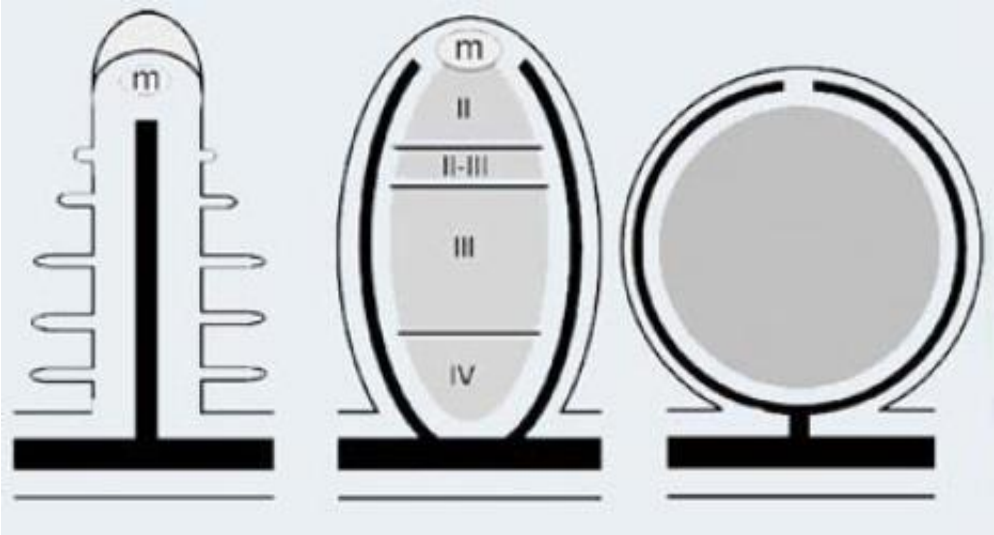


Figure1.2.1 Comparison between lateral root, (right), indeterminate legume nodule (middle) and determinate legume nodule (left). The vascular system is given in black. The vascular system of legume nodules is embedded in the nodule parenchyma that forms a turgor-controlled oxygen diffusion barrier. The indeterminate legume nodule with a meristem in its tip (I) forms a developmental gradient of infected cells in the tissue containing the infected cells. II, zone of prefixation (infection); II-III, interzone;

III, zone of nitrogen fixation; IV, zone of senescence (nomenclature according to Vasse *et al*., 1990).

The symbiosis between legumes and the nodulating symbionts begins with a specific exchange of signal compounds, when the bacterium detects flavonoids and other compounds released by the host plant functioning as inducers of the rhizobial *nod* genes (Stougaard, 2000; Prell & Poole, 2006), e.g. *Medicago truncatula* and their nodulating symbionts *Sino-rhizobium meliloti* (Monahan-Giovanelli *et al*., 2006) (Fig. 1.2.2**)**. These activate transcriptional regulators which then induce the expression of bacterial *nod* genes essential for the biosynthesis of the lipooligosaccharide signaling molecule, nod factor which is required for nodulation (Mulligan & Long, 1989; Brewin, 1991; Kondorosi *et al*., 1991; Denarie *et al*., 1996; Stougaard, 2000) (Fig. 1.2.2**)**. This initiates a complex signalling pathway involving calcium spiking the in root hairs, resulting in root hairs curling and trapping the rhizobia (Gage, 2004) (Fig. 1.2.3**)**. A plant derived tubule filled with dividing and growing bacteria (infection threads, ITs) first develops in an infected root hair and then transverse several cell layers to deliver bacteria to the root cell in the developing nodule (Stougaard, 2000; Brewin, 2004; Gage, 2004) (Fig. 1.2.3**)**. Bacteria exit the ITs through infection droplets, thus entering the cytoplasm of the nodule cells and differentiate into nitrogen fixing bacteroids (Stougaard, 2000; Gage, 2004) (Fig. 1.2.3**)**.

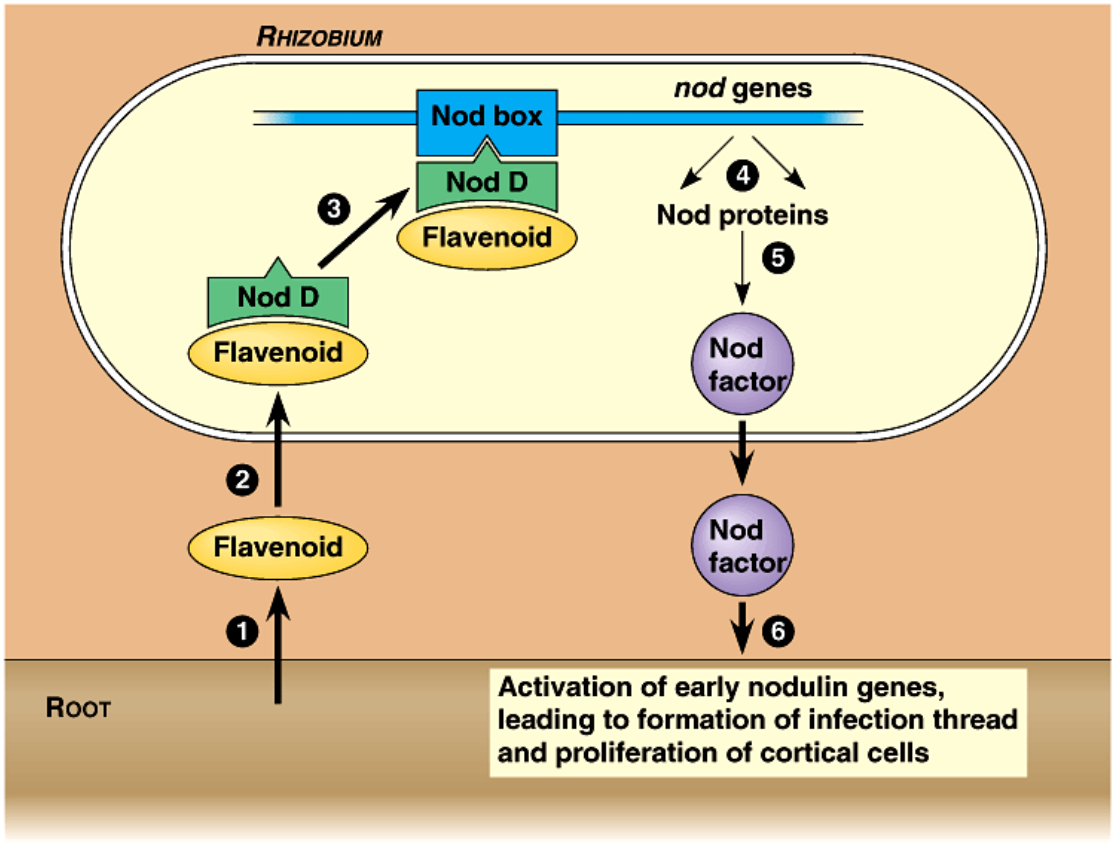
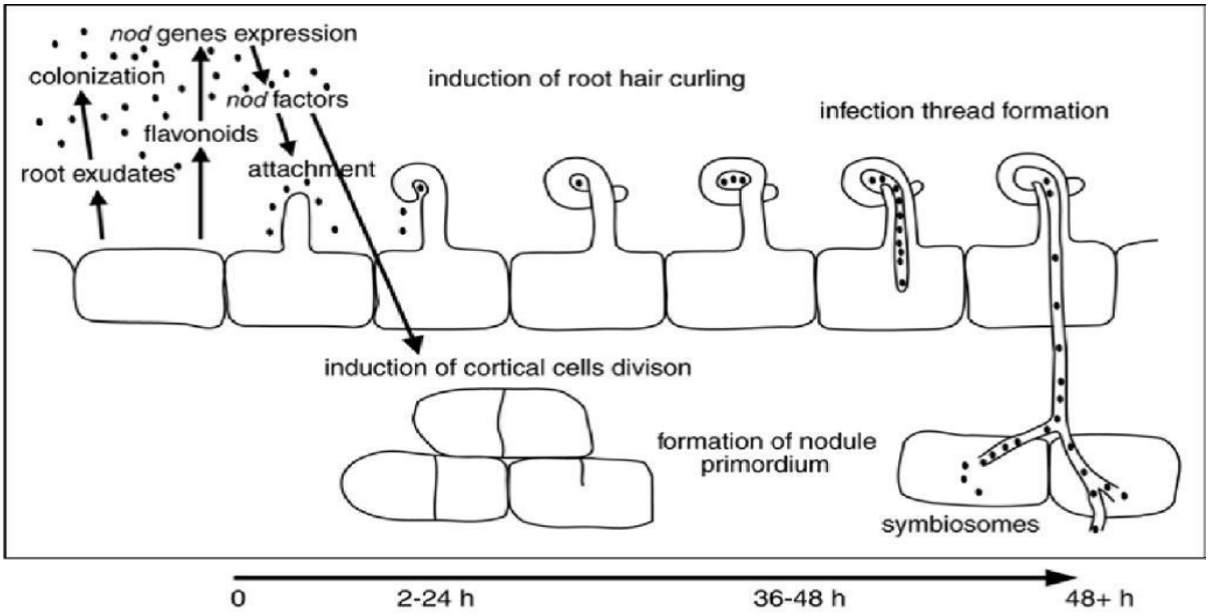


Figure 1.2.2 Schematic diagrams showing the initial infection process, from establishing symbiosis through the root hairs. Root exudates, nodulin gene expression leading to formation of infection threads and proliferation of cortical cells (Cobley, 1956; Amâncio & Stulen, 2004).

(a)



(b)

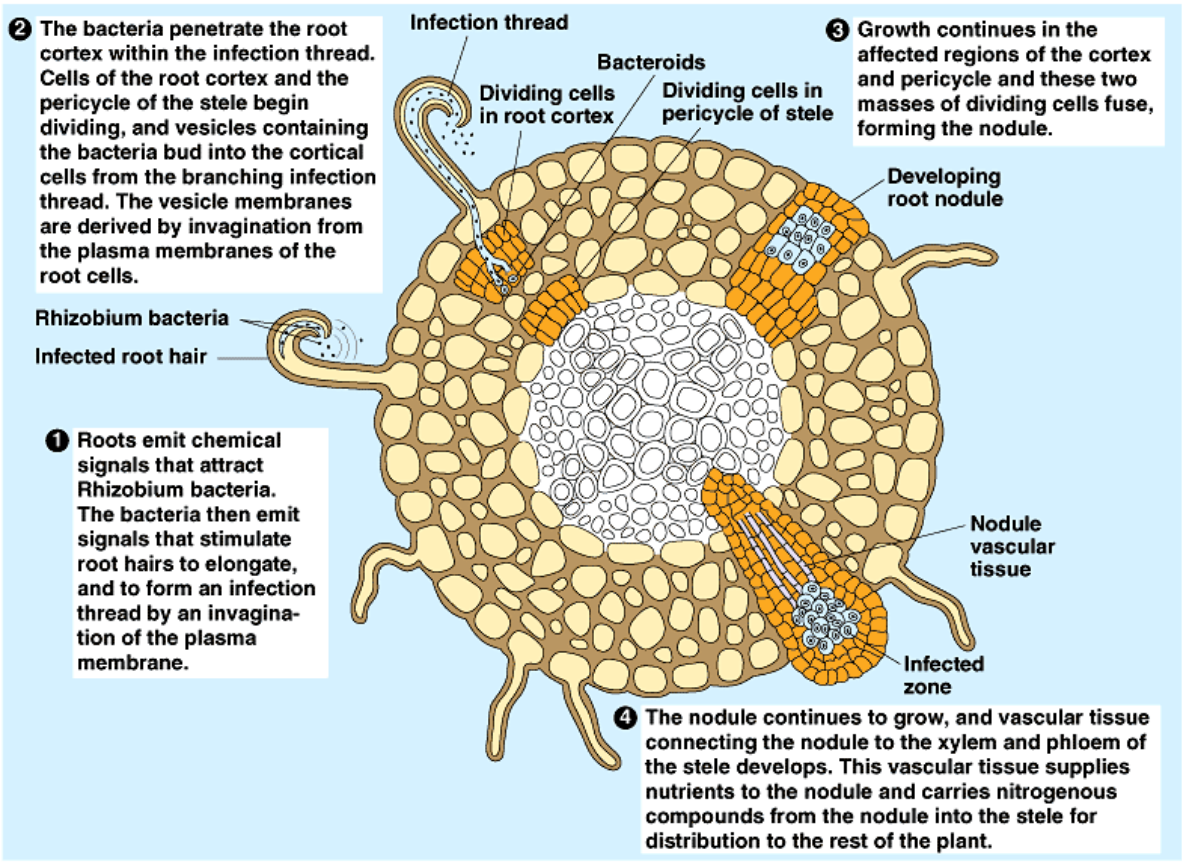


Figure 1.2.3 (a and b) Schematic diagrams showing the infection process, from establishing symbiosis through the root hairs, flavonoids exudation by roots,

expression of *nod* genes, colinization and the simultaneous formation of the nodule primordium (Cobley, 1956; Amâncio & Stulen, 2004).

1.3 Carbon and energy cost during N_2 fixation

Nitrogen fixation is the most energetically expensive reaction known to occur in any plant cell (Raven & Johnson, 2008). In order to obtain reduced N_2 as ammonia, the plant trades the N fixed for reduced carbon that is used to sustain bacterial physiology as well as is required to produce the 16 adenosine triphosphates (ATPs) required by nitrogenase for N reduction. Each symbiotically fixed ammonium molecule utilizes 8 ATPs for the reaction (Valentine *et al.*, 2011). The cost of symbiotic N_2 reduction in legumes was estimated to be between 2 and 3 mg carbon (C) per mg fixed N, varying according to the species and probably also specific genotypes (Valentine *et al*., 2011). Symbiotic N_2 fixation is assumed to require significantly more energy per N fixed than NO_3^- uptake and reduction (Valentine *et al*., 2011). In this regard, theoretically C costs of N_2-fixation ranges between 3.3 to 6.6 g $C.g^{-1}$ N, depending on the legume-rhizobia combination, whereas NO_3^- reduction should not exceed 2.5 g $C.g^{-1}$ N. Although differences in C costs may be statistically insignificant as they are small, when integrated over the whole growth cycle, the costs may be significant (Minchin & Witty, 2005). The high sensitivity of the N_2 fixation process to varying environmental conditions may be attributed to the carbon and energy costs (Mengel, 1994).

1.4 Environmental factors that affect symbiotic nitrogen fixation

The great evolutionary advantage of N_2-fixing legume species is the fact that they can easily succeed in low N environments where other species struggle (Bordeleau & Prevost, 1994; Valentine *et al*., 2011). Symbiotic N_2 fixation is dependent on the *Rhizobium* strain, and their interaction with the pedoclimatic factors, especially the environmental factors associated with the acid soil complex of high aluminium and manganese, low calcium and phosphorus (Bordeleau & Prevost, 1994). Abiotic stresses account for major reductions in N_2 fixation, where more than 50% of legume crops are lost worldwide due to drought, salinity, aluminium toxicity and nutrient deficiencies (Valentine *et al*., 2011). Different *Rhizobium* strains vary in their tolerance to environmental conditions, for example temperature changes affect the competitive ability of *Rhizobium* strains (Bordeleau & Prevost, 1994). Soybean and chick pea rhizobia were reported to be tolerant to 340 mM NaCl, with fast growing strains being more tolerant than slow growing strains. Zahran (1999) extended their work on the halotolerant strains of cowpea, observing that the rhizobial cells responded to high salt stress by changing their morphology. Some strains tolerated extremely high levels of salt, but showed a significant decrease in the symbiotic efficiency under salt stress (Bordeleau & Prevost, 1994; Zahran, 1999). Laboratory studies have also shown that sensitivity to moisture stress varies for a variety of rhizobial strains, so it can be assumed that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host (Zahran, 1999). The competitiveness and persistence of rhizobial strains are not expected to show their full potential for N_2 fixation if exposed to limiting factors (Zahran, 1999).

1.4.1 Role of phosphorus (P) deficiency associated with acid soils on N_2 fixation

After N, P is the second most limiting nutrient for vegetative growth, especially in legume plants (Jebara *et al*. 2004). Inorganic P is known to regulate bioenergetic processes in plants (Rychter *et al*., 1992; Le Roux *et al*., 2006; Plaxton & Tran, 2011). In the case of legumes, more P is required by symbiotic than non-symbiotic plants. Symbiotic N fixation (SNF) has a high demand for P, with up to 20% of total plant P being allocated to nodules during N_2 fixation. The process consumes large amounts of energy, such that the energy generating metabolism is depended upon the availability of P (Schulze *et al*., 1999). Nodule biomass is strongly correlated to P availability to plants. It was reported that nodules require about 3 times more P than the surrounding root tissues (Vadez *et al*., 1997; Jebara *et al*., 2004) and are severely reduced by P deficiency, resulting in a major reduction in nodule size. This was confirmed by Olivera *et al*. (2004), who reported that an increase in P supplied to host legume plants led to a 4-fold increase in nodule mass. The effects of P deficiency may be direct, as P is needed by nodules for their growth and metabolism, or indirect. The high requirement of P may be linked to its role in nodule carbon and energy metabolism; therefore as the deficiency may affect the supply of carbon to the nodules, the bacteria will have greater respiratory demand on the host plant during N fixation (Sar & Israel, 1991; Valentine *et al*., 2011). This coincides with findings of Le Roux *et al*. (2009), Magadlela *et al*. (2014) and Vardien *et al*. (2014) who showed that the nodule construction cost and growth respiration of different legume plants increased with P deficiency. Thus P deficiency can lead to a reduction of both nodulation and SNF (Drevon & Hartwig, 1997; Magadlela *et al*., 2014; Vardien *et al*., 2014).

Phosphorus deficiency and Aluminium (Al^{3+}) toxicity are associated with each other in acid soils, and they both have major effects on legume plant growth and function (Ward *et al*., 2010). Few data have been reported on this, nevertheless it has been stated that different bacterial strains can tolerate acidity better than others, and tolerance may vary amongst strains within a species (Lowendorff, 1981; Vargas & Graham, 1988; Bordeleau & Prevost, 1994; Zahran, 1999). It appears that acid tolerance in rhizobia depends on the ability to maintain an intercellular pH, even at acid external pH, to maintain symbiotic nitrogen fixation (Graham *et al.*, 1994). Strains of rhizobia differ significantly in tolerance to P deficiency. Very few rhizobia grow well at low pH associated with P deficiency (Graham *et al.*, 1994). It has been reported that slow growing *Rhizobium* and *Bradyrhizobium* strains appear to be more tolerant to low P than fast growing strains (Graham *et al*., 1994; Zahran, 1999). This was also reported by Muofe & Dakora (1999), who found indigenous *Bradyrhizobia* associated with *Aspalathus linearis* (Burm.f.) (R.Dahlgren) to be naturally tolerant to acidity. This legume also displayed the ability to modify its rhizosphere pH in order to promote symbiotic establishment. Furthermore, members of the Psoraleae form root nodules with a wide range of N_2 fixing bacteria, including *Rhizobium*, *Mesorhizobium* and *Burkholderia* strains and obtained 60-80% of their N nutrition from symbiotic N_2 fixation (Kanu & Dakora, 2012). Indicating their nodulation and adaptation to the low nutrient, acidic soils of the Cape fynbos (Kanu & Dakora, 2012). Buekus *et al*. (2013) furthermore reported that diversity of *Burkholderia* species are well adapted to the nutrient poor acidic soils of the Cape fynbos and are able to nodulate various fynbos legumes species, including *Hypocalyptus*, *Virgilia* specifically *oroboides*, *Podalyria* and *Cyclopia*. It has been stated that tropical legumes are more tolerant to soil acidity

and deficiency of nutrients than temperate legumes (Andrew & Norris, 1961; Norris, 1965; Norris, 1967).

Since 40% of the world's arable soil is considered acidic including the Cape fynbos soils, P deficiency is commonly reported along soil acidity (Ward *et al*., 2010). They are collectively considered as inseparable factors that limit crop productivity on such soils (Ward *et al*., 2010). Nodulated legumes are self-sufficient at acquiring nitrogen, but are particularly sensitive to a wide range of other environmental limitations, including P deficiency (Le Roux *et al.*, 2007). The effect of the relationship between low P supply and N_2 fixation on legumes remains unclear, primarily because of the indifference in responses to P deficiency by some legume species. The growth of N_2-fixing trees is often limited by the available supply of P in the soil and any factor limiting growth may also limit the rates of N_2 fixation (Le Roux *et al*., 2007). The productivity of tropical forest plantations is commonly limited by the supply of nitrogen in the soil, especially in situations where phosphate limitations have been improved by fertilization (Binkley *et al*., 2003). Most of the agricultural soils in India and other countries around the world are P deficient (Naeem *et al*. 2010). Phosphorus fertilizers are usually applied to overcome the P deficiency, particularly in the case of legumes, which have comparatively a higher characteristic potential of phosphorus consumption than other crops (Naeem *et al*., 2010).

Despite these problems, there are legume species that grow specifically in the very acidic fynbos soil environments. These plants have evolved adaptations to obtain adequate P under these conditions (Vance *et al*., 2003).

1.4.2 Adaptations to P deficiency

Plants have been reported to have evolved two broad adaptations to P deficiency. One adaptation is aimed at conservation of use of P, while the other is directed towards enhanced acquisition and uptake of P (Lajtha & Harrison, 1995; Raghothama, 1999; Horst *et al.*, 2001; Vance, 2011). Adaptations that conserve the use of P involve a decrease in growth rate, increased growth per unit of P uptake, remobilization of internal inorganic P (Pi), modification in carbon metabolism that bypass P-requiring steps and alternative respiratory pathways (Schachtman *et al*., 1998; Plaxton & Carswell, 1999; Raghothama, 1999; Uhde-Stone *et al*., 2003a and b). Legumes have furthermore evolved adaptations for growth under P-deficient soil conditions (Dinkerlaker *et al*., 1995; Keerthisinghe *et al*., 1998; Neumann & Martinoia, 2002). The plants have coordinated different gene expressions that enable them to cope under these conditions, such as the development of cluster roots (proteoid roots), root and nodule exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters and the symbiotic relationship with arbuscular mycorrhizal (AM) fungi (Gilbert *et al*., 1999; Nuemann *et al*., 1999; Neumann and Mortinoia, 2002 ; Uhde-Stone *et al*., 2003a; Vance *et al*., 2003; Barea *et al*., 2005a,b). Johnson *et al*. (1994) and Dinkerlaker *et al*. (1995) regarded P deficiency as a major inducing factor for proteoid roots in *Lupinus albus*. This concurs with Nuemann *et al*. (1999), who found that proteoid root formation in white lupin was the first visible symptom during P deficiency. The exudation of organic acids and acid phosphatase solubilize bound forms of P, increasing the availability of P and micronutrients in cluster roots zones. This is supported by the findings of Neumann *et al*. (1999), who found that acid phosphatases released from white lupin roots during P deficiency may be involved in mobilization of P_i from the organic soil P fraction. Nuemann *et al*. (1999)

observed an increased exudation of organic acids, such as malic acid and citric acid, during severe P deficiency in white lupin proteoid roots. Moreover, the formation of cluster roots results in an increase in nodule number, because of the increased root surface area for P uptake (Lamont, 1983). It is also well established that AM fungi are able to benefit the nutrition of the host primarily through enhanced uptake of P (Jakobsen *et al*., 1994; Smith & Read, 1997; Mortimer *et al*., 2008, 2009). Apart from these adaptations stated, indigenous legumes that occur naturally in low nutrient ecosystems, such as in the Cape fynbos, may have other unique adaptations to grow in such a low P environment.

1.5 Fynbos ecosystems of the Cape Floristic Region

Most plants, including legumes in the Cape Floristic Region (CFR) (Goldblatt & Manning, 2000) of South Africa seem to have developed the adaptations, exudation of organic acids, increased acid phosphatase activity, cluster roots formation and arbuscular mycorrhizal symbiosis, as they grow well in a very poor P environment. Situated at the southern tip of the African continent, between latitudes 31° and 34° S, the CFR represents less than 4% of the total area of the African continent (Goldblatt & Manning, 2000; Linder, 2003). The CFR is one of the world's richest regions in terms of botanical diversity. About 9000 species of vascular plants are native to this area, of which almost 69% are endemic (Goldblatt & Manning, 2000). The families Asteraceae and Fabaceae are the largest and second largest lineages in this region, respectively, and collectively contribute about 20% of the total number of species. Fynbos and Renosterveld represent two of the main vegetation types present within

the CFR, with fynbos confined to the sandstone derived soils, while Renosterveld is mostly restricted to richer shale derived soils.

The fynbos bearing sandstone derived soils are typically very acidic and nutrient poor, particularly with regard to N and P (Kruger *et. al*., 1983). In this, they bear a resemblance to the soils of the Western Australian heathlands rather than other Mediterranean-climate regions (Groves, 1983; Mitchell *et al*., 1984). Phosphorus availability has been shown to vary in these different Cape fynbos soil types. Witkowski & Mitchell (1987) demonstrated that strand veld soils had the highest available P of 70.0 μg P g soil^{-1}, followed by limestone with 6.6 μg P g soil^{-1} and mountain fynbos sandstone with 1.1 μg P g soil^{-1}. The fynbos vegetation of the southwestern Cape is pirophytic and thus dependent on frequent fires. This has a major influence on the cycling of nutrients in this environment (Cowling, 1992). The range of nutrient cycling patterns in the CFR at large is expected to be wide, because of the diversity of soil types present in the biome, each carrying different and characteristic vegetation types (Cowling, 1992). Significant amounts of nitrogen are lost during the thermal volatilization, but exactly how it is replaced in the most nutrient poor soils preferred by fynbos vegetation is unknown (Cocks & Stock, 2001). The availability of N and P in the soils of this ecosystem has been well-studied. N and P in this ecosystem seem to have different nutrient patterns and these two elements are suggested to be most likely to limit primary production of legumes endemic to the fynbos (Cowling, 1992). Plants in the fynbos possess specialized nutrient uptake strategies (Lamont, 1983) and internal nutrient cycling pathways (Mitchell *et al*., 1986). Maseko and Dakora (2013) further said, plant enzymes, root exudates, cluster roots and mycorrhizal symbiosis are the drivers of P nutrition in native legumes growing in P deficient soils of the Cape fynbos in southern Africa. Nutrient

availability in fynbos communities plays an important ecological role in determining species distributions and community composition (Kruger *et al*., 1983).

The Cape fynbos legumes might simulate model legume species and may have developed alternate N assimilation and metabolising pathways during N_2 fixation to conserve energy during P nutrient deficiencies.

1.6 Nitrogen (N) assimilation

The N_2 fixation product ammonia or ammonium (NH_3/NH_4^+) is secreted to the plant for incorporation into amides, glutamine and asparagine, in many legumes that have indeterminate nodules or into ureides, which are purine derivatives, mainly in tropical legumes with determinate nodules, which both requires energy as ATP. These serve as major nitrogen carriers in plants (Lam *et al*., 1996; Dubois *et al*., 2003; Prell & Poole, 2006). The major assimilating pathway is through glutamine synthetase (GS) and glutamate synthase (GS-GOGAT) (Prell & Poole, 2006). The first step of assimilation of (NH_3/NH_4^+) is the ATP-dependent reaction with glutamate to form glutamine, catalysed by glutamine synthetase (GS) (Leegood *et al*., 1995; Keys, 2006; Forde & Lea, 2007).

$$NH_3/NH_4^+ + \text{Glutamate} + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i + H^+$$

The second enzyme involved in assimilation is glutamate synthase, also known as glutamate: 2-oxoglutarate amidotransferase (GOGAT). This is a reduction reaction where there is a transfer of the amide amino group from glutamine to 2-oxoglutarate to yield two molecules of glutamate (Lea and Miflin, 2003; Lea & Miflin, 2011).

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). However Oliveria *et al*. 2004 suggested, in common bean (*Phaseolus vulgaris*), that P treatment reroutes ammonium assimilation in nodules to amine production (asparagine) instead of forming ureides. Furthermore early literature also indicated that mineral nutrient deficiencies might have induced alternate pathways, stimulating large increases in asparagine concentration (Stewart & 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. However this is puzzling since the subsequence reaction of AAT and asparagine synthetase (AS) to form asparagine is an ATP requiring enzyme.

Although not strictly on the direct route of NH_3/NH_4^+ assimilation, nitrogen is often diverted from glutamine to asparagine during periods of a wide range of stress conditions (Sieciechowicz *et al*., 1988; Lea *et al*., 2007). The major route of asparagine synthesis involves the initial assimilation of NH_3/NH_4^+ to the amide, glutamine. Glutamate from the GS-GOGAT cycle can be incorporated to aspartate through aspartate amino-transferase (AAT) (*Hanks et al*., 1981; Olivera *et al*., 2004). Then the enzyme asparagine synthetase (AS) catalyses the ATP-dependent transfer of amides amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine (Romagni & Dayan, 2000; Lea *et al*., 2007; Lea & Miflin, 2011).

Glutamine + Aspartate + ATP →Glutamate + Asparagine + AMP + PP_i

The genes encoding plant AS (*AS1* & *AS2*) were isolated from peas, where northern blot analysis indicated that the expression of both genes was repressed by light in the leaves, but in roots *AS2* was expressed essentially and only *AS1* was repressed by

light (Tsai & Coruzzi, 1991). The expression of genes encoding AS by dark and repression by light concurred with early studies that showed that the accumulation of asparagine was stimulated by darkness (Lea *et al*., 2007). This was observed in soybean deprived of P for 20 days, considerable amounts of asparagine accumulated in the roots and stem (Rufty *et al*., 1993). Higher asparagine concentrations were also detected in the roots and shoots of young tobacco plants deprived of a P supply for 10 days (Rufty *et al*., 1990). Similarly asparagine accumulated in the nodules and roots of white clover subjected to decreasing concentrations of P (Almeida *et al*., 2000). In agreement with the increased asparagine concentration during nutrient deficiencies, a study on a non-model legume tree *V. divaricata (Adamson)*, an endemic legume showed similar characteristics, the accumulation of asparagine, under P deficient conditions (Magadlela (2013), M.Sc. thesis, Stellenbosch University, South Africa).

Several authors have come up with speculative theories due to the change in the amino acid biosynthesis. It has been mentioned by Lea *et al*. (2006) that, asparagine is a substrate for a few enzymatic reactions its soluble form, it therefore forms an ideal storage and transport N compound and accumulates under a range of nutritional stressed conditions, particularly in legume plants (Lea *et al*., 2006). Furthermore Almeida *et al*. (2000) said increased accumulation of asparagine may be a result of the regulation of the N feedback mechanism induced during P deficiency. However, it is also likely that during P deficiency, one of the biochemical adaptations of legumes, is increasing organic acid exudation (Tesfaye *et al*., 2007), that any excess oxaloacetate may be available for the transamination reaction glutamate to produce aspartate, where aspartate with glutamine produce asparagine (Prell & Poole, 2006).

In contrast, Le Roux *et al*. (2009) showed that in soybean nodules, there was an accumulation of ureides relative to amino acids under P starvation. Ramos *et al*. (2005) also found a five-fold increase ureides accumulation in nodules of water stressed soybean plants than watered soybean plants. The ureide strategy may be more conserving of photosynthates than the amide strategy (Todd *et al*., 2006). This concurs experimental derteminations 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. 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 value deduced were 5 ATP per N for allantoin or allantoate compared with 12 for asparagine, calculated from know biochemical pathways and assuming that the starting compounds are ammonium and phosphoglycerate. Even though there are other associated costs with the ureide strategy (Winkers *et al*., 1987), it can be concluded that the ureide strategy for N assimilation might be more cost effective than the amine strategy. The different N assimilation strategies might be adopted by fynbos legumes to conserve energy during nutrient deficiencies.

Although the incorporation of bacterial N during amino acid metabolism is now known to involve enzymes, GS and GOGAT in the reduction of (NH_3/NH_4^+) (Dubois *et al*. 2003), there may be another enzyme in the reduction of NH_3/NH_4^+. This other enzyme is the glutamine dehydrogenase (GDH), a mitochondrial NAD (H)-dependent enzyme, however in a number of species, an NADP (H) dependent activity found, associated with the chloroplast (Leech & Kirk, 1968; Lea & Thurman, 1972; Stewart

et al., 1980; Dubois *et al*., 2003). The GDH protein is an hexamer composed of two subunit polypeptides that differ in mass and charge, α and β subunits with seven isoenzymes (Magalhaes *et al*., 1990; Loulakakis *et al*. 1994; Purnell *et al*., 2005). The GDH protein has always been known for its capacity to fix ammonium *in vitro*, utilising organic acid 2-oxogluterate to synthesize glutamate which led to a number of proposals that GDH could operate in the direction of ammonium assimilation but k_m for NH_4^+ of GDH aminating activity is 100 time higher than of GS, therefore GDH might plays a negligible role in assimilation of NH_3/NH_4^+ (Yamaya & Oaks, 1987; Oaks, 1995; Melo-Oliveria *et al*., 1996; Frechilla *et al*., 2002). However later experiments demonstrated that GDH might operate in the direction glutamine deamination (Yamaya & Oaks, 1987; Fox *et al*., 1995; Oaks, 1995; Aubert *et al*., 2001). It was therefore concluded that excess GDH is rather involved in the recycling of carbon molecules by supplying 2-oxoglutarate to tissue that are becoming carbon limited (Robinson *et al*., 1992; Aubert *et al*., 2001; Miflin *et al*., 2002). Studies on *Daucus carota*, demonstrated that increases in GDH activity have been observed following carbohydrate starvation (Robinson *et al*., 1992; Athwal *et al*., 1997). This is also supported by the work of Melo-Oliveira *et al*. (1996) by a study of GDH genes in *A. thaliana* that showed an expression of one of the GDH genes, *gdh1-2/gdh2-1*, where it was at its highest in dark adapted or sucrose starved plants. Similar experiments have been performed on GDH on transformed tobacco and corn plants overexpressing bacterial GDH, both plants showed an increased tolerance to water stress, accompanied with increase biomass and yield (Ameziane *et al*. 1998; Ameziane *et al*. 2000). Therefore GDH functions in the catabolic direction, where it might help fuel the TCA cycle under stress conditions to balance cellular carbon: nitrogen ratios (Miflin & Habash, 2002; Dubois *et al*., 2003). Despite conclusive

evidence that GDH plays an important role in N and carbon metabolism, as a stress related enzyme in plants, no fundamental theory and regulatory behaviours of amino acid synthesis pathways have been presented, explaining the anabolic and catabolic functions of GDH due to nutrient deficiencies, specifically P in non-model legumes native to Cape fynbos nutrient poor soil ecosystems. Therefore substantial evidence still needs to be explored on the role of GDH, particularly in endemic legume plants that have been reported to be adapted to nutrient poor soils at the Cape fynbos.

1.7 Study species *Virgilia divaricata*

The Fabaceae is one of the most species-rich families in Fynbos (Goldblatt & Manning, 2000). *Virgilia* is a taxonomically isolated genus within the Fabaceae subfamily Papilionoideae (Van Wyk, 1986; Lewis, *et al*., 2005), and it is endemic to the fynbos in the southwestern and southern coastal regions of the CFR (Greinwald *et al*., 1989). *Virgilia* includes only two species, *V. divaricata* (Adamson) and *V. oroboides* (P. J. Bergius) Salter. These trees are small to medium-sized, with a bushy, rounded to broadly conical habit, with branches growing close to the ground (Goldblatt & Manning, 2000). *V. oroboides* is limited to the southwestern Cape coastal regions, from the Cape Peninsula to Swellendam, while *V. divaricata* occurs in the southern and eastern Cape, from Knysna to Port Elizabeth. They prefer well-drained soils and are tolerant to wind. As is the case with other legume species, the number of *Virgilia* individual decreases as fynbos vegetation matures (Power *et al*., 2010). During the present study the focus species was *V. divaricata* which was found to maintain its nutrition and biomass better so than *V. oroboides* under P deficiency (Magadlela *et al*., 2014, 2015). Furthermore, *V. divaricata* has been reported to grow

across a wide range of habitats including the mature fynbos even in the absence of fire and has been described as a forest precursor and it enhances fynbos soils fertility (Coetsee & Wigely, 2013).

1.8 Problem statement and proposed research

Power *et al*. (2010) identified a decrease in legume numbers with increasing veld age, the decrease may be ascribed to the intense energetic costs of N_2 fixation, which potentially limits the ability of competition in low light or low macro-nutrient (e.g. P) availability. Cocks and Stock (2001) suggested that post fire changes in the soil nutrient dynamics could be one of the most important factors that limit legumes in fynbos. The post fire environment provides a non-permanent flush in nutrient availability, especially in terms of phosphorus (Power *et al*., 2010). However, amongst other legumes, indigenous fynbos legumes *V. divaricata* are adapted for growth in these soils (Coetsee & Wigely, 2013; Vardien *et al*., 2014; Magadlela *et al*., 2014, 2015) and may display high phosphorus-use efficiency and specialized P and N uptake strategies. Therefore these adaptations enable indigenous fynbos legumes like *V. divaricata* to fix atmospheric N_2 under these low macro-nutrient soil conditions of the fynbos (Muofe & Dakora, 1999; Maistry, 2010; Power *et al*., 2010; Kanu & Dakora, 2012; Vardien *et al*., 2014; Magadlela *et al*., 2014, 2015).

More information is needed on the dynamics of P and N uptake and utility in legume plants in a wider range of fynbos soils, especially with regards to their influence on spatial variation in vegetation structure and nutrient cycling (Lynch & Ho, 2005; Maistry, 2010; Power *et al*., 2010). A better understanding of metabolic and

ecological costs associated with P and N acquisition and metabolising strategies is needed for the management and future conservation of fynbos legume species.

Therefore more information is needed to understand the mechanisms of legume adaptations to fix N_2 in a low P environment. Furthermore it is important to better understand the effects of P deficiency on the root nodule symbiotic partners and function of *V. divaricata*. This associated to the energy status during N assimilation and metabolism. The current research may also provide genetic insights into these physiological adaptations in *V. divaricata*, which may in turn be applied successfully in agriculture. The genes responsible for this adaptation to low P may be used in genetic modification, where they can be expressed in crop plants to enhance their growth in P-poor farm soils. This is highly relevant, given predictions that the world is going to run out of P-fertilizer in 30-50 years' time (Vance, 2003).

Forme mentioned studies on legume plants indigenous to Mediterranean-type ecosystem of the fynbos observed whole-plant physiological events, without any knowledge of the molecular biological components that underpin these phenotypic responses (Muofe & Dakora, 1999; Maistry, 2010; Power *et al*., 2010; Kanu & Dakora, 2012; Vardien *et al*., 2014; Magadlela *et al*., 2014, 2015). Most research presented throughout the litereature has been done on model legumes, which are herbaceous crops, with an annual growth form. Therefore, the use of a slow growing legume tree from a Mediterranean-type ecosystem to study the role of P deficiency in N nutrition is an interesting model because it is more representative of legumes from nutrient-poor, Mediterranean-type ecosystems.

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Chapter 2

General introduction

2.1 General introduction

2.1.1 *Virgilia*

The fynbos ecosystem in the Cape Floristic Region (CFR) is home to two *Virgilia* species, *Virgilia divaricata* (Adamson) and *V. oroboides* (Berg) (Van der Bank *et al*., 1996; Goldblatt & Manning, 2000; Coates Palgrave, 2002). Both are tree species in the family Fabaceae, with shiny to hairy, pinnate leaves and attractive mauve to pink, pea-shaped flowers and leathery pods (Van Wyk, 1986; Goldblatt & Manning, 2000). Both of these species occur in a range of fynbos from forest margins, besides streams or on river banks, hillside thickets on the narrow strip along the southeastern coast of South Africa (Greinwald *et al*., 1989; Van der Bank *et al*., 1996; Coetsee & Wigley, 2013). They are valued by gardeners as useful ornamental trees, as they are attractive, and suitable for both domestic gardens and big landscaped areas (Mbambezeli *et al*., 2003). *Virgillia* trees prefer well-drained soils, and are tolerant to wind. They have dense foliage relatively close to the ground, so they are useful as pioneer species for privacy and for wind protection (Mbambezeli *et al*., 2003). Their Afrikaans common name is Keurboom, which means ‘choice tree’ (Mbambezeli *et al*., 2003). In earlier times their wood was in high demand to be used as yokes on oxen. It was also used for spars, wagon-bed planks and rafters, and can be used for furniture (Goldblatt & Manning, 2000; Mbambezeli *et al*., 2003).

2.1.2 *Virgilia divaricata*

V. divaricata is a fast growing nitrogen fixing tree species often found along forest margins of the southern Cape. This tree species has been reported to be abundant

particularly after fires, but is able to grow in a wide range of soils the fynbos even in the absence of fire and has been characterised as a forest precursor (Coetsee & Wigley, 2013). Moreover, soils taken from *V. divaricata* stands had higher N and P value than the adjacent fynbos soils, enriching soils in a similar manner to the invasive accacias (Coetsee & Wigley, 2013). Furthermore compared to *V. oroboides*, *V. divaricata* is reported to grow well in fynbos P deficient characterised soils of the Cape fynbos by alterations in biomass allocations and improved efficiency of N acquisition and utilisation (Magadlela *et al*., 2014, 2015). In addition, these legume plants are able to maintain an effective symbiosis with N_2 fixing bacteria (*Burkholderia sp*.) to fix atmospheric N_2 in these environmental conditions (Goldblatt & Manning, 2000; Maistry, 2010; Magadlela *et al*., 2014). This implies that the legume has a functional tolerance for variable soil N and P levels.

2.1.3 Symbiotic N_2 fixation

Symbiotic N_2 fixation by the legume-*Rhizobium* symbiosis is a finely regulated process that involves significant carbon and energy and is vital to nutrient cycling in the biosphere (Sar & Israel 1991; Drevon 1997; Olivera *et al*. 2004; Valentine *et al*. 2011). Phosphorus deficiency is reported to impair both nodulation decreasing symbiotic N_2 fixation in legumes, thus affecting photosynthesis, respiration, growth and organic acid supply and production of the host plant (Sar & Israel, 1991; Drevon, 1997; Olivera *et al*., 2004; Valentine *et al*., 2011). The nodules of legume host plants require comparatively high amounts of P as an energy driver during N_2 fixation (Sar & Israel, 1991; Drevon, 1997; Olivera *et al*., 2004; Valentine *et al*., 2011). Most studies related to P deficiency during symbiotic nitrogen (N_2) fixation have been

conducted on fast growing, herbaceous legume plants. In contrast, few such studies have been conducted on Mediterranean type legume trees, especially legume trees indigenous to the P deficient soils of the Cape fynbos soil ecosystems. Given the nutrient poor state of these soils, fynbos legumes, specifically *V. divaricata* may display specific adaptive strategies to function under these challenging conditions (Vardien *et al*., 2014; Magalela *et al*., 2014, 2015).

Evaluating the physiological and biochemical reactions of *V. divaricata* during prolonged periods of P deficiency will aid our understanding of the carbon economy costs and efficiency of these plants to fix atmospheric N and energy status to assimilate N from different sources for plant use is important. Rhizobial strains experience different growing conditions, where nodulation, efficiency and capacity to fix atmospheric nitrogen are dependent on the soil pH, nutrient availability, climate and soil environmental conditions (Bordeleau & Prevost, 1994; Zahran, 1999). Determining the composition of the N_2 fixing bacterial population and the contribution of these bacteria to N_2 fixation in the P deficient environments of the Cape Fynbos, will enable us to recognise bacterial species that are able to fix atmospheric N_2 in these soil conditions. Legumes in nutrient poor soil environments may utilise two modes of nitrogen acquisition, either via symbiotic nitrogen fixation or via root acquisition of combined mineral nitrogen (Hellsten & Huss-Danell, 2000; Gentili & Huss-Danell, 2002; Gentili & Huss-Danell, 2003). Both root and nodule uptake and metabolism of nitrogen will impose a drain on energy and host carbon reserves. Such energy status and carbon economy during N assimilation associated with the N metabolising enzymes need to be investigated in order to enable better management of these trees with or without their symbiotic partners.

2.1.4 Nitrogen 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 & Miflin, 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 indicates that mineral nutrient deficiencies might have induced alternate pathways, stimulating large increases in asparagine concentration (Stewart & 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 atmosphere during P deficiency. This meaning that aspartate aminotransferase (AAT) may play a role in N and carbon metabolism, as 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 N_2 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 & 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 & Haaker, 1991; Wallsgrove *et al*., 1983). Glutamate dehydrogenase (GDH) had been originally regarded as the main ammonium assimilatory enzyme (Leech & Kirk, 1968; Lea & Thurman, 1972).

Lea & Miflin (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-oxoglutrate and the reverse catabolic reaction of the oxidative deamination of glutamate (Leham *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).

2.2 Hypothesis

Phosphorus deficiency alters the primary inorganic nitrogen assimilation and subsequent metabolism in the root nodules of *Virgilia divaricata*.

2.3 Research questions

1. What are the root nodule microbial profiles (N_2 fixing microbes) on P deficient plants of *V. divaricata* relying on two N sources (soil nitrogen (N) supplied as NH_4NO_3 and atmospheric N_2)?

2. During various P supply, does supplied N (NH_4NO_3) compete with atmospheric N_2?

3. Does various P supply affect carbon costs and bacterial efficiency?

4. Are there any N metabolic changes during N assimilation in the root and nodules during P deficiency?

3. What are the major branch points in amino acid biosynthesis during N assimilation and the controlling factors regulating these branch points in P deficient *V. divaricata*, supplied with soil N (NH_4NO_3) and atmospheric N_2?

4. Which glutamate dehydrogenase (GDH) transcripts are expressed during N metabolism in P deficient *V. divaricata* plants?

2.4 Research objectives

a) This study identified the rhizobial species that nodulate and fix atmospheric N_2 in *V. divaricata* nodules and their N_2 fixing efficiency during P deficiency. Moreover, assessed the C and N metabolism and the N product exported via xylem for plant use (Chapter 4).

b) In addition this study determined whether P deficiency affects the metabolic status of root and nodules and the consequent impact on the routes of N metabolism and assimilation in a fynbos legume, *V. divaricata* (Chapter 5).

c) Thereafter this study identified the glutamate dehydrogenase (GDH) transcripts expressed during N metabolism associated with P deficiency in *V. divaricata* (Chapter 6).

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Chapter 3

Pilot Growth Experiment

3. Pilot growth experiment

3.1 Introduction

The Mediterranean-type ecosystem of the Cape fynbos, in southern Africa mainly grows on sandstone-derived soils, which are typically very acidic and nutrient poor, specifically with regard to phosphorus (P) (Kruger *et al*., 1983, Goldblatt & Manning, 2000). Many studies have been conducted in this ecosystem of the Cape fynbos, these focusing on the growth and adaptations of indigenous legume plants in the Cape fynbos soils but very limited studies focused on *Virgilia divaricata* during P deficiency (Kanu & Dakora, 2012; Muofhe & Dakora, 1999; Power *et al*., 2010; Spriggs & Dakora, 2008). *V. divaricata* an indigenous Cape fynbos legume, which has been documented to be found along forest margins but has been reported to grow in a wide range of habitats this including the mature fynbos even in the absence of fire and has been characterised as a forest precursor (Coetsee & Wigley, 2013). Therefore, the aim of this growth experiment was to investigate *V. divaricata*'s performance during various P supply and subsequently select the two P levels which will set a baseline of the P conditions to be used throughout the study.

3.2 Methods and materials

Seeds of *V. divaricata* were obtained from Kirstenbosch Botanical Gardens, Cape Town, South Africa, and scarified using an acid-scarification method that entails soaking the seeds in sulfuric acid (H_2SO_4) for 30 minutes and then rinsing them 10 times in distilled (Magadlela *et al*., 2014). Thereafter seeds were treated overnight with diluted smoke water solution, which was also obtained from Kirstenbosch Botanical Gardens (Magadlela *et al*., 2014). The seeds were germinated in natural fynbos soils, randomly collected where *V. divaricata* grows and were used as natural

inoculum. Seeds were germinated and grown under the conditions in the glasshouse of the Department of Botany and Zoology, University of Stellenbosch, South Africa. The range of midday irradiances was between 600-800 µmol m^{-2} s^{-1} and the average night/day temperatures were 15-25 °C.

After the seedlings nodulated, they were transferred into pots with sterile quartz sand and initially watered with deionized distilled water for a week, to allow acclimatization. Thereafter, seedlings were supplied with a quarter strength Long Ashton nutrient solution (pH 5.8), which was modified to contain 500 µM NH_4NO_3 as the nitrogen source and one of the following P levels (1000; 500; 100; 25; 5 µM) as $NaH_2PO_4.2H_2O$ (Hewitt, 1966). The P and N levels were chosen in accordance to the CFR soil analysed for P and N nutrient, from the forest, forest margins to the fynbos ecosystem along the Knysna forest, southern coast of South Africa. The addition of 1000 µM P as a treatment was to examine if *V. divaricata* plants would show any toxicity signs during growth at that concentration of P. Seedlings were supplied with a nutrient solution once a week and watered with sufficient 200 ml of distilled H_2O in between the nutrient solution supply.

Saplings were harvested after 90 days after transplanting into the sand culture. On harvesting, the plant material was placed in a drying oven at 40 °C for a week and dry weight of plants were recorded. The dried material was milled with a tissue-lyser and the milled samples were analysed for their P concentrations, using an inductively coupled plasma-mass spectrometry (ICP-MS) with suitable standards by a commercial laboratory (Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa). Stress was dertemined according to biomass and P nutrient concentration.

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 (6-8) were separated using post-hoc Tukey's LSD (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences between treatments.

3.3 Results and discussions

Figure 1 (a and b) shows that the P supply increases, biomass and the total plant P concentration of *V. divaricata* increased. Greater stress was experienced by plants at the lowest level of P (5 µM) compared to the other P concentrations, thereafter 25 µM P. This data further presents that both biomass and P concentration are maintained at 100 µM P compared to 500 µM P. At 1000 µM P level, biomass and total plant P concentration are accumulated. Therefore at 1000 µM P, plants did not show any toxicity signs. This P range experiment data of 500 and 5 µM P is in alignment former studies on *V. divaricata* (Magadlela *et al*., 2014; 2015), where plants showed stress when supplied with 5 µM P and sufficient growth when supplied with 500 µM P.

These results are consistent with results from previous studies, where limited P nutrition reduced plant biomass and P accumulation in plants, while the inverse was observed where P supply is optimal (Sa & Israel, 1991; Al Niemi et al., 1998; Muofhe & Dakora, 1999; Almeida et al., 2000; Olivera et al., 2004; Hernández et al., 2007). Therefore the P levels (5 and 500 µM) were selected to serve as the baseline conditions for subsequent experiments.

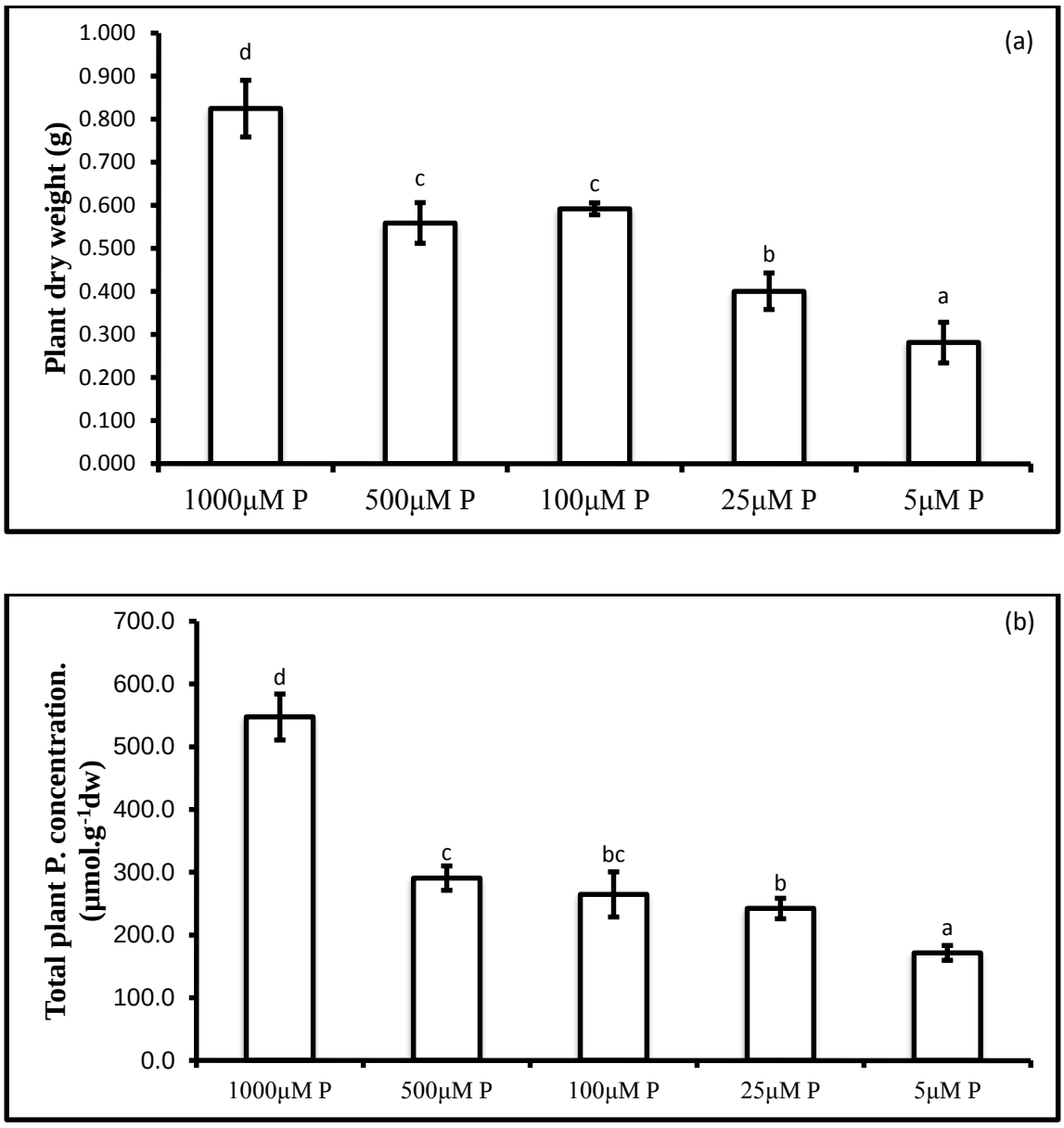

Fig. 1. (a) Plant dry weight and (b) Total plant P concentration of *Virgilia divaricata* saplings grown in sand culture under a P concentration range (1000; 500; 100; 25; 5 µM) for 90 days. Values are means (n=10) with standard error bars. Different letters indicate that the treatments are significantly different from each other . *P≤0.05.

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Chapter 4

Does P deficiency affect nodule bacterial composition and N source utilisation in a legume from nutrient-poor Mediterranean-type ecosystems?

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Does P deficiency affect nodule bacterial composition and N source utilisation in a legume from a nutrient-poor Mediterranean-type ecosystem?

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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, a nutrient-poor ecosystem in the Cape Floristic Region of South Africa. Although this implies that the legume has a wide functional tolerance for variable soil N and P levels, it is not known how the plant utilizes inorganic N under variable P supply. The aim of this experiment was therefore to identify the nodulating bacterial species and their biological N_2 fixing (BNF) efficiencies in *V. divaricata* during P deficiency. Furthermore, to integrate plants C and N metabolism to the N product exported via xylem to the shoots. Plants were grown at sufficient and low P levels, with four concentrations of inorganic N supply. At both levels of P, soil N supply reduced the reliance of legumes on BNF. Although the bacterial composition of nodules remained unchanged by P and N supply, the nodule function was greatly altered. In this regard, plants reliant on only N_2 at both P levels had higher and more efficient BNF, which resulted in greater plant N. This may have resulted from two physiological strategies at high and low P, when plants relied only on N_2 fixation. The decline in both sugars and organic acids may imply a reduced energy supply to the bacteroid during P stress. Furthermore altered bacteroid function may be inferred from BNF, and the N compounds synthesized and exported. At high P, plants exported more amino acids relative to inorganic N and ureides in their xylem sap, whereas at low P the plants exported more ureides relative to amino acids and NH_4. The bacterial tolerance for changes in P and N via nodule metabolites and xylem export might be a major factor that underpins the growth of *V. divaricata* under these variable soil conditions.

Keywords: P deficiency, legume, N_2 fixation, nodule bacteria, xylem export, metabolites

Introduction

Among the plant families indigenous to the fynbos, Leguminosae is one of the most species-rich (Goldblatt and Manning, 2000). Nevertheless it has been reported that legume species are mostly absent in the mature fynbos in the Cape Floristic Region (CFR). The fynbos is adapted to regular fires in order to maintain both the ideal microclimate and to disrupt the nutrient cycle associated with a tree dominated vegetation (Manders et al, 1992; Coetsee and Wigely, 2013). Cock and Stock (2001) have therefore suggested that post-fire changes in soil nutrient dynamics could be one of the most important factors limiting legume abundance in mature fynbos. Fynbos legumes are considered to be short lived, post fire colonizers in the ecosystem due to a temporary flush of nutrient availability, specifically phosphorus (P), which may sustain legumes until it is exhausted (Brown and Mitchell, 1986). In addition to the post fire soil nutrient dynamics, fynbos soils bear resemblance to soils of the Western Australian heathlands (Groves, 1983), which are leached, acidic sandy soils associated with low nutrient concentration, specifically with regards to P (Wisheu et al, 2000; Herppich et al, 2002). Fynbos soils contain about 58-77% organic P (Straker, 1996), but most of it is unavailable to plants due to complexion with cations (Ca, Fe), and under acidic conditions P ions can easily precipitate with cations (Dakora and Phillips, 2002) or bind to organic compounds by microbial action (Vance et al, 2003). Therefore P is generally present in micro molar concentrations or less for plant use in the fynbos soils (Maseko and Dakora, 2013).

The P concentrations of fynbos soils are extremely low to drive P-requiring metabolic processes, therefore P deficiency forms a critical constraint for plant growth,

especially for legume plants, as P is the vital energy driver during symbiotic nitrogen fixation (Maseko and Dakora, 2013; Sulieman et al, 2013). Generally legume plants require neutral to slightly acidic soils for growth and might experience problems with nodulation if the pH drops to a very acidic state. Soil acidity and nutritional disorders, including P deficiency have been reported to adversely affect not only legume plant growth but also the legume-rhizobia symbiosis as well as nitrogen-fixing efficiency (Lie 1981; Munns 1986; Graham 1992; Magadlela et al, 2014). Various legume fynbos species have been reported to implement different mechanisms so as to enhance P acquisition, in order to improve their capacity to fix atmospheric nitrogen (N_2). These mechanisms include specialized root adaptations such as root clusters, root exudates, root-mycorrhizal symbiosis, increasing phosphatase excretion in roots and nodules to mobilize P (Lamont, 1982; Allsopp and Stock, 1994; Muofhe and Dakora 1999; Horst et al, 2001; Vance, 2001; Dakora and Phillips, 2002; Spriggs et al, 2003; Power et al, 2010; Maseko and Dakora, 2013; Vardien et al, 2014). Muofhe and Dakora (1999) have reported that *Aspalathus linearis* (Brum.f) (Dahlg), nodulated by species in the genus *Bradyrhizobium*, and which is naturally tolerant to acidity as low as pH 3 also displayed the ability to modify the pH of its rhizosphere in order to promote symbiotic establishment (Muofhe and Dakora 1999). Elliot and colleagues (2007) also presented evidence that *Cyclopia* species can effectively be nodulated by *Burkholderia tuberum*. Members of the Psoraleae also form root nodules with a wide range of N_2 fixing bacteria, including *Rhizobium*, *Mesorhizobium* and *Burkholderia* strains and obtained 60-80% of their N nutrition from symbiotic N_2 fixation (Kanu and Dakora, 2012). Indicating their nodulation and adaptation to the low nutrient, acidic soils of the Cape Fynbos. Beukes et al. (2013) furthermore reported that diverse *Burkholderia* species are able to nodulate various fynbos

legumes species, including *Hypocalyptus*, *Virgilia* (specifically *oroboides*), *Podalyria* and *Cyclopia* and that these beta-rhizobia appear to be well-suited to the nutrient poor acidic soils of the Cape fynbos. Though these studies show nodule promiscuity and N_2 fixing efficiency of the legume plant and rhizobial partner/s, none regarded how soil nutrient dynamics, such as P deficiency, affect the rhizobial partner/s, nor the efficiency of the interaction. Fynbos legumes are expected to dominate the fynbos ecosystem as they possess a competitive advantage (their ability to fix atmospheric nitrogen), therefore their absence in mature fynbos is somewhat puzzling.

However *Virgilia divaricata* Adamson is reported to invade mature fynbos even in the absence of fire and has been described as a forest precursor, this species also enhances fynbos soil fertility (Coetsee and Wigely, 2013). *V. divaricata*, closely related to *V. oroboides* (P.J. Bergius) Salter, both indigenous to the CFR, are confined to the southwestern and southern coastal regions (Greinwald et al, 1989). Although some work has been done on the symbioses of Cape fynbos legumes and how it is affected by nutrient deficiencies, such as P deficiency (Muofhe and Dakora 1999; Dakora and Phillips, 2002; Spriggs et al, 2003; Power et al, 2010; Maseko and Dakora, 2013; Beukes et al, 2013; Magadlela et al, 2014, 2015; Vardien et al, 2014) little is known of how P deficiency affects the composition and efficiency of rhizobial species in the nodule, integrated with plants carbon (C) and nitrogen (N) metabolism. Symbiotic N_2 fixation involves the mutually beneficial exchange of reduced C from the plant for reduced N from the bacteria (Udvardi and Day, 1997).

Legumes fix atmospheric N_2 via bacterial symbiosis located in the nodules, such N fixation is fuelled by the provision of reduced C (sugars) from host plant fixed by

shoots (Molero et al, 2014). C fixed by shoots is invested in nodule metabolism required for nitrogenase activities and as carbon skeleton for amino acid synthesis (Marino et al, 2006). Nodulated legume can be classified in two groups based on the assimilation of fixed N (ammonia), and subsequent transport to the stems and leaves of the plant. It is generally accepted that in temperate legumes assimilate ammonium (NH_4) is catalyzed into amides, by glutamate synthetase (GS) into glutamine (Hirel and Lea, 2001). Recent studies have indicated that GS enzyme is able to regulate alternative metabolic pathways during N metabolism, and NH_4 can be used by aspartate synthetase (AS) instead of glutamine as nitrogen source for asparagine synthesis (Shi et al, 1997; Barsch et al, 2006). While for tropical leguminous species, it has been demonstrated that the principal compounds for transport and storage of fixed nitrogen in nodulated plants are ureides, allantoin and allantoic acid (Christensen and Jochimsen, 1983; Triplett, 1986).

As rhizobia acquire large amounts of ammonium to the host plants, great research efforts have been dedicated in order to understand the regulation and mechanisms of ammonium assimilation to model plants (Desbrosses et al, 2005; Barsch et al, 2006; Molero et al., 2014). Despite the considerable importance of such C and N relationship, little research efforts have been dedicated to understand the relationship in indigenous legume plants that are adapted to the low nutrient, acidic soils of the Cape fynbos, as P deficiency might cause metabolic variability.

Therefore this study aims to identify the rhizobial species that nodulate and fix atmospheric N_2 in *V. divaricata* and their N_2 fixing efficiency during P deficiency. Moreover, this study aims to assess the integration of the C and N metabolism, and the N product exported via xylem to the stems and leaves of the *V. divaricata*.

Material and Methods

Plant material and growth conditions

Seeds of *V. divaricata* were obtained from Kirstenbosch Botanical Gardens (Cape Town, South Africa). Seeds were scarified using an acid treatment that entailed soaking the seeds in 95-99% sulphuric acid (H_2SO_4) for 30 min and then rinsing them 10 times with distilled water. Thereafter seeds were treated overnight with diluted smoke water solution (also obtained from Kirstenbosch Botanical Gardens, Cape Town, South Africa). The seeds were germinated in fynbos soil samples, randomly collected from 9 plots in a range (from forest margins soils to mature fynbos soils) where *V. divaricata* grows at Knysna, southern coast, South Africa and these soil samples were used as natural inoculum. Germinated seeds were grown under the following glasshouse conditions: the range of midday irradiances was between 600-800 µmol m^{-2} s^{-1} and the average night/day temperatures were 15-25°C. After seedlings nodulated, they were transferred to pots with sterile quartz sand and initially watered with distilled water for a week, to acclimatize. Hereafter seedlings were supplied with quarter strength Long Ashton nutrient solution (pH 5.8), modified with either high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P seedlings were then supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source or exclusively relied on atmospheric N_2 fixation. Seedlings were supplied with nutrient solution once a week and watered with 200 ml of distilled H_2O in between nutrient solution supply. The experiment was split between treatments, low P and sufficient P and the N sources in both P levels (500 µM NH_4NO_3 as soil nitrogen (N) source or exclusively reliant on atmospheric N_2 fixation). The combination of the P

concentrations and N sources resulted in four treatments, with 35 replicates each treatment.

Harvesting and nutrient analysis

Harvesting (of xylem sap) occurred at 180 days after seedling emergence, during which the sap was extracted between 06:00-09:00 am from five plants in each treatment, using a pressure chamber instrument (http://www.pmsinstrument.com/products/model-600-pressure-chamber-instrument). The harvested xylem sap was then diluted 10 times and stored at -80˚C.

Thereafter plants were harvested, where fresh nodules were separated from the roots of 10 plants per treatment, surface sterilized by immersion in 70% ethanol for 30s, and in a 3% sodium hypochlorite solution for 3 minutes and were subsequently washed at least five times with sterile water, thereafter stored for bacterial extraction and sequencing.

During harvest ten plants were stored at -80˚C for metabolite profiling of polar metabolites in the roots and nodules. The remaining plants were separated into sections (nodules, roots, stems and leaves), where after the plant material was placed in a drying oven, at 50˚C for 10 days, in order to record the dry weights (DW) of each section. The dried material was ground with a tissue-lyser and were analysed for their respective C and P concentrations and $\delta^{15}N$ analyses 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 the Archeometry Department, University of Cape Town, South Africa).

Xylem sap analysis

These analyses included the determination of total amino acids, ureides, ammonia and nitrate according to Rosen (1957), Trijbels and Vogels (1966), Emmet (1968) and Cataldo et al. (1975) respectively.

Fynbos soil DNA extraction and purification

For microbial analyses, 9 soil subsamples of the fynbos soils used as the natural inoculum were analysed for bacterial composition using Denaturing Gradient Gel Electrophoresis (DGGE) at a commercial laboratory (MicroScie, University of Pretoria, Pretoria, South Africa). At MicroScie fynbos soils were maintained at 4°C until DNA could be extracted. Total DNA was extracted directly from 0.5 g of each soil sample using the NucleoSpin®Soil Kit (Macherey-Nagel). The DNA concentration was determined by agarose gel electrophoresis.

16S rDNA PCR

A portion of the 16S rDNA gene was amplified for (DGEE) through PCR analysis using identical methods as in Magadlela et al. (2015).

Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR product was subjected to DGGE analyses following the methods described by Muyzer et al. (1993). Twelve microlitres (ca. 250 ng) of 16S rDNA PCR product were loaded per lane using 40-55% denaturing gradient gels. Gels were run at 70 V for 17 hours at a constant temperature of 60°C. Image analysis was performed using the Gel2K (Norland, 2004) programme and fingerprints were analysed in a cluster analyses using CLUST (Norland, 2004). Of 58 dominant bands that were different between samples were compared and analysed for population diversity determination.

DGGE band sequencing

Sequencing the DGGE bands from the gel using the K/M primers above provided tentative species identification. The bands were outsourced to Inqaba Biotec (Pretoria, South Africa) for µpPCR (re-amplification) and sequencing. The 16S rDNA sequences obtained after amplification were all subjected to a BLAST analysis (Altschul et al, 1990) on the GenBank database and matching hits were selected.

Bacterial extraction, sequencing and phylogenetic analysis

Presumptive root nodule forming and N_2-fixing bacteria were isolated from the stored nodules (from ten plants per treatment) and grown on yeast mannitol agar (YMA), incubated at 28˚C. Culture purity was verified by repeated streaking of single colony isolates. A portion of the 16S rDNA gene was amplified for all the pure bacterial colonies through PCR reactions using the primers 27F (5'AGA GTT TGA TCC TGG CTC AG3') (Suau et al., 1999) and 485R (5'TAC CTT GTT ACG ACT TCA CCC

CA3') (Logan et al., 2000). Each 50 µl PCR reaction contained sterile milliQ water, 10x PCR reaction buffer (Inqaba Biotech, Pretoria), 10 µM of the respective primers, 2 mM dNTPs, 25 mM $MgCl_2$, 250 µM Super-Therm Taq DNA polymerase (Inqaba Biotech, Pretoria), 10 mg/ml BSA and diluted bacterial pure colony. Prokaryotic DNA amplification was performed on BioRad Mini Opticon thermal cycler (BioRad, South Africa) using the following protocol: initial denaturation for 5 min at 94°C, 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and elongation for 2 min at 72°C, followed by a final elongation step of 10 min at 72°C. The amplicons were viewed on a 1% agarose gel, and were confirmed to be of the expected size (1500 base pairs). Thereafter the 10 amplicons in each treatment were sequenced directly at The Central Analytical Facilities, Sequencing Facilities (Stellenbosch University, South Africa). The raw sequences files were viewed, edited (where necessary) and aligned using the following the software programmes BioEdit and Geneious version 8.0.2 created by Biomatters (http://www.geneious.com/). With regards to strain selection for the maximum-likelihood 16S rRNA phylogeny; all available type strain sequences for the environmental *Burkholderia* species were included. The type strain information as well as links to the database accession numbers can be found on the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net; Euzéby, 1997; Parte, 2013). There are however species included in the dataset which were not yet available on the list as of 14 October 2015, but for which published information is available *B. metalliresistens* (Guo et al., 2015), *B. ginsengiterrae* and *B. panaciterrae* (Farh et al., 2015) *B. rinojensis* (Cordova-Kreylos et al., 2013), *B. kirstenboschensis* (Steenkamp et al., 2015), *B. dipogonis* (Sheu et al., 2015), *B. humisilvae*, *B. solisilvae* and *B. rhizosphaerae* (Lee and Whang, 2015).

The original sequence trace files was manually edited with the software ChromasLite v2.01 (Technelysium, Queensland, Australia) and BioEdit v7.0.5 (Hall, 199). To verify the genus placement of the isolates *Blastn* searches were performed in GenBank ® (National Centre for Biotechnology Information http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990; Benson et al., 2004). To align the resulting dataset MAFFT v7 was used (Multiple Alignment using Fast Fourier Transformation; http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) and specifically the Q-INS-i strategy in order to take the secondary structure of the RNA molecule into consideration (Katoh and Standley, 2013). The best-fit evolutionary model (and parameters therefore) was determined with the programme jModelTest v2.1.7 (Dariba et al., 2012). Particulars for the chosen model appears next to phylogeny. A maximum-likelihood phylogenetic analysis was performed with PHYML v3.1 (Guindon, 2010; Guindon and Gascuel, 2003), with the default settings and branch support was calculated based upon 1,000 pseudoreplicates (Felsenstein, 1985).

Calculations of percentage nitrogen derived from the atmosphere (%NDFA)

The $\delta^{15}N$ analyses were carried out on the ground whole plant oven dried material at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of $\delta^{15}N$ was calculated as δ=1000‰ (Rsample/Rstandard), where R is the molar ratio of the heavier to the lighter isotope of the samples and standards are as described 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 $\delta^{15}N$ 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- $(NH_4)_2SO_4$. The %NDFA was calculated according to Shearer and Kohl (1986): %NDFA = 100((δ^{15}Nreference plant - δ^{15}Nlegume)/ (δ^{15}Nreference plant –B)). Where the reference plant was *Virgilia divaricata* grown under the same glasshouse conditions. The B value is the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *V. divaricata*, grown in a N-free culture. The B value of *V. divaricata* was determined as -2.58‰.

GC-MS extraction and metabolite profiling of polar metabolites such as sugars, amino acids and organic acids.

For sugars, amino acids and organic acids metabolite profiling using GC-MS, 10 plants harvested plants approximately 100 mg homogenized tissue (roots and nodules) was extracted with 1400 µl 100% pre-chilled methanol and 60 µl of ribitol was added as internal standard. The mixture was incubated for 15 min at 70°C, centrifuged for 10 min at 13000 rpm and the supernatant transferred to clean microcentrifuge tubes. To the supernatant, 750 µl chloroform and 1500 µl deionized water (dH_2O) was added, the samples vortexed for 1s and centrifuged at 4000 rpm for 15 min. From the polar phase, 150 µl was then dried down under vacuum and the dried residue subsequently re-dissolved and derivatized for 90 min at 30ºC in 40 µl 30 $mg.ml^{-1}$ methoxyamine hydrochloride (in pyridine). Subsequently samples were

trimethylsilylated by a 30 min treatment at 37ºC containing 140 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). n-Alkane retention time standard mixture was also added prior to trimethylsilylation. Samples were then injected splitless into a GC quadropole MS (ThermoFinnigan, UK) and run according to specifications of Roessner et al. (2001).

The analysis of primary metabolites was performed on a gas chromatograph (Agilent technologies network GC system, model 6890N), coupled to a Agilent technologies inert XL EI/CI Mass Selective Detector (MSD), model 5975B (Agilent Technologies Inc., Palo Alto, CA). The GC MS system was coupled to a CTC Analytics PAL autosampler. Separation of the metabolites (sugars, amino acids and organic acids) was performed on a Restek 12723-127 column (30 m, 0.25 mm ID, 0.25 µm film thickness), from Agilent Technologies. Analyses were carried out using helium as the carrier gas with a flow rate of 1.0 ml/min. The injector temperature was maintained at 250°C and the oven temperature programmed as follows: 150°C for 1 min; and then ramped up to 310°C at 7 °C/min and held for 2 min and finally ramped up to 320°C at 15 °C/min and maintained for 7 min. The total running time was 33.52 min. The MSD was operated in full scan mode and the source and quad temperatures maintained at 240°C and 150°C, respectively. The transfer line temperature was maintained at 280°C. The mass spectrometer was operated under electron impact mode at ionization energy of 69.92 eV, scanning from 50 to 650 m/z.

Data pre-processing for baseline correction, scaling and alignment was conducted with MetAlign software, with parameters as specified in MetAlign v 200410

(www.metalign.wur.nl/UK/). For targeted metabolite analyses such sugars, amino acids and organic acids, authentic standards and calibration curves were constructed and metabolite identities and annotations cross-checked with the Golm metabolome database (csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd).

Carbon and nutrition cost calculations

1) Construction costs, C_W ($mmolCg^{-1}DW$), were calculated according to the methods proposed by Mortimer et al. (2005), modified from the equation used by Peng et al. (1993):

$$C_W = (C + kN/14 \times 180/24)\ (1/0.89)\ (6000/180)$$

Where C_W is the construction cost of the tissue ($mmolCg^{-1}DW$), C is the carbon concentration ($mmolCg^{-1}$), k is the reduction state of the N substrate (k=-3 for NH_3) and N is the organic nitrogen content of the tissue ($g^{-1}DW$) (Williams et al. 1987). The constant (1/0.89) represents the fraction of the construction costs that provides reductant that is not incorporated into the biomass (Williams et al. 1987; Peng et al. 1993) and (6000/180) converts units of g glucose DW^{-1} to $mmolCg^{-1}DW$.

2) Specific N absorption rate (SNAR) ($mgNg^{-1}rootDWd^{-1}$) is the calculation of the net N absorption rate per unit root DW (Nielson et al. 2001):

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

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

3) Specific Nitrogen utilization rate (SNUR) ($gDWmg^{-1}Nd^{-1}$) is a measure of the DW gained for the N taken up by the plant (Nielson et al. 2001):

$$SNUR = [(W_2 - W_1/ t_2 - t_1)] \times [(\log_e M_2 - \log_e M_1)/ (M_2 - M_1)]$$

Where W is the plant DW and M is the N content.

4) Belowground allocation represents the fraction of new biomass partitioned into new roots and nodules over the given growth period. This was calculated according to Bazzaz (1997):

$df/dt = RGR\ (\partial\text{-}B_r/B_t)$

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

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 (6-8) were separated using post-hoc Tukey's LSD (SuperAnova for Macintosh, Abacus Concepts, USA) ($P \leq 0.05$). Different letters indicate significant differences between treatments.

Results

Bacterial species identity and diversity

PCR amplification of the nearly complete 16S rDNA gene was successful for both the fynbos soil inoculums as well as the *V. divaricata* root nodules. The GenBank BLAST results placed the majority of amplified sequences in the beta-rhizobium

genus *Burkholderia* (isolates with high sequence similarity were included in the phylogenetic analyses; Fig. 1). The results included several isolates of *B. phytofirmans* and frequently the undefined species, *Burkholderia* sp. N362 (Fig. 1). The remained of the isolates amplified from the soil are undefined isolates belonging to the alpha-rhizobium genus *Bradyrhizobium* (or belonged to the broader Bradyrhizobiaceae). All of the isolates from this study formed a monophyletic and highly supported (93% support) group (in which there was very little sequence diversity), together with *Burkholderia spp*. N362, FM-A and CYEB-3 as well as a an isolate labelled *B. phytofirmans* isolate PSB48 (Fig. 1). *Burkholderia dipogonis* LMG28415T and *B. phytofirmans* PsJNT (as well as *B. phytofirmans* isolate G44-5) formed an unsupported sister group to the isolates from this study. With regards to the placement of our isolates in relation to other nodulating South African isolates; the closest species were *B. dilworthii* WSM3556T and *B. rhynchosiae* WSM3937T (Fig. 1).

P and N nutrition

Low P levels caused a reduced P accumulation during growth in *V. divaricata* irrespective of the N sources (Table 1). Despite the effects of P supply, low P supplied plants amended with combined N maintained their percentage N derived from the atmosphere. There was a reduction in plants that were exclusively reliant on atmospheric N during low P supply compared to high P (Table 2). Furthermore external N supply reduced plants reliance on atmospheric N_2, which is presented by the reduction of % N derived from atmospheric N_2 irrespective of P supply (Table 2). This reduced plant reliance to atmospheric N_2 is also presented by the N fixing

efficiency per unit nodule mass (Table 2) and specific atmospheric N absorption rate (SNAR) (Table 2). N fixing efficiency per unit P was influenced by P supply, with high P supplied plants showing a reduction in efficiency when compared to low P plants. Even though low P plants showed an increase in N_2 fixing efficiency, low P plant exclusively reliant on atmospheric N_2 had a higher N_2 fixing efficiency than low P plants amended with external N (Table 2).

High P plants amended with external N accumulated more total plant N concentration than low P plant amended with external N, this was the same in plants exclusively reliant on atmospheric N_2 (Table 2). Plants also showed variation in N concentration from different sources (Table 1). Furthermore plants also showed no significant difference in specific soil N absorption rate (SNAR) between the treatments except the high P plants that were exclusively reliant on atmospheric N_2, which showed an increase in SNAR (Table 2). Low P plants amended with external N showed an increase in specific N utilization rate (SNUR), while there was no significant difference between the other treatments.

Nodule metabolites

The variation in P and N supply affected nodule metabolites. During low P there was reduction in the sucrose and glucose concentration in the plants that are exclusively reliant on atmospheric N_2, where it was maintained in all other treatments (Table 3). This pattern was the same for the organic acids, malic acid and succinate during P stress in plants, irrespective of the form of N at each P level, except the significant

difference in malic acid between plants amended with external N. Furthermore there was an increase in the primary amino acids, glutamic acid and aspartic acid in the high P plants exclusively reliant on atmospheric N_2 than the other treatments. Glutamic acid concentration in the low P nitrogen mended plants showed an increase compared to high P nitrogen amended plants and low P atmospheric N_2 reliant plants (Table 3).

In the roots the major variation were seen on the principal sugar, sucrose and organic acids, malic acid. Where there is a significant difference between the low P plants. The low P supplied plants exclusively reliant on atmospheric N_2 with much higher accumulation of sucrose, though there was no significant difference compared to high P plants irrespective of N supply (Table 3). Furthermore there was an increase in malic acid concentration in the low P plants compared to high P supplied plants, exclusively reliant on atmospheric N_2. In contrast high P supplied plants amended with external N had a higher accumulation of malic acid than low P plants supplied with the same N environment (Table 3).

N assimilation

There was a change in the root nodule N export product into the xylem sap. *V. divaricata* exported mostly amino acids, then ammonium and ureides (Table 2) from the root nodule to the xylem in all treatments. The high P supplied plants showed an increase in amino acid concentration when compared to the low P plants. The high P plants exclusively reliant on atmospheric N_2 had the highest amino acid

concentration, followed by high P plants amended with external N, then low P plants amended with N and the low P plants exclusively reliant on atmospheric N_2 (Table 2). Also noteworthy was the fact that the plants that were amended with external N had increased ureide concentrations compared to the other plants (Table 2).

High P plants amended with external N had the highest ammonium exported to the xylem. Low P plants exclusively reliant on atmospheric N_2 had the lowest ammonium exported and there was no significant difference between the other treatments.

V. divaricata plants were able to alter their export products to favor ureides, more than ammonium and amino acids during low P supply in comparison to high P supplied plants (Table 2).

Biomass and growth kinetics

In spite of P supply, the growth of the plants was maintained, this is shown by the biomass of the plants (Table 1). Nevertheless plants amended with external N accumulated the most nodule biomass irrespective of the P supply compared to plant exclusively reliant on atmospheric N_2. Root biomass followed the same pattern, while there was no significant difference between shoot biomass (Table 1). There was no significant difference in relative growth rate and root allocation through the treatments (Table 1). N supplies seem to have affected nodule allocation, as there is a significant difference when different N sources are used (Table 1). High P plants amended with external N show an increase in C construction costs in comparison to low P plants grown in the same N environment. There is no significant difference in plants solely reliant on atmospheric N_2, irrespective of the P supply. High P plants

amended with external N show an increased growth respiration while there is no significant difference between the other treatments (Table 1). There is an increased root: shoot ratio in low P supplied plants, more significantly in plants exclusively reliant on atmospheric N_2 with no significant difference in the plants amended with external N.

Discussion

It appears as though isolates from the undefined *Burkholderia* sp. N362 are able to form functional nodules with the host *Virgilia divaricata* (Fig. 1). These isolates appear (as far as can be determined based upon 16S rRNA phylogenetic analyses) to remain the preferred symbiont of *V. divaricata* even during P deficiency and in spite of the altered nodular sugar and organic acid concentrations. However approaches such as Multi-Locus Sequence Analysis (MLSA) and rep-PCR analysis should provide better clarification on the species identity of these isolates and whether or not it changes during treatments as used in this study. Our results do indicate that nodule function was greatly influenced by P deficiency, as evidenced by the reduced reliance on biological N_2 fixation (BNF) and the alteration of xylem export products in organic and inorganic N forms.

The initial fynbos soil inoculum exposed the roots to a variety of soil bacteria, some of which could potentially be capable of nitrogen-fixation (either as endophytes or rhizobia) and plant growth promotion. The genus *Burkholderia* was however dominant in both the soil samples as well as in the resulting nodules. Indigenous fynbos legumes such as *Psoralea*, *Hypocalyptus*, *Podalyria*, *Cyclopia* and *Virgilia oroboides* (closely related to *V. divaricata*), are reportedly nodulated by a variety of

bacteria including species of *Burkholderia* (Elliot et al, 2007; Kanu and Dakora, 2012; Beukes et al, 2013). Thus far nodulating species of *Burkholderia* have been shown to be prominent in the Cape fynbos (Beukes et al, 2013; Lemaire et al, 2014); and although it has been hypothesized that physical factors such as soil acidity or elevation (Bontemps et al, 2010; Lemaire et al, 2014) predisposes these legumes to associations with beta-rhizobia, perhaps it is the effect of the most basic factor such as soil dynamics and the ability of members of the *Burkholderia* to better withstand changes in these dynamics.

Some of the nodule isolates showed high 16S rRNA sequence similarity to an isolate of *Burkholderia phytofirmans*. The type strain of this species (PsJN^{T}) does not possess the ability to fix atmospheric nitrogen or form nodules (Weilharter et al., 2011); however we would not be able to rule out the possibility that isolates of this species could potentially acquire both or either of these abilities through horizontal gene transfer (Springael and Top, 2004; Taghavi et al, 2005; Tuanyok et al, 2007; Juhas et al, 2009). This *Burkholderia* species is however a well-known endophyte (a bacterium present in plant tissue without causing harm; Bacon and Hinton, 2006) with plant growth-promoting properties which appear to be due to the effect this bacterium has on plant growth hormones (Kurepin et al, 2015). Endophytic plant growth-promoting *Burkholderia* isolates (lacking a *nodA* locus) has been found in *Lespedeza* root nodules (Palaniappan et al, 2010). The second *Burkholderia* isolate to which the root nodule isolates showed high similarity (*Burkholderia* sp. N362) does in fact possess the full complement of *nod* and *nif* genes (W.Y. Chan pers. comm.). These isolates remained persistent in the nodules, despite the P-deficient changes in nodule's metabolic profile. Carbon supplied to the bacteroid to fuel BNF is ultimately derived

from photosynthates, in the form of sucrose, transported to the nodules via phloem, this sucrose is further metabolized to produce malate via Phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) (Streeter, 1981; Reibach and Streeter, 1983; Vance et al., 1985; Streeter, 1985; Rosendahl et al, 1990; Lodwig and Poole, 2003). During P deficiency, the reduction in the concentration of sugars (sucrose and glucose) and organic acids (malate and succinate) in nodules reliant solely on BNF, may indicate a reduced energy supply to the bacteroids. Nonetheless, this rhizobial partner was able to fulfill its function despite drastic changes in external nutrient supply or internal nodule metabolic changes. The bacterial composition of the resulting nodules remained therefore unchanged, in spite of the initial soil inoculum containing a variety of possible N_2-fixing and nodulating partners and the change in soil dynamics during growth (Figure 1; 16S rRNA phylogeny). The constant host preference for this specific *Burkholderia* isolate (from soils across the native range of the legume) may indicate a very specific host-symbiont relationship and could be indicative of ecosystem-specific co-evolution. In spite of the tight symbiotic relationship, the functional consequence of these altered nodule bacteroid functions, was evidenced by the N_2 fixation and the consequent N metabolism and export.

In this regard, the higher N acquisition in P-stressed legumes exclusively dependent on N_2, was likely due to the efficient functioning of the bacteroids in the nodules. The BNF efficiency per nodule mass investment was higher when N was solely derived from atmospheric N_2 and not amended with soil N. However there was a reduction in the atmospheric N_2 fixation during low P supply, which may be related to the reduced organic acid fuel found in nodules (Silsbury et al, 1986; Israel, 1987; Nelson and Edie, 1991; Qiao et al, 2007). In spite of this reduction, BNF efficiency per unit P was

always higher in low P treated plants, implying that the nodules can function efficiently with low P supply. This concurs with other studies where P-stress induced a higher BNF efficiency in the host plants (Magadlela et al, 2014; Vardien et al, 2014; Magadlela et al, 2015). In view of this reduction BNF, the increased efficiency per unit P may have been based on the nodule capacity to use C and energy more sparingly in order to maintain this efficiency.

One possibility is the alterations of the N export products from the nodules into the xylem sap. In this regard, the nodules were able to alter the export products to favor more ureide export during P stress. The benefit of ureide export during P stress is that a more N-dense form of N is being exported to shoots, compared to AA or NH_4 (Atkins, 1991; Todd et al., 2006). Furthermore the ureide exporting strategy may conserve more photosynthate than the amide export strategy. Studies have shown that ureide export is generally more carbon economical, requiring as little as 1.4 g C g^{-1} fixed N compared with amide exporters with minimum of 3.9 g C g^{-1} fixed N (Atkins, 1991; Todd et al., 2006). The preference of more ureides exported during P stress concurs with a study by Le Roux et al. (2009), where legumes accumulated more ureides relative to amino acids during P stress. The potentially lower costs of ureide export would have affected the plant's C budget, and have a positive impact on growth.

In spite of P stress, growth of the plants was not affected, as these C and energy saving mechanisms appear to mitigate the stressful growth conditions and could play a part in plant adaptation to P-poor soils. This concurs with previous work done on *V.*

divaricata, inoculated with natural fynbos soils (Magadlela et al., 2014). The legume plant maintained its biomass by the alteration in biomass allocation to nodules and their improved efficiency in N acquisition and utilization (Magadlela et al., 2014). In contrast, similar work done on *V. divaricata* plants inoculated with a pure *Burkholderia* strain, where a decreased plant biomass was reported during P stress (Vardien et al., 2014). This difference might be related to N_2 fixing efficiency of the nodules and the associated costs of BNF to the host. The differences in the ratio of ureides: AA and the ureides: NH_4 ration, between low P and high P plants may explain why the growth respiration is lower in low P than high P supplied plants (Atkins, 1991; Todd et al., 2006). This alteration in N export may account for the costs of growth, thereby allowing similar biomass productions.

Conclusion

The bacteroid tolerance for changes in P and N supply is evident in nodule metabolite changes and N xylem export products. These functional tolerances may be major factors that underpin the growth of *V. divaricata* under these variable soil conditions, ranging from forest margins to mature fynbos. Moreover, this single bacteroid species preference of *V. divaricata* may imply a functional co-existence in a variable nutrient environment.

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Table 1 Biomass, growth kinetics and plant mineral concentrations of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source or exclusively reliant on atmospheric N_2. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

	Treatments			
Parameters	500 µM		5 µM	
	N+Bact	Bact	N+Bact	Bact
Dry weights				
Plant (g)	7.67±0.860 a	6.72±0.903 a	8.97±0.483 a	6.82±0.535 a
Shoot (g)	4.40±0.040 a	5.21±0.582 a	5.08±0.172 a	4.68±0.319 a
Roots (g)	2.42±0.884 ab	1.07±0.288 a	3.00±0.508 b	1.65±0.239 ab
Nodules (g)	0.85±0.135 ab	0.44±0.041 a	0.90±0.213 b	0.49±0.100 ab
Growth kinetics				
Relative growth rate ($mg.g^{-1}.day^{-1}$)	7.66±0.860 a	6.71±0.90 a	8.96±0.480 a	6.81±0.530 a
Root allocation ($mg.g^{-1}.day^{-1}$)	4.34±0.050 a	5.09±0.59 a	5.02±0.170 a	4.60±0.320 a
Nodule allocation ($mg.g^{-1}.day^{-1}$)	0.09±0.030 b	0.02±0.001 a	0.08±0.040 b	0.03±0.010 a
C construction costs ($mmolC.g^{-1}dw$)	0.13±0.001 c	0.124±0.001 b	0.108±0.001 a	0.125±0.001 b
Growth respiration (µmol $CO_2.day^{-1}$)	140.6±35.0 b	58.5±21.29 a	51.0±9.002 a	54.1±9.363 a
Root:shoot	0.55±0.040 bc	0.20±0.030 a	0.60±0.120 c	0.35±0.040 b
Plant Mineral Concentrations				
P conc ($\mu mol.g^{-1}$ dw)	88.9±0.620 c	101.2±2.27 d	55.0±1.420 b	34.9±0.820 a
N conc fixed from atm ($mmolN.g^{-1}$ dw)	1.21±0.150 ab	1.33±0.360 c	1.05±0.140 a	1.18±0.310 bc
N conc assimilated by roots ($mmolN.g^{-1}$ dw)	0.83±0.130 ab	1.35±0.350 b	0.66±0.111 ab	0.83±0.310 a

Table 2 Nitrogen (N) data of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source or exclusively reliant on atmospheric N_2. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

	Treatments							
	500 µM				5 µM			
Parameters	N+Bact		Bact		N+Bact		Bact	
percentage N. derived from the atm (%NDFA)	52.9±1.004	a	82.6±0.465	c	50.6±3.697	a	73.7±2.049	b
N. fixing efficiency (mmolN.g^{-1}.g^{-1} nodule)	1.47±0.193	a	3.13±0.840	b	1.22±0.176	a	2.63±0.833	b
N. fixing efficiciency (mmolN. µmol^{-1}P.g^{-1})	0.60±0.012	a	0.81±0.021	a	0.93±0.084	b	2.17±0.102	c
Total plant N conc. (mmol N.g^{-1} DW)	2.05±0.071	b	2.68±0.021	c	1.71±0.045	a	2.01±0.074	b
Specific N. absorption rate (atm) (mmolN.g^{-1}nodule.d^{-1})	0.037±0.01	a	0.194±0.03	c	0.025±0.01	a	0.112±0.02	b
Specific N. absorption rate (soil) (mmolN.g^{-1}roots.d^{-1})	0.010±0.004	a	0.031±0.009	b	0.004±0.002	a	0.007±0.002	a
Specific N. utilization rate (g DW.mg^{-1}N.d^{-1})	1.369±0.27	a	0.583±0.11	a	3.632±0.49	b	1.031±0.24	a
Amino acids concentration (mmol. µL^{-1})	0.048±1.8E03	c	0.068±4.0E-03	d	0.031±7.8E-04	b	0.019±1.0E-03	a
Ureides concentration (mmol. µL^{-1})	0.0006±3E-05	b	0.0004±4E-06	a	0.0006±3E-05	b	0.0004±2E-05	a
Ammonium concentration (mmol. µL^{-1})	0.005±4E-05	c	0.0032±4E-05	b	0.0031±0E+00	b	0.0027±4E-05	a
Ureide:Amino acids	0.0129±0.001	b	0.006±0.0004	a	0.021±0.001	c	0.023±0.003	c
Amino acids:NH4	9.653±3E-01	b	21.09±1E+00	c	10.164±2,5E-01	b	6.899±3,4E-01	a
Ureide:NH4	0.125±6E-03	b	0.004±3E-05	a	0.207±9E-03	d	0.156±9E-03	c

Table 3 Metabolite data (sugars, amino acids and organic acids) of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source or exclusively reliant on atmospheric N_2. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05).

		Treatments							
Plant organ	**Parameters**	500 µM				5 µM			
		N+Bact		Bact		N+Bact		Bact	
Roots	**Sugars (µmol.g^{-1}dw)**								
	Sucrose	109.39±9.00	ab	89.03±12.50	ab	30.71±4.20	a	151.23±10.20	b
	Glucose	1.40±0.20	a	1.59±0.60	a	1.08±0.10	a	2.82±1.40	ab
	Fructose	5.86±0.60	ab	5.01±1.30	ab	6.15±0.20	b	6.69±0.80	b
	Xylose	0.19±0.10	a	0.04±0.01	a	0.01±0.01	a	0.06±0.01	a
	Ribose	0.19±0.01	a	0.19±0.10	a	0.16±0.01	a	0.43±0.20	ab
Nodules									
	Sucrose	174.34±51.50	b	307.08±59.80	c	137.87±22.70	b	102.73±0.50	ab
	Glucose	8.50±4.20	c	9.75±1.80	c	7.90±0.80	bc	1.42±0.10	a
	Fructose	3.13±0.70	ab	6.94±2.50	b	4.46±0.70	ab	2.13±0.20	a
	Xylose	0.18±0.10	a	0.08±0.01	a	0.07±0.01	a	0.03±0.01	a
	Ribose	1.24±0.70	bc	1.38±0.20	c	0.57±0.10	abc	0.19±0.01	a
Roots	**Amino acids (µmol.g^{-1} dw)**								
	Glutamic acid	0.01±0.01	a	0.02±0.01	a	0.03±0.001	a	0.13±0.09	ab
	Aspartic acid	0.01±0.001	a	0.06±0.03	a	0.02±0.01	a	0.11±0.06	ab
	Alanine	2.54±1.38	b	0.64±0.29	a	1.43±1.26	ab	1.92±1.04	ab
	Lysine	0.00±0.00		0.001±0.001	a	0.002±0.001	a	0.02±0.001	a

	Threonine	0.00±0.00		0.01±0.01	a	0.01±0.001	a	0.12±0.001	b
	Cystine	0.01±0.001	a	0.01±0.001	a	0.01±0.001	a	0.02±0.001	a
	Glycine	0.43±0.03	ab	0.46±0.06	ab	0.48±0.05	ab	0.57±0.14	ab
	Proline	0.08±0.01	ab	0.08±0.03	ab	0.08±0.01	ab	0.13±0.05	ab
	Luecine	0.02±0.001	a	0.08±0.03	a	0.06±0.01	a	0.10±0.06	a
	Valine	0.004±0.001	a	0.02±0.01	ab	0.02±0.001	ab	0.01±0.001	ab
	Isoleucine	0.003±0.001	a	0.01±0.001	a	0.01±0.001	a	0.01±0.001	a
Nodules									
	Glutamic acid	0.03±0.02	a	0.45±0.01	c	0.19±0.06	b	0.08±0.02	ab
	Aspartic acid	0.01±0.001	a	0.19±0.03	b	0.05±0.01	a	0.02±0.01	a
	Alanine	3.08±2.94	b	0.25±0.10	a	0.11±0.05	a	0.06±0.02	a
	Lysine	0.03±0.001	a	0.08±0.001	b	0.02±0.001	a	0.01±0.001	a
	Threonine	0.04±0.02	a	0.06±0.01	a	0.03±0.001	a	0.01±0.001	a
	Cystine	0.04±0.001	a	0.01±0.001	a	0.01±0.001	a	0.01±0.001	a
	Glycine	0.81±0.26	bc	1.15±0.06	c	0.64±0.11	ab	0.40±0.03	a
	Proline	0.19±0.08	b	0.34±0.03	c	0.19±0.04	b	0.05±0.001	a
	Luecine	0.15±0.07	a	0.32±0.03	b	0.11±0.02	a	0.05±0.01	a
	Valine	0.06±0.03	b	0.04±0.01	ab	0.03±0.001	ab	0.01±0.001	a
	Isoleucine	0.03±0.01	a	0.03±0.001	a	0.01±0.001	a	0.01±0.001	a
Roots	**Organic acids (µmol.g^{-1}dw)**								
	Malic acid	4.27±0.39	bc	1.49±0.49	ab	0.85±0.09	a	5.27±1.30	c
	Succinate	0.20±0.04	a	0.09±0.01	a	0.09±0.02	a	0.30±0.11	ab
Nodules									
	Malic acid	11.16±1.82	c	11.40±0.49	c	4.29±0.77	ab	2.53±0.31	a
	Succinate	0.68±0.33	bc	0.70±0.01	c	0.35±0.08	abc	0.10±0.01	a

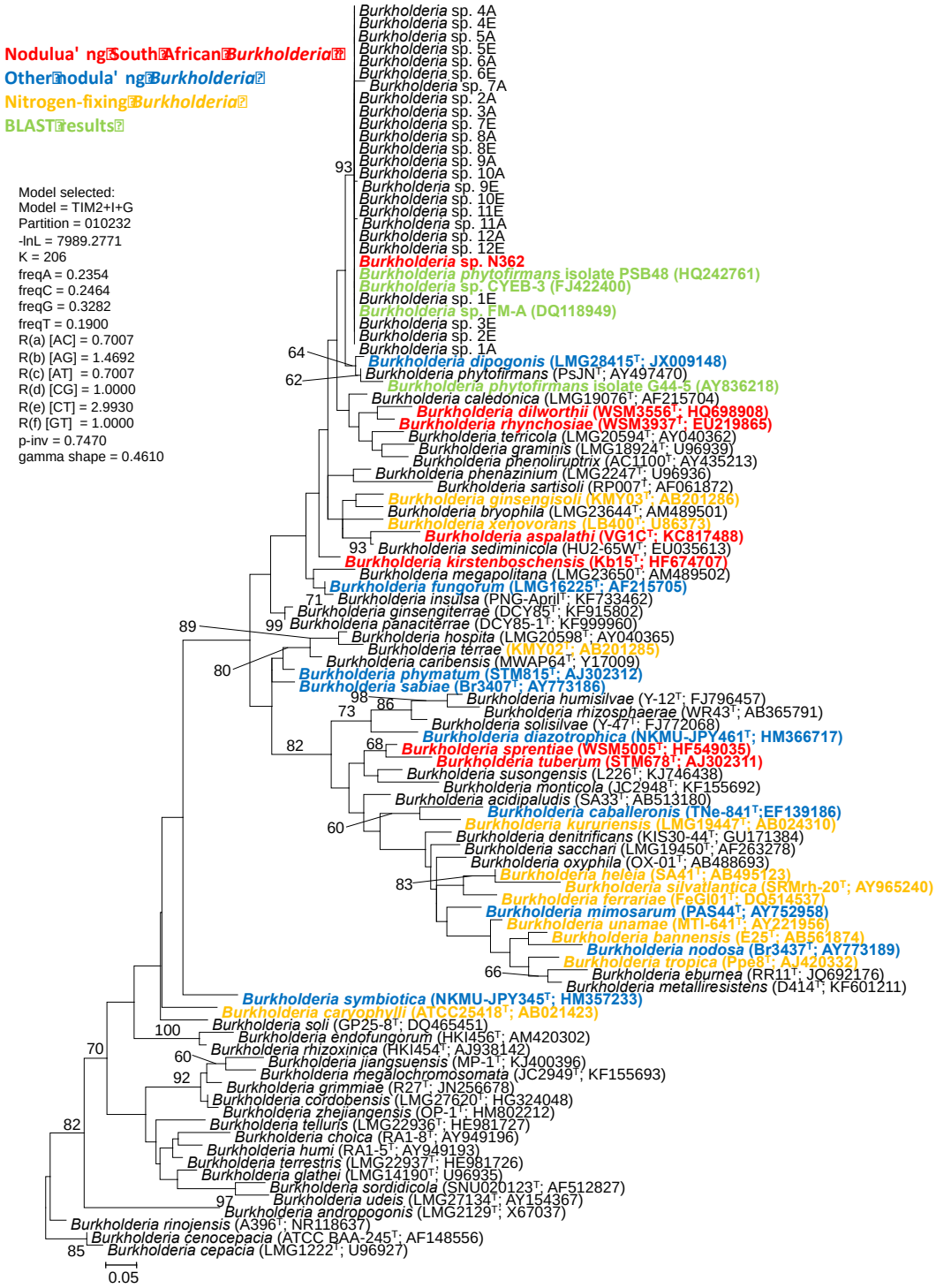

Figure 1 A 16S rRNA maximum-likelihood phylogeny for the 'environmental' *Burkholderia* isolates. Only bootstrap support of ≥ 60% are indicated. The sequence information for the

type strains of *B. cepacia* and *B. cenocepacia* were used as the outgroup. Nitrogen-fixing, nodulating and nodulating South African species are all indicated in various colours, with the isolates resulting from BLAST searches are in green. **(Nodulating South African *Burkholderia*, Other nodulating *Burkholderia*, Nitrogen-fixing *Burkholderia*, BLAST results)**. Listed in parentheses next to each species is their type strain number (if applicable) and GenBank accession number.

Chapter 5

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

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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 PPi-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, N_2 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; AAT, aspartate amino transferase; 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 complexion 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* (Adamson) 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*, 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 Miflin 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 N_2 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 Miflin (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-oxoglutrate 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 (H_2SO_4) 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 µmol m^{-2} 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 $NaH_2PO_4.2H_2O$ and 500 µM NH_4NO_3.

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 50 mM KH_2PO_4 buffer, pH 7.5, containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpyrrolidine

(PVP). Complete Protease Inhibitor Cocktail tablet (Roche) was dissolved in the buffer. The homogenate was then centrifuged at 3,000 g for 5 min at 4 °C, thereafter the supernatant was centrifuged at 30,000 g for 20 min 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 100 mM KH_2PO_4 buffer, pH 7.5, 100 mM KNO_3, 2 mM NADH and root or nodule extract, then incubated at 30 °C for 30 min. Thereafter the reaction was stopped by the addition of 1% (w/v) of sulphanilamide in 1.5 M HCl and 0,02% (w/v) of n-l-napthy-ethylenediamine dihydrochloride solution. All samples centrifuged at 500 g for 5 min to remove any suspended matter and activities measured spectrophotometrically, monitoring the absorbance at 540 nm.

GS activities were assayed following the methods of Kaizer and Lewis (1986), in a reaction medium containing 250 µl of imidazole (pH7.2), 20 mM $MgSO_4$, 25 mM hydroxylamine, 100 mM glutamate, 10 mM 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 500 nm.

GOGAT activities were assayed spectrophotometrically, monitoring the oxidation of NADH at 340 nm at 30°C for 15 min according to Groat and Vance (1981). The assay medium consisted of 1mM Na_2-EDTA, 25 mM 2-oxoglutarate, 10 mM aminooxyacetate, 0.15 mM NADH, 10 mM L-glutamate and 0,1% (v/v) 2-mercaptoethanol in 50 mM potassium phosphate buffer, pH 7.5.

AAT activities were determined according to González *et al*. (1995) in a reaction medium containing 4mM $MgCl_2$, 10 mM aspartic acid, 0.2 mM NADH, 1 mM 2-oxoglutarate and 50 mM Tris-HCl buffer, pH 8.0.

Animating GDH activities were assayed according to Glevarec *et al*. (2004) by following the oxidation of NADH at 340 nm. The reaction mixture contained Tris-HCl (100 mM, pH 8), 1 mM $CaCl_2$, 13 mM 2-oxoglutarate, 50 mM $(NH_4)_2SO_4$ and 0.25 mM NADH. Deaminating-GDH activity was assayed by following the reduction of NAD. The reaction mixture contained Tris-HCl buffer (100 mM, pH 9) supplemented with 1 mM $CaCl_2$ (pH 9), 33 mM Glu and 0.25 mM NAD or NADP.

For activities of Xanthine dehydrogenase (XDH) and uricase the extraction medium consisted of 25 mM TES-KOH buffer, pH 7.5, containing 0.15 M sorbitol and the macerates were centrifuged at 3500 g for 15 min. XDH and uricase activities were determined following the procedure of Schubert (1981). For the XDH activity the reaction medium consisted of 3.5 mM NAD^+, and 0.5 mM hypoxanthine in 50 mM TES-KOH buffer, pH 8.4. For uricase

assay, the reaction medium consisted of 50 mM uric acid in 85 mM glycine-KOH buffer pH 9.0.

Inorganic phosphate

A frozen sample (about 0.5 g) was homogenized in 1 ml 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 10,000 g 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 0.5 M H_2SO_4 (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 (9 g 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 15,000 g for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M $KHCO_3$ to approximately pH 5.0. The supernatant was then centrifuged at 10,000 g for 10 min 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 2.5 mL of water containing 10% (v/v) ^2H_2O, neutralized to pH 7.5 and

buffered with 50 mM 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 $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town, South Africa. The isotopic ratio of $\delta^{15}N$ was calculated as δ=1000‰ (Rsample/Rstandard), 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 $\delta^{15}N$ 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 $(NH_4)_2SO_4$.

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

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

Where *B* is the $\delta^{15}N$ 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) (mg N. g^{-1} root DW. d^{-1}) is the calculation of the net N absorption rate per unit root DW (Nielson *et al*., 2001):

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

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). 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 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 initial amount of N, 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 (P*i*) 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 PPi-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 $NH_4^+NO_3^-$ as an inorganic N source, the increases in NR activity, deamininating 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 GHD

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 of 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

Phosphorus 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.

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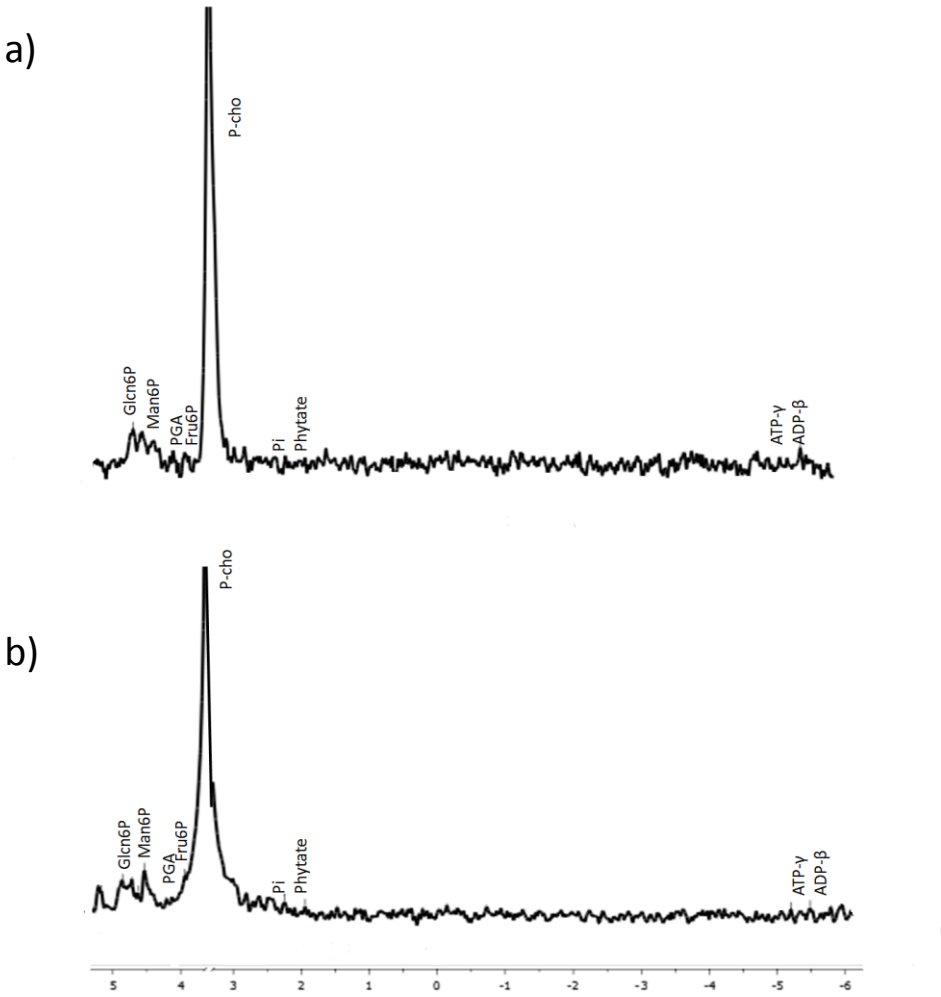
Table 1 Total plant dry weights, total plant P concentrations and uricase and xanthine dehydrogenase (XDH) activities of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

		Treatments			
Total Plant		500 µM P		5 µM P	
	Dry weight	0,56±0,042	b	0,28±0,031	a
	P concentration (µmol.g^{-1})	348,2±58,96	b	171,4±11,89	a
Organ	**Ureide activities** (µmol.min^{-1}.mg^{-1} protein)				
Nodules	XDH	0,096±0,007	b	0,005±0,001	a
	Uricase	0,283±0,033	b	0,095±0,001	a
Roots	XDH	0,033±0,006	a	0,048±0,005	b
	Uricase	0,291±0,018	a	0,330±0,021	a

Table 2 N metabolising enzyme ratios of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

Organ	**N metabolising enzyme ratios**	**Treatments**			
		500 µM P		5 µM P	
Nodules	GOGAT:Uricase	0,12±0,02	a	0,58±0,02	b
	GOGAT:XDH	0,374±0,09	a	10,6±1,56	b
	GDH-A:GDH-D	0,73±0,13	b	0,19±0,05	a
	AAT: Uricase	0,014±0,00	a	0,014±0,00	a
	AAT:XDH	0,043±0,01	a	0,23±0,03	b
Roots	GOGAT:Uricase	0,27±0,05	b	0,14±0,02	a
	GOGAT:XDH	2,50±0,38	b	0,94±0,09	a
	GDH-A:GDH-D	1,12±0,22	b	0,52±0,08	a
	AAT: Uricase	0,14±0,01	b	0,07±0,01	a
	AAT:XDH	1,38±0,30	b	0,48±0,07	a

GOGAT, glutamate synthase/ glutamate 2-oxoglutarate aminotransferase; XDH, xanthine dehydrogenase GDH-A, glutamate dehydrogenase aminating; GDH-D, glutamate dehydrogenase deaminating; AAT, Aspartate amino transferase.



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

Figure 1 Representative NMR spectra of *Virgilia divaricata* (Adamson) a) nodule and b) root tissue, grown under high P conditions. Peak areas of spectra were used to derive 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.

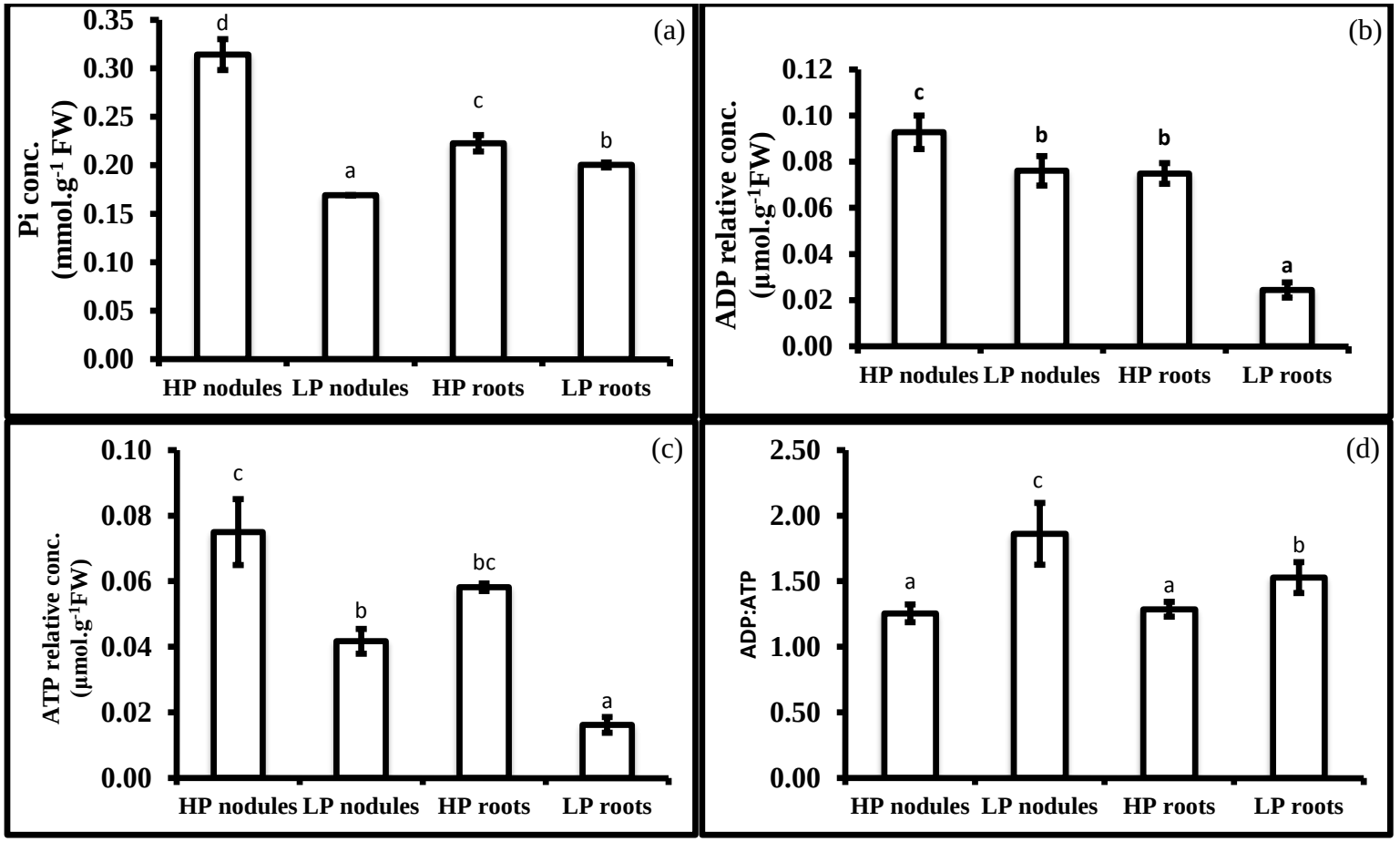


Figure 2 (a) Pi concentration, (b) ADP and (c) ATP relative concentrations and (d) ADP and ATP ratio of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

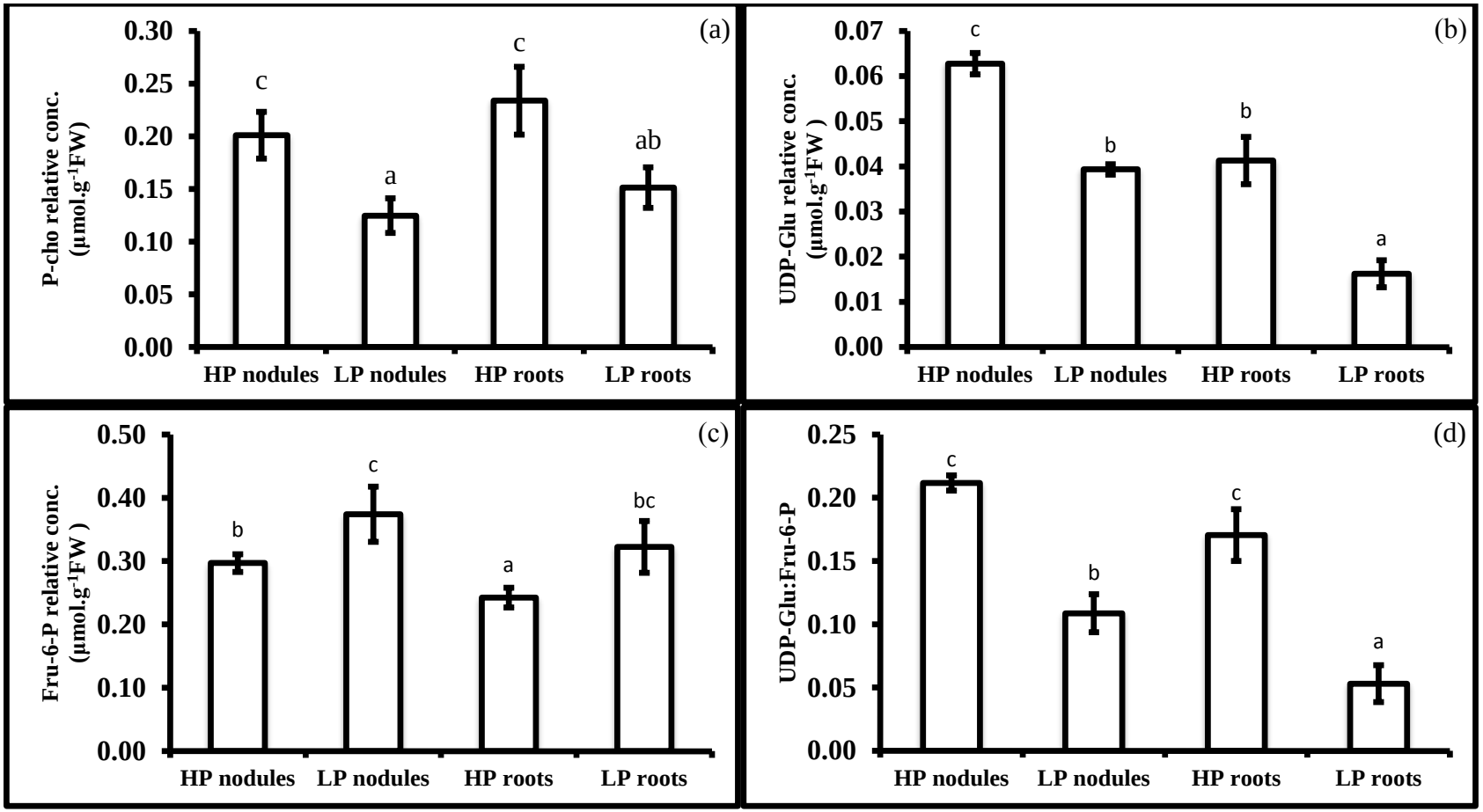


Figure 3 (a) P-cho, (b) UDPG, (c) Fru-6-P relative concentrations and (d) UDPG and Fru-6-P ratio of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

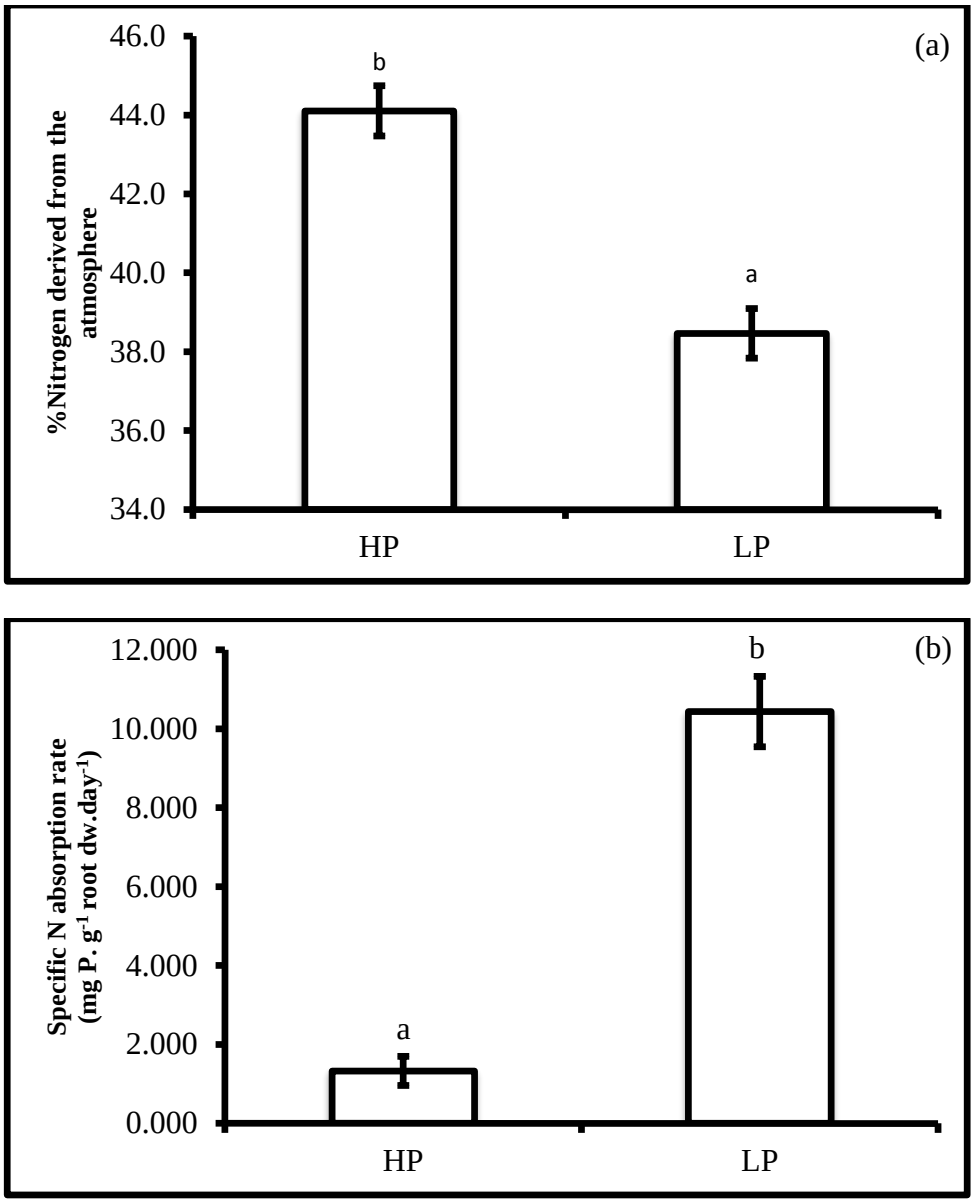


Figure 4 (a) Percentage N derived from the atmosphere (%NDFA) and (b) Specific N absorption rate (SNAR) of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments (*P<0.05).

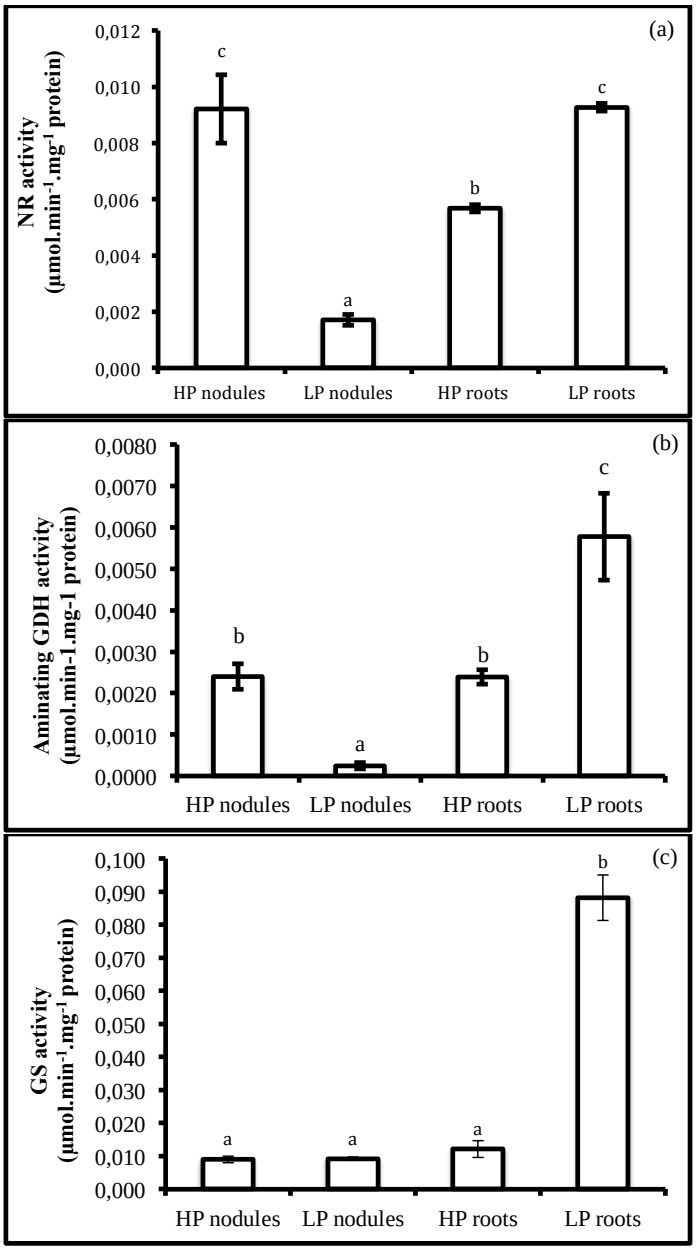


Figure 5 (a) NR, (b) Aminating GDH, (c) GS activities of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

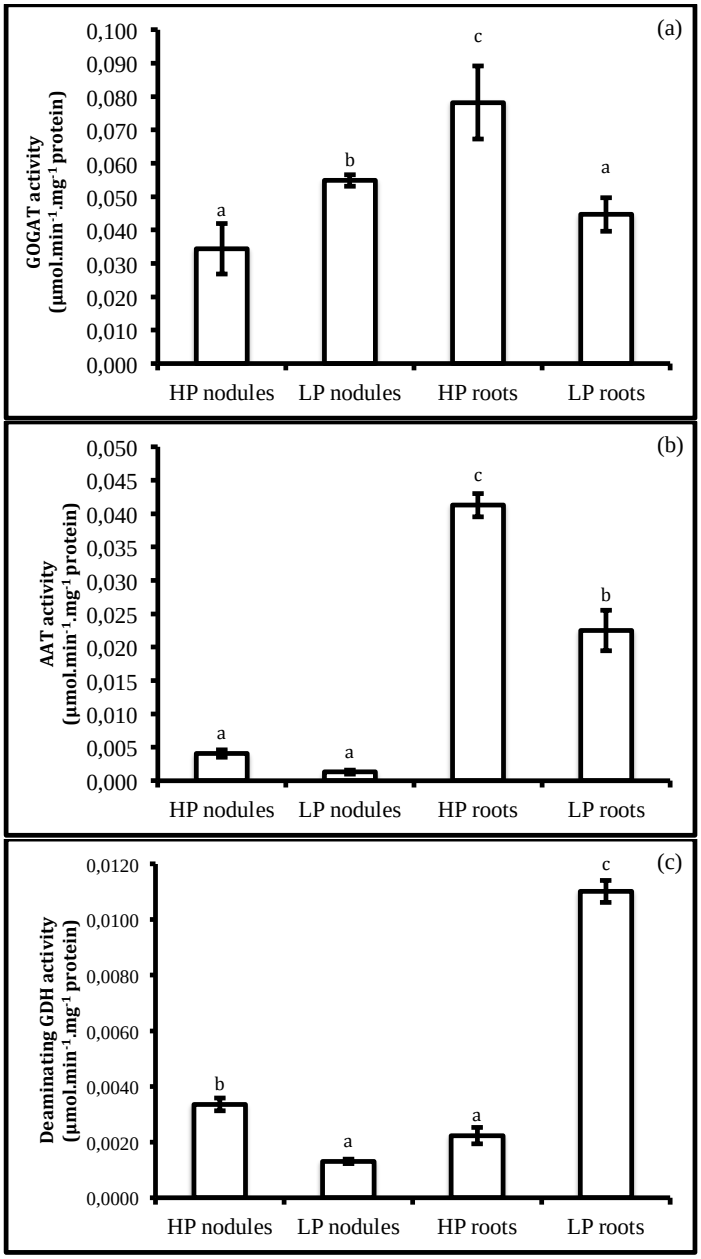


Figure 6 (a) GOGAT, (b) AAT and (c) deaminating GDH activities of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences among the treatments. (*P<0.05)

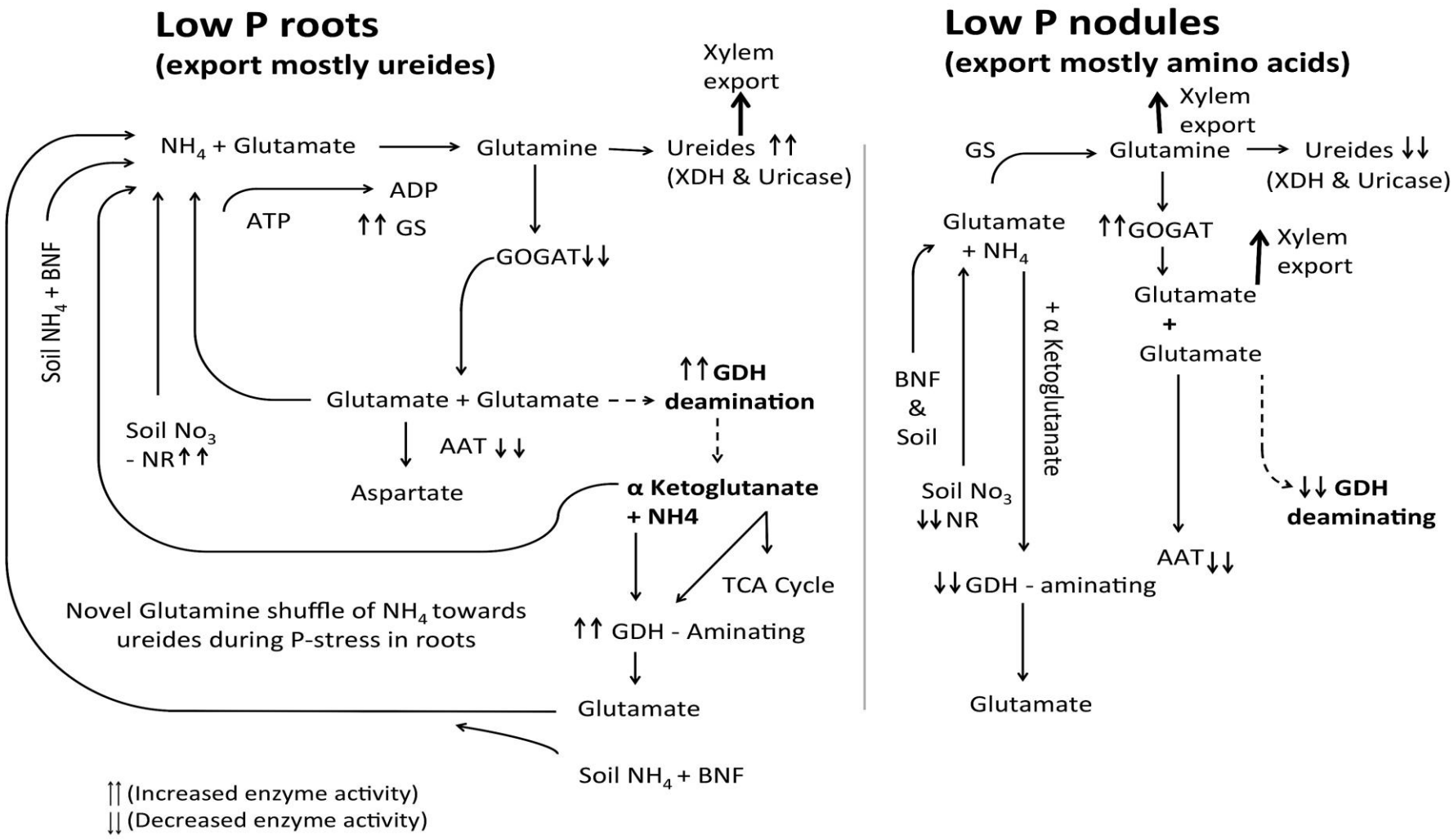


Figure 7 Proposed routes of N metabolism in (a) roots and (b) nodules of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source.

Chapter 6

Glutamate dehydrogenase is essential in the adaptation of *Virgilia divaricata* legume indigenous to the nutrient-poor Mediterranean-type ecosystems of the Cape fynbos?

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Glutamate dehydrogenase is essential in the adaptation of *Virgilia divaricata*, a legume indigenous to the nutrient-poor Mediterranean-type ecosystems of the Cape fynbos.

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Abstract

Glutamate dehydrogenase (GDH) serves as a link between C and N metabolism as it is capable of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia. GDH might contribute to the functional tolerance of *Virgilia divaricata* to variable soil N and P levels in the mature fynbos. To better understand the complex nature of GDH in *V. divaricata*, the aim of this experiment was to identify the GDH transcripts, their relative expressions and activity in P-stressed roots and nodules during N metabolism. The analysis of the GDH cDNA sequences in *V. divaricata* revealed the presence of *GHD 1* and *GHD 2* subunits, these corresponding to the *GDH1*, *GDH-B* and *GDH3* genes of legumes and non-legume plants. The relative expression of *GDH1* and *GDH2* genes was analysed in the roots and nodules, our results indicate that two subunits were differently regulated depending on the organ type and P supply. Although both transcripts appeared to be ubiquitously expressed in the roots and nodules, the *GDH1* transcript evidently predominated over those of *GDH2*. Furthermore, the higher expression of both GDH transcripts in the nodules than roots in this study may play a role in the ability of nodules to regulate and conserve their internal P better than roots during P deficiency. With regards to GHD activity, both aminating and deaminating GDH activities were induced during P deficiency. This might be to assimilate N to conserve energy and regulate internal C and N in the roots and nodules.

GDH1 and *GDH2* transcript expression and GDH activity variation maybe essential aminating NH_4 and for regulating the plant C and N balances by recycling amino acid during P deficiency in *V. divaricata*.

Keywords: *Virgilia divaricata*, Glutamate dehydrogenase (GDH), Gene expression, aminating, deaminating.

Introduction

Carbon (C) and nitrogen (N) metabolism is fundamental to plant growth and development (Miyashita & Good, 2008). The enzyme, glutamate dehydrogenase (GDH) seems to play a pivotal role during the complex co-ordination of C and N metabolism in plants (Miyashita & Good, 2008). GDH serves as a link between C and N metabolism as it is capable of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia (Melo-Oliveira *et al*., 1996; Miyashita & Good, 2008). A primary function of GDH for assimilating ammonium has been less supported, since a more efficient pathway (GS-GOGAT pathway) for this process has been established by Lea and Miflin (1974). However, several authors still propose that this enzyme may play an alternative role to the GS-GOGAT pathway in the ammonium assimilation under specific physiological conditions that make ammonium concentration increase (Melo-Oliveira *et al*., 1996; Masclaux-Daubresse et al., 2002). Even more, there is evidence in favor of the catabolic function of GDH in glutamate metabolism, to help fuel the tricarboxylic acid (TCA) cycle under carbon insufficiency and help the cellular carbon: nitrogen ratios during stress conditions (Melo-Oliveira *et al*., 1996; Ficarelli *et al*., 1999; Dubois *et al*., 2003; Purnell *et al*., 2005; Miyashita and Good, 2008). Therefore GDH may play an essential role in the during N assimilation and co-ordination of C and N metabolism in *Virgilia divaricata* growing in the nutrient limited soils of the Cape fynbos.

V. divaricata is an indigenous forest-margin legume, growing in nutrient richer soils, but is reported to invade the mature fynbos (Coetsee and Wigley, 2013). The mature fynbos is characterised by strongly leached, acidic sandy soils with low nutrient concentrations, specifically in regards to phosphorus (P) (Coetsee and Wigley, 2013; Maseko and Dakora,

2013). Even though the fynbos is adapted to regular fires to maintain both its ideal microclimate for the vegetation and to disrupt the nutrient cycle associated with tree dominated vegetation, post fire changes in the soil nutrient dynamics could be one of the most important factors limiting legume in the mature fynbos (Manders *et al*., 1992; Coetsee and Wigley, 2013). The legume plant, *V. divaricata*, invades the mature fynbos even in the absence of fire and is described as a forest precursor. Furthermore, it enhances the fertility of fynbos soils (Coetsee and Wigley, 2013).

Fynbos soils contain relatively 58-77% organic P (Straker, 1996), with most of it not available for plant use due to complexion with cations (calcium and iron). Under acidic conditions, P ions can easily precipitate with cations (Dakora and Phillips, 2002) and can also be bound to organic compounds by microbial action (Vance *et al*., 2003; Uhde-Stone *et al*., 2003). Phosphorus concentration of the fynbos soils, is generally available in micromolar or lower concentrations, and these P concentrations are extremely low to drive the P requiring metabolic processes, including N metabolism (Maseko and Dakora, 2013). As such, P deficiency forms a critical constraint for plants, especially fynbos legume plants, as P is critical during N_2 fixation, N assimilation and metabolism during plant growth (Maseko and Dakora, 2013; Sulieman *et al*., 2013). P deficiency affects N nutrition consequently the C costs of plants during growth (Magadlela *et al.*, 2014). Therefore GDH may play a role during N metabolism and carbon provision, as it is capable of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia (Melo-Oliveira *et al*., 1996).

The GDH protein is a hexamer comprised of two subunits polypeptides that differ slightly in mass and charge. The largest subunit (43.0 kDa) designated α, is encoded by the gene *GDH2*

while the second subunit (42.5 kDa) designated β, is ecoded by the gene *GDH1* (Loulakakis & Roubelakis-Angelakis, 1991, 1996; Loulakakis *et al*., 1994; Purnell *et al*., 2005; Lehmann *et al*., 2011). GDH gene families coding for both subunits of the enzyme have been characterised in *Arabidopsis thaliana* and *Nicotiana plumbaginifolia* (Turano *et al*., 1997; Ficarelli *et al*., 1999). Regulation of GDH gene expression may occur at various levels, and it has been established that a substantial constituent in this regulation is not only the metabolic environment, but also the varied expression of the subunits of GDH (Terce-Laforgue *et al*., 2004; Fontaine *et al*., 2006; Labboun, *et al*., 2009; Lehmann *et al*., 2011). Considering this pivotal role of varied GDH subunit expression of *GHD1* and *GDH2* (Lehmann *et al*., 2011), it is an intruiging possibility that this may also play a role in GDH regulation in the nodulated root systems of a legume from a nutrient-poor ecosystem.

Given the importance of GDH in linking C and N metabolism during P stress, the aim of this study was to identify for the first time the *GHD1* and *GDH2* transcripts and their regulation in a legume from a P-poor ecosystem. This was achieved via the transcript identification, quantitative expression levels and enzyme activities in P-stressed *V. divaricata* roots and nodules.

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 (H_2SO_4) 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 (Magadlela *et al*., 2014). 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 µmol m^{-2} 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 500µM P (HP) and 5µM P (LP) as $NaH_2PO_4.2H_2O$ and 500µM NH_4NO_3 (Hewitt, 1966).

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.

Enzymatic assay

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 KH_2PO_4 buffer, pH 7.5, containing 2mM EDTA, 1.5% (w/v) soluble casein, 2mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpyrrolidine (PVP). The 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 of 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.

Glutamate dehydrogenase 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 $CaCl_2$, 13mM 2-oxoglutarate, 50mM $(NH_4)_2SO_4$ 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 $CaCl_2$ (pH 9), 33mM Glu and 0.25mM NAD or NADP.

PCR, cloning and sequencing

A pair of primers was used for the reverse transcriptase-polymerase chain reaction (RT-PCR) for gene subunits. The primers used for *GHD1* were as follows: RTfgdh1 5′-GAGTATATCAGGACAACGGTTTGTCA-3′ and RTrgdh1 5′-GGATCACCACCATGGAATCCTT-3′ (Lehman *et al*., 2011) and for *GDH2* were as follows: forward *GDH2*, 5-GGATTCATGTGGGAAGAGGA-3′ and reverse *GDH2* 5’-GCGACTCGGTTAACTCCAAG-3′ (Miyashita and Good, 2008). Total RNA (Fig. 1A) was isolated using the RNeasy Mini Kit (QIAGEN, Qiagen Strasse 1, 40724 Hilden, Germany) according to the manufacturer’s protocol. Genomic DNA was removed using the DNase 1, RNase-free (Thermo Scientific, Johannesburg, South Africa) according to the manufacturers protocol. Subsequently, first-strand cDNA was synthesized using AMV reverse transcriptase (RevertAid TMH Minus First Strand cDNA Synthesis kit, Fermentas, Vilnius, Lithuania) and oligo(dT)18 anchor. PCR was carried out on BioRad Mini Opticon thermal cycler (BioRad,

South Africa) in a volume of 50 µL, containing 5 µL of the first-strand cDNA, 1x GoTaq PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25U of 5U/µL GoTaq G2 Flexi DNA polymerase (Promega, USA). The PCR program was as follows: 94°C for 1 min, 94°C for 30s, 55 °C for 30s, 72°C for 30s, for 35 cycles and a final extension for 5 min at 72°C and viewed in a 1,2 % agarose gel electrophoresis (Fig. 1B).

Amplification products were cloned (Fig. 1C) using the pGEM-T easy plasmid vector (Promega) according to the manufacturer's protocol, thereafter sequenced at the Central Analytical Facilities, Stellenbosch University, South Africa. The obtained raw sequences files were viewed, edited (where necessary) and aligned using the following the software programmes BioEdit and Geneious version 8.0.2 created by Biomatters (http://www.geneious.com/). The aligned GDH sequences were all subjected to a BLAST analysis on the GenBank database and matching hits were selected.

With regards to the maximum-likelihood *GDH1* and *GDH2* cluster analysis, a range of Fabaceae sequences and out-group sequences and *GDH 1* (177 base pairs) and *GDH2* (141 basepairs) sequences were aligned using the following the software programmes BioEdit and Geneious version 8.0.2 created by Biomatters (http://www.geneious.com/). The aligned sequences for both *GDH 1* and *GDH 2* subunits were subjected through MrBayes 3.2 (Ronquist *et al*., 20120), for a 1,000000 generations using the NST mixed command to model over model space and a gamma correction for amongst site variation. A burn-in of 25% was used and all parameters converged at an estimated sample sizes greater than 500.

Quantitative real-time PCR was performed using cDNA normalized to 20ng/reaction (1µL), using KAPA SYBR FAST Universal One-Step qRT-PCR kit (KAPABIOSYSTEMS, Cape Town, South Africa) according to manufactures protocol. The primers used for *GHD1* were as follows: RTfgdh1 5′-GAGTATATCAGGACAACGGTTTGTCA-3′ and RTrgdh1 5′-

GGATCACCACCATGGAATCCTT-3′ (Lehman *et al*., 2011) and for *GDH2* were as follows: forward *GDH2*, 5-GGATTCATGTGGGAAGAGGA-3′ and reverse *GDH2* 5'-GCGACTCGGTTAACTCCAAG-3′ (Miyashita and Good, 2008). The analysis was carried out in an Applied Biosystems Step-One Plus RT-PCR (Thermo Fisher Scientific) using the following program: 30s at 95°C, followed by 40 cycles 3s at 95°C and 20s at 50°C. In each assay for one of the two targets, triplicate standard curve were prepared for the appropriate primer set using the diluted plasmid samples. Furthermore, standards, cDNA samples and negative control were analyzed in three repeats in each assay. To quantify the transcript of each gene, the copy numbers of each target were determined by the standard curve constructed using the diluted plasmid samples. The determined copy numbers of *GDH1* and *GDH2* were then normalized the copy numbers of *Actin2* transcripts.

Results

GDH activity assay

During N assimilation, aminating GDH show an increased activity in roots during low P supply compared to roots supplied sufficient P (Table 1). The contrary was observed in the nodules, where low P nodules showed reduced activity compared to high P nodules. Deaminating GDH showed the same trend of activity in the roots and nodules as the aminating GDH. Deaminating GDH activity show a significant increase in the roots during P deficiency while the inverse was observed in the nodules (Table 1).

Virgilia divaricata GDH gene sequencing

In the current study only two previously known genes, *GDH1* and *GDH2*, were investigated. The cDNA encoding GDH in *V. divaricata* was identified. The aligned subjected to GenBank BLAST, *GDH1* nucleotide sequence in *V. divaricata* showed approximately 92%, 90%, 90%, 88%, 88%, identities with *Lupinus luteus GDH1* (AY681352.1), predicted *Glycine max* GDH1-like (LOC100789509) (XM_006603650.1), *Glycine max GDH1* (NM_001249475.1) and *Medicago truncatula* NADP-specific *GDH* (XM_003618924.2), *Lupinus luteus* clone Ylgdh-L.07 GDH (AY871065.1) respectively. The *GDH2* nucleotide sequence in *V. divaricata* showed approximately 97%, 94%, 92%, 89%, 87% identity to predicted *Glycine max GDH1*-like (LOC100789509) (XM_006603650.1), predicted *Elaeis guineensis GDH* (XR_830269.1), predicted *Vitis vinifera GDH-B* (XM_010655427.1), predicted *Beta vulgaris* subsp. *vulgaris GDH-B* (XM_010673749.1) and *Cicer arietinum* probable *GDH3* (XM_004489651.2) respectively.

With regards to the maximum-likelihood cluster, isolates of *V. divaricata GDH1* (Fig. 3) formed a monophyletic cluster with a legume, *Lupinus luteus*, which forms a clade with other legumes such as *Cicer arietinum* and *Glycine max*. Where *GDH2* (Fig. 4) isolates formed a monophyletic cluster with a legume *Lotus japonicum*, which forms a clade with *Cicer arietinum*, *Glycine max* and a non-legume species, *Prunus persica*.

Target GDH transcript organ-dependent expression

In this study, the expression of *GDH1* and *GDH2* results indicated that *GDH1* is highly expressed in roots supplied with sufficient P compared to low P supplied plants (Fig 2a). In the nodules, low P supplied nodules showed slight increase in *GDH1* expression than high P

nodules (Fig 2a), though there was no significant difference. Regarding *GDH2*, in the roots there was no significant difference between the P treatments, while low P supplied nodules showed higher *GDH2* expression than high P supplied nodules (Fig 2b). Furthermore, both GDH transcript are highly expressed in the nodules compared to roots (Fig. 2)

Discussion

During limited P supply *V. divaricata* showed different GDH responses in the roots and nodules, via aminating and deaminating enzyme activies and *GDH1* and *GDH2* transcript expression. The increased GDH activities in the roots and variation of *GDH1* and *GDH2* subunits expression in the different organs suggest that GDH might have contributed to the coordinating the C and N metabolism in nodulated root systems of *V. divaricata* in low P soils.

Although GDH transcripts have been extensively studied using biochemical, genetic and physiological approaches in model plants (Miyashita and Good, 2008; Lehmann *et al*., 2011). No studies on the GDH protein has been done on Cape fynbos legume that have evolved to grow in both P richer and poorer soils, like *V. divaricata* (Coetsee and Wigley, 2013; Maseko and Dakora, 2013). P deficiency in *V. divaricata* consequently affects N nutrition, carbon (C) costs and allocation during plant growth (Magadlela *et al*., 2014). Studies on GDH focused on carbon starvation (Miyashita and Good, 2008) and ammonium tolerance (Sarasketa *et al*. 2014), a few focused on P stress, example Qui *et al*. (2009) looked at responses of rice to N and P deprivation. Based on recent literature on GDH genes, in *Arabidopsis* four genes described as GDH have been identified, including two previously described by Turano et al.

(1997) *GHD1* (At5g18170) and *GDH2* (At5g07440) as well as two assumed ones *GDH3* by Purnell et al. (2005) and Miyashita and Good (2008), (Atg03g0390, encoding the β-subunit and GDH gene encoding NADP(H)-dependent GDH, At1g551720), four GDH genes in rice by Qui *et al.* (2009) and by Lehmann *et al*. (2011) in yellow Lupine. In this study we have found that at least two GDH transcripts are present in *V. divaricata*. Our analysis of cDNA sequences coding for GDH revealed that both *GDH1* and *GDH2* are present in the roots and nodules and expressed differently within the organs and between the organs (roots and nodules) due to varying P supply. The phylogenetic comparison of these *GDH1* and *GDH2* sequences, indicate that these genes are conserved within the legume group. It is interesting to note that the *GDH1* largely clustered with the group of amino acid exporting legumes, while the *GDH2* largely clustered with the ureide exporting legumes. Recent work from our group indicated that under P stress, that nodules synthesise mostly amino acid, compared to the ureide synthesis of roots (Magadlela *et al*., 2015). It may therefore be that the role of alpha subunit of the enzyme GDH, as encoded for by *GDH2*, can possibly play a role in the ureide synthesis, by means of the GDH provision of the substrate glutamate.

The results in this study concur with findings by Lehmann *et al*. (2011). They found GDH coding cDNA sequences in yellow lupine were expressed differently in various organs during the developmental stages, comparing the yellow lupine sequences to *Arabidopsis* confirmed that they correspond to the products of *GDH1* and *GDH2* genes (Lehmann *et al*., 2011). Furthermore Miyashita and Good (2008) results indicated that the expression of GDH genes was differently regulated depending on the organ or tissue types and cellular C availability in *Arabidopsis thaliana.* The differences in GDH subunits (alpha and beta) expression levels between the roots and nodules with varying P supply might play a role in the assimilation of ammonium (NH_4^+).

Since two enzyme systems (GS/GOGAT and GDH) have the capacity to assimilate NH_4^+, it has been proposed that GDH may provide an alternative pathway under particular physiological conditions. This may occur when GS/GOGAT is not be able to achieve its function, and then the mitochondrial NAD(H) dependent GDH may become prominent, by assimilating NH_4^+ by utilising the organic acid 2-oxoglutarate to synthesise glutamate (Melo-Oliveria *et al*., 1996; Miflin and Habash, 2002). For instance, the up-regulation of GDH in response to elevated ammonium levels suggest that GDH is important in the detoxification of ammonium by assimilating some of the ammonium ions (Tercé- Laforgue *et al*., 2004a,b). Furthermore, the assimilation of ammonium via GDH confers a saving in energy (ATP and NADPH) compared with GS/GOGAT cycle (Helling, 1998; Qui *et al*., 2009). In the current study, low P roots showed an enhanced aminating GDH activity, possible due to aminating GDH becoming more prominent or sharing NH_4^+ assimilating capacity with GS/GOGAT. This is because the roots were supplied with inorganic N and therefore, the legume plants were not only reliant on atmospheric fixed N by *Burkholderia sp*. but also on the mineral N source (NH_4NO_3). The contribution of GDH to N assimilation during P deficiency might be an energy saving strategy as recently found by this group (Magadlela *et al*. 2015). This concurs with findings by Sarasketa *et al*. (2014) in *Arabidopsis thaliana* fed with both NH_4^+ and NO_3^- aminating GDH activities were induced.

In addition to GDH assimilating N, deaminating GDH in roots of P stressed plants may also engage in the recycling of amino acids during P limitation.

GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to yield a keto-acid and NH_3, which can be separately recycled for utilisation in Krebbs Cyle

respiration and amide formation (Miflin and Habash, 2002). The increased deaminating GDH activity in the P stressed roots indicates that glutamate is broken down to form 2-ketoglutarate and NH_4^+. Although this has not been established in P stressed plants roots and nodules before, studies have implied that in times of stress, particularly carbon starvation, there maybe a strong demand to obtain carbon from amino acids to feed into the tricarboxylic acid (TCA) cycle, this achieved by deaminating GDH (Lea and Miflin, 1980; Miflin and Habash, 2002; Miyashita and Good, 2008). This might be the case in *V. divaricata* plants during P stress, as it has been reported that P stress not only affects biomass and N nutrition in *V. divaricata* but also carbon (C) costs and allocation during plant growth (Magadlela *et al*., 2014; 2015).

In a recent study by Magadlela *et al*. (2015) on *V. divaricata* grown under the same P conditions as the current study, nodules maintained a lower percentage decline in ATP and ADP than the roots. This implies that the nodules have a strategy to regulate their internal P and conservation, minimizing the effects of P deficiency (Magadlela *et al*., 2015). The higher expression of both GDH transcripts in the nodules than roots in this study might play a role in the ability of nodules to regulate and conserve their internal P during P deficiency.

Conclusion

The changes in the GDH transcript expression and activity might be essential for regulating the plant C and N balances during plant growth during P deficiency in *V. divaricata*. These findings indicate that the control of C and N metabolism is important for the success of *V.*

divaricata in soils ranging from nutrient rich soils in the forest to nutrient poorer soils in the fynbos.

Acknowledgements

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Table 1 GDH-A and GDH-D enzyme activities (µmol.min^{-1}.mg^{-1} protein) of *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source. Values are presented as means (n=10). The different letters indicate significant differences between the treatments in each row. (*P<0.05)

Organ	GDH enzyme activity and ratios	Treatments			
		500 µM P		5 µM P	
Nodules	GDH-A	0.0024±0.0003	b	0.0002±0.0001	a
	GDH-D	0.0034±0.0002	b	0.0013±0.0001	a
Roots	GDH-A	0.0024±0,0002	a	0.006±0,0010	b
	GDH-D	0.0022±0.0003	a	0.0110±0.0004	b

GDH-A, glutamate dehydrogenase aminating; GDH-D, glutamate dehydrogenase deaminating.

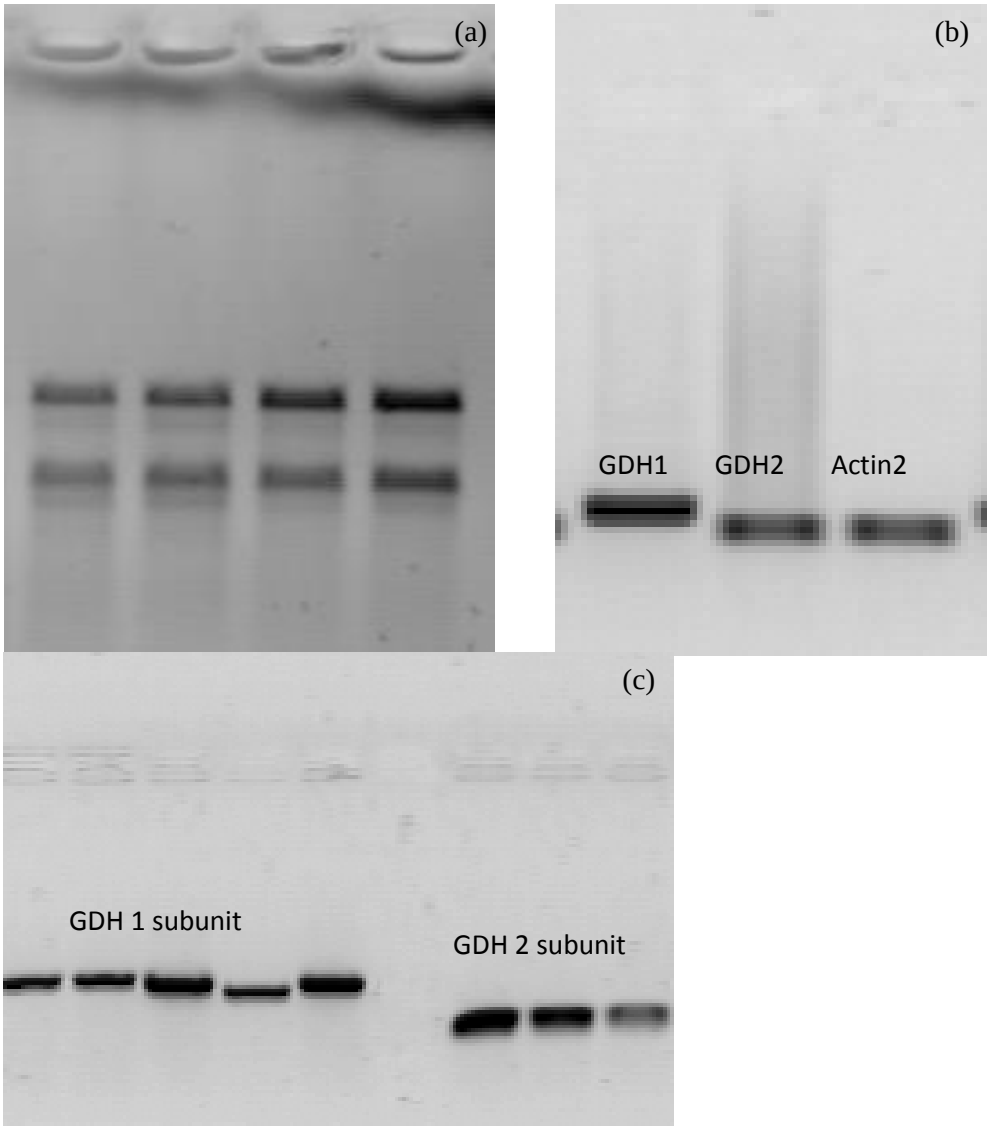

Figure 1 (a) Total RNA extracts from roots and nodules, (b) cDNA post PCR amplified products of *GDH1* and *GDH2* subunit including a reference gene Actin2, (c) Cloned products in pGEM-T easy plasmid vector (Promega) visualized in a 1,2 % agarose gel electrophoresis.

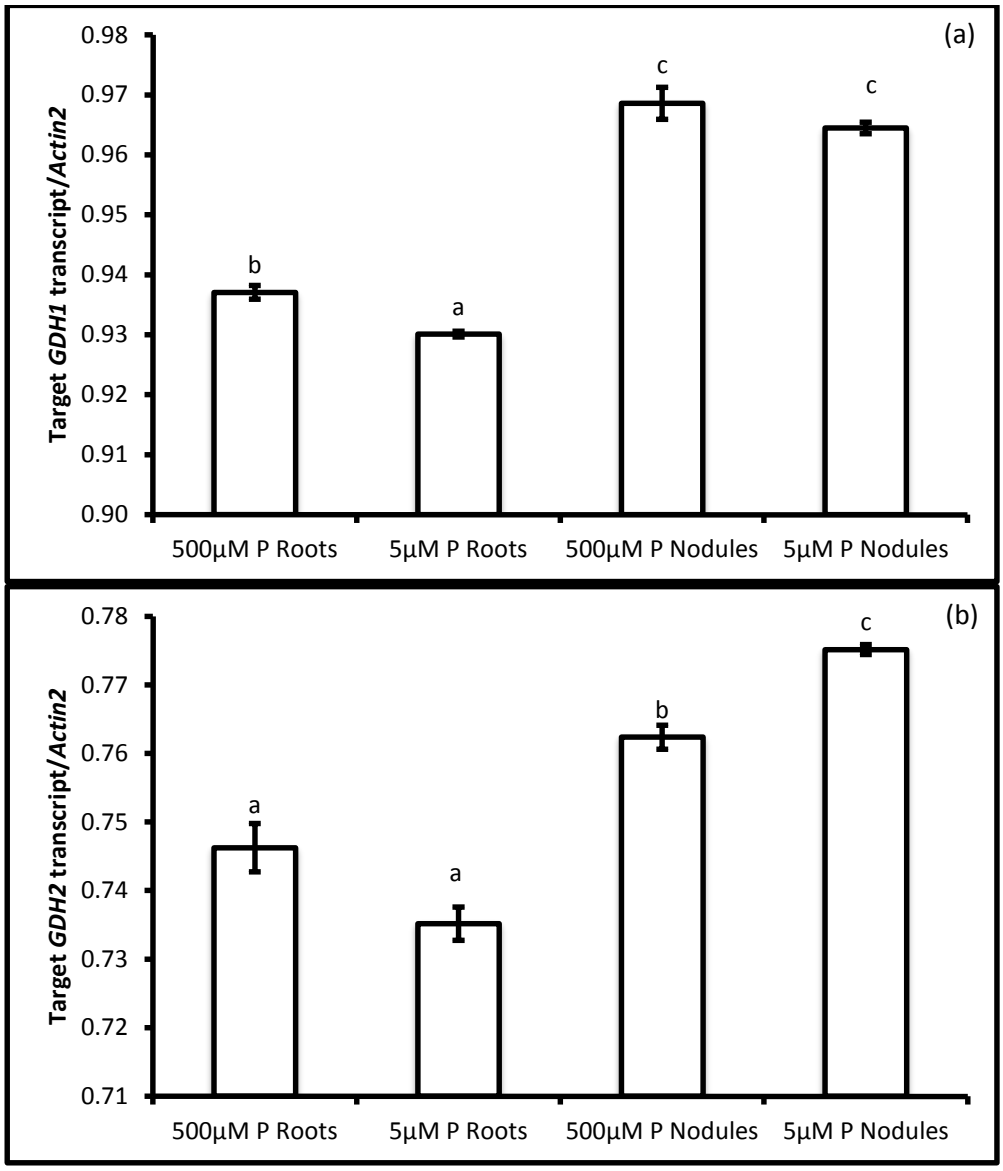

Figure 2 Levels of expression of the two *Virgilia divaricata* GDH genes in roots and nodule, grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4.2H_2O$. Both the high and Low P plants were either supplied with either 500 µM NH_4NO_3 as soil nitrogen (N) source. The abundance of each transcript was measured by quantitative real-time RT-PCR (qRT-PCR) and normalized against *Actin2*. Values are presented as means of triplicate samples. The different letters indicate significant differences among the treatments. (*P<0.05)

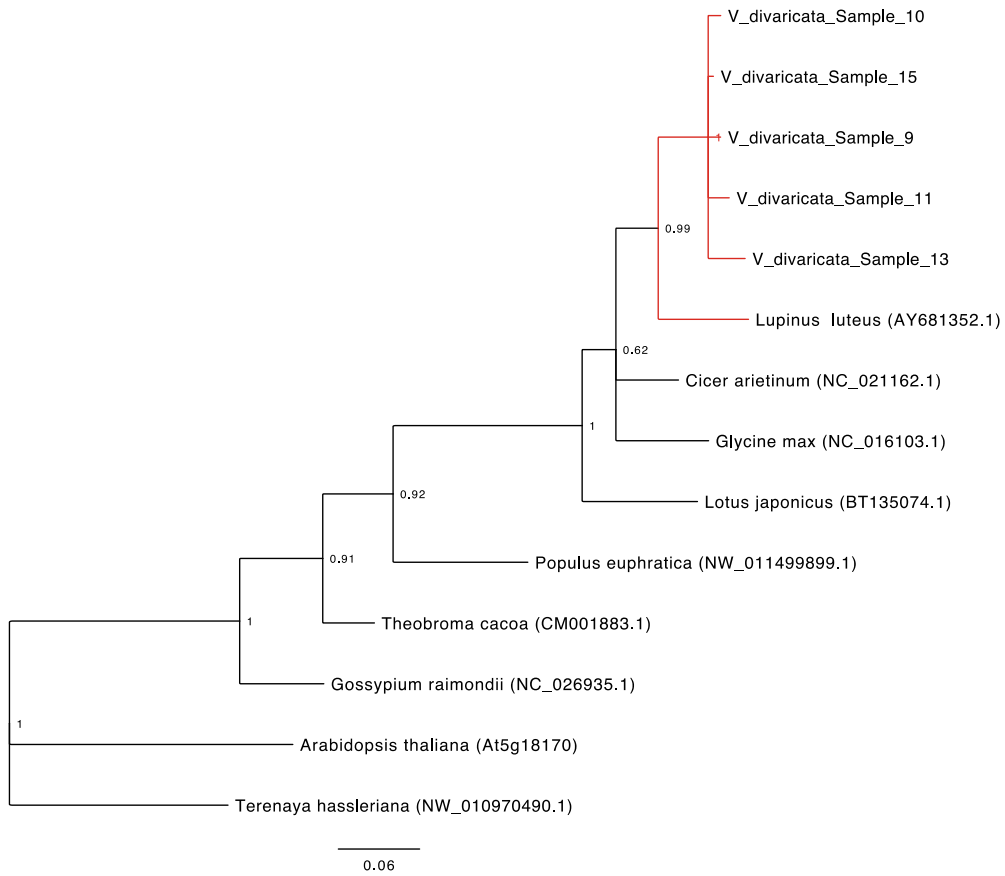

Figure 3 Evolutionary relationships of *GDH1* subunit encoded by *Virgilia divaricata* cDNAs. The aligned sequences of *GDH1* subunits were subjected through MrBayes 3.2 (Ronquist *et al.*, 20120), for a 1,000000 generations using the NST mixed command to model over model space and a gamma correction for amongst site variation. A burn-in of 25% was used and all parameters converged at an estimated sample sizes greater than 500. Accession number is shown in parentheses, according to the sequences deposited in GenBank (NCBI, EMBL/GenBank/DDBJdatabase).

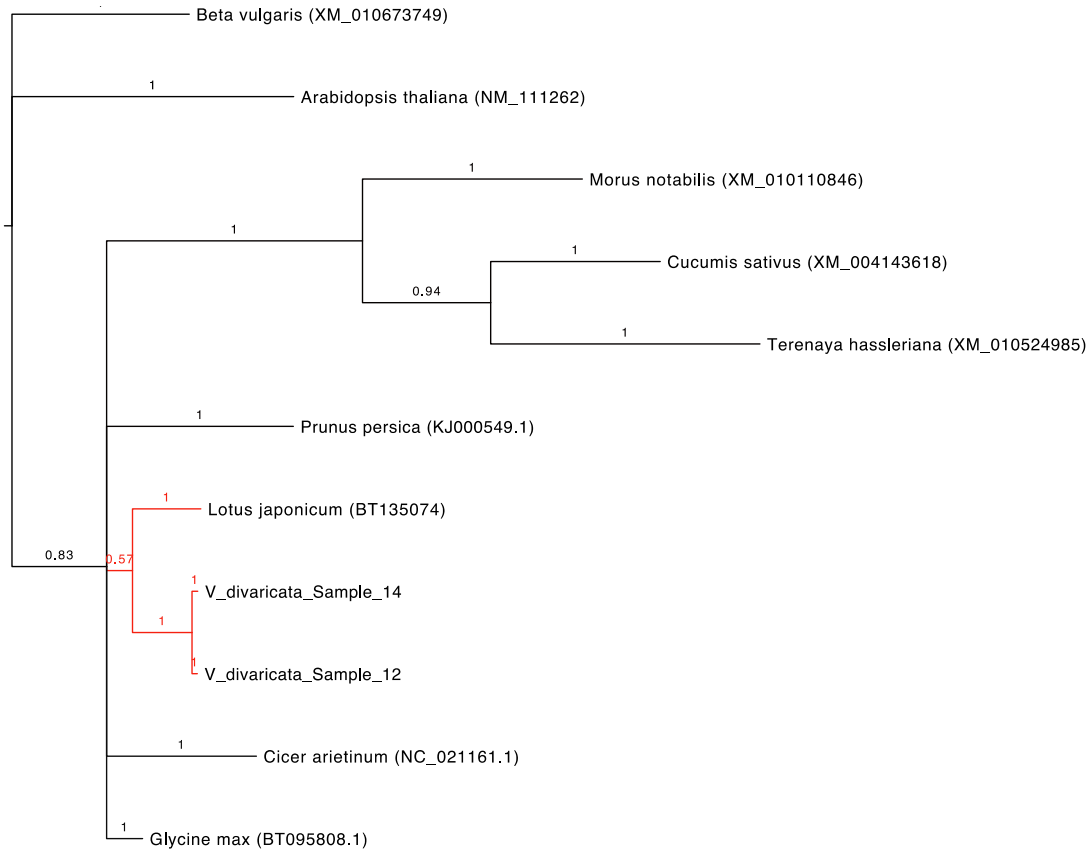

Figure 4 Evolutionary relationships of *GDH2* subunit encoded by *Virgilia divaricata* cDNAs. The aligned sequences of *GDH2* subunits were subjected through MrBayes 3.2 (Ronquist *et al*., 20120), for a 1,000000 generations using the NST mixed command to model over model space and a gamma correction for amongst site variation. A burn-in of 25% was used and all parameters converged at an estimated sample sizes greater than 500. Accession number is shown in parentheses, according to the sequences deposited in GenBank (NCBI, EMBL/GenBank/DDBJdatabase).

Chapter 7

General Discussion and Conclusion

7.1 General Discussion and Conclusion

The fynbos ecosystem in the Cape Floristic Region (CFR) is largely known for its characteristic leached, acidic sandy soils that are nutrient poor, typically low in P and N (Spriggs and Dakora, 2008; Power *et al*., 2010; Maseko and Dakora, 2013). Both these nutrients have primary importance for plant growth, that not withstanding, it has the most diverse flora comprising about 9,030 vascular plant species with 68,7% endemic, these including member of the Fabaceae (Goldblatt and Manning, 2002; Maseko and Dakora, 2013). Members of the Fabaceae that are endemic to the Cape fynbos depend heavily on N_2 fixation for their N nutrition (Spriggs and Dakora, 2008; Power et al., 2010; Maseko and Dakora, 2013). However it is generally thought that low P availability can reduce nodulation and N_2 fixation in legumes (Muofhe and Dakora, 1999; Magadlela *et al*., 2014, 2015; Vardien *et al*., 2014). Nevertheless the endemic legume plants seem to have evolved adaptations listed in Table 7.1 to obtain adequate P or conserve internal P under these P deficient conditions (Vance et al, 2003; Maseko and Dakora, 2013; Magadlela *et al*., 2014, 2015; Vardien *et al*., 2014).

Clearly there is a need for additional information on the dynamics of P and its influence on N uptake and metabolism in legume plants in a wider range of the Cape fynbos soils. Especially regarding their influence on spatial variation in vegetation structure and nutrient cycling. Therefore a better understanding of metabolic and ecological costs associated with N acquisition and metabolising strategies is needed for the management and future conservation of Cape fynbos legumes in the P poor environment.

We therefore need to understand the mechanisms of legume adaptations to fix N_2, assimilate and metabolise N in a low P environment. Furthermore to better understand the effects of P deficiency on the root nodule symbiotic partners and function of *V. divaricata*, associated to the energy status during N assimilation and metabolism. This research also provides genetic insights of these physiological adaptations, which may in turn be applied successfully in agriculture. The genes responsible for this adaptation of *V. divaricata* to low P may be used in genetic modification, where they can be expressed in crop plants to enhance their growth in P-poor farm soils. This is highly relevant, given predictions that the world is going to run out of P-fertilizer in 30-50 years' time (Vance, 2003).

Former, most mentioned studies on non-legume and legume plants native to Mediterranean-type ecosystem of the Cape fynbos observed whole-plant physiological events, with a few studies listed in Table 7.1 which included knowledge of the molecular biological components and mechanisms that underpin these phenotypic responses (Muofhe and Dakora, 1999; Kanu and Dakora, 2012; Vardien *et al*., 2014; Magadlela *et al*., 2014, 2015). Most research presented in chapter 1 (Literature review) and Table 7.2 on N metabolism is done on non-legume plants and model legume plants, which are herbaceous crops, with an annual growth form. Therefore, our use of a legume tree from a Mediterranean-type ecosystem to study the role of P deficiency in N nutrition is a relevant model because it is more representative of tree legumes from nutrient-poor, Mediterranean-type ecosystems. It is therefore pertinent for *V. divaricata* to be used as a model legume, which should be proposed for exposure to modern genomics tools.

This study was undertaken to increase knowledge and understanding of how P deficiency affects root nodule microbial profiles (N_2 fixing microbes) relying on two N sources (soil nitrogen (N) supplied as NH_4NO_3 and atmospheric N_2). Nitrogen metabolic changes during N assimilation in both the root and nodules associated with the carbon and metabolic costs. Furthermore the major branch points in amino acid biosynthesis during N assimilation. In addition the glutamate dehydrogenase (GDH) transcripts expressed during N metabolism. This was determined through a series of experiments using a legume tree from a Mediterranean-type ecosystem *V. divaricata* as a model legume, which has shown a wide range of tolerances in terms of soil conditions and growth environments.

The study in chapter 4 aimed to identify the rhizobial species that nodulate and fix atmospheric N_2 in *V. divaricata* and their N_2 fixing efficiencies during P deficiency. Moreover, this study assessed the integration of the C and N metabolism and the N product exported via xylem to the shoots. It appeared as though *Burkholderia* sp. are able to form functional nodules with the host *V. divaricata* even during P deficiency but with a reduction of reliance on biological N_2 fixation. Therefore bacterial composition of the resulting nodules remained unchanged, the constant host preference of these *Burkholderia* isolates may indicate a very specific host-symbiont relationship and could be indicative of ecosystem-specific co-evolution. Indigenous fynbos legumes such as *Psoralea*, *Hypocalyptus*, *Podalyria*, *Cyclopia* and *V. oroboides* (closely related to *V. divaricata*), are reportedly nodulated by a variety of bacteria including species of *Burkholderia* (Elliot *et al*., 2007; Kanu and Dakora, 2012; Beukes *et al*, 2013). Thus far nodulating species of *Burkholderia* have shown to be prominent in the Cape fynbos (Beukes *et al*, 2013; Lemaire *et al*, 2014); and

although it has been hypothesized that physical factors such as soil acidity or elevation (Bontemps *et al*, 2010; Lemaire *et al*, 2014) predisposes these legumes to associations with beta-rhizobia, perhaps it is the effect of the most basic factor such as soil dynamics and the ability of members of the *Burkholderia* to better withstand changes in these dynamics.

Furthermore the decrease in both sugars (sucrose, glucose, fructose, and ribose) and organic acids (malic acid and succinate) concentrations in the nodules may imply a reduced energy supply to the bacteroid during P stress. This might have resulted in the reduced N_2 fixation (Silsbury *et al*., 1986; Israel, 1987; Nelson and Edie, 1991; Qiao *et al*., 2007). Our findings further showed that altered bacteroid function may be inferred from BNF, and the N compounds synthesized and exported. At high P, plants exported more amino acids relative to inorganic N and ureides in their xylem sap, whereas at low P the plants exported more ureides relative to amino acids and NH_4. The potentially lower costs of ureide export would have affected the plant's carbon and energy budget, this having a positive impact on biomass accumulation (Atkins, 1991; Todd et al., 2006). The bacterial tolerance for changes in P and N via nodule metabolites and xylem export might be a major factor that underpins the growth of *V. divaricata* under these variable P soil conditions. Although findings in chapter 4 indicated that P deficiency doesn't affect bacterial composition but the metabolite profile affecting atm N_2 fixation and N product exported. The energy status and its impact on the specific N metabolising enzymes involved in the routes of N assimilation remained unknown. Therefore this was addressed in the next chapter.

In chapter 5, we aimed to determine whether P deficiency affects the metabolic status of roots and nodules and the consequent impact on the routes of N assimilation. The results showed that based on the adenylates data, P stressed nodules maintained their P status better than P stressed roots. Although these findings are for a tree legume from a nutrient-poor habitat, they concur with findings by Le Roux *et al*. 2006 for a model herbaceous perennial legume, Lupin. 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). 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 (Zavaleta-Pastor *et al*. 2010; Lambers *et al*. 2012), and increased reliance on the PPi-dependant metabolism of sucrose via UDPG and to Fru-6-P. It is know that during P deficiency, the metabolic bypasses in glycolysis would be engaged, 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 (Theodorou and Plaxton 1993; Plaxton 2010). P stressed roots exported mostly ureides as organic N (Schubert 1986; Atkins 1991; Todd *et al*. 2006), and recycled amino acids via deamination glutamate dehydrogenase (GDH) (Miyashita and Good 2008). 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 (Le Roux *et al*. 2006; Vardien *et al*. 2014). This resulted in the roots and nodules of *V. divaricata*, metabolising N differently during P stress, meaning that these organs may contribute differently to the success of this plant in soils ranging from forest to

fynbos. The role of GHD during P deficiency further necessitates the exploration at transcript level, in chapter 6 we investigated glutamate dehydrogenase (GDH) gene subunits and their relative expression levels during limited P.

Therefore, in chapter 6, this study was undertaken to better understand the complex nature of GDH in *V. divaricata*. The aim of this experiment was to identify the GDH transcripts, their relative expressions and activity in P-stressed roots and nodules during N metabolism. The analysis of the GDH cDNA sequences in *V. divaricata* revealed the presence of *GHD 1* and *GHD 2* subunits, these corresponding to the *GDH1*, *GDH-B* and *GDH3* genes of legumes and non-legume plants (Purnell *et al*., 2005; Miyashita and Good, 2008; Qui *et al*., 2009; Lehmann *et al*., 2011). Furthermore the relative expression of *GDH1* and *GDH2* genes analysed in the roots and nodules with varying P, indicate that two subunits (*GHD 1* and *GHD 2*) were differently regulated depending on the organ type and P supply (Miyashita and Good, 2008; Qui *et al*., 2009; Lehmann *et al*., 2011). Although both transcripts appeared to be ubiquitously expressed in the roots and nodules, the *GDH1* transcript evidently predominated over those of *GDH2*. The higher expression of both GDH transcripts in the nodules than roots in this study may play a role in the ability of nodules to regulate and conserve their internal P better than roots during P deficiency (Le Roux *et al*. 2006; Vardien *et al*. 2014; Magadlela *et al*., 2015). With regards to GHD activity, both aminating and deaminating GDH activities were induced during P deficiency. This might be to assimilate N to conserve energy and regulate internal C and N in the roots during P deficiency (Melo-Oliveria *et al*., 1996; Miflin and Habash, 2002).

GDH tissue expression and activity variation maybe essential for regulating the plant C and N balances during plant growth during P deficiency this contributing to the success of *V. divaricata* in soils ranging from nutrient rich soils in the forest to nutrient poorer soils in the fynbos.

The collective studies revealed that *V. divaricata* may employ contrasting adaptations in order to maintain N nutrition and conserve energy during P deficiency. Different mechanisms have been reported in Cape fynbos endemic non-legumes and legume plants, listed in Table 7.1, and on model plants on different N metabolising mechanisms employed assimilate N and conserve energy listed in Table 7.2. These adaptation presented in this study on *V. divaricata* include the persistence of their symbiotic partners (*Burkholderia* sp.) during P deficiency. The persistence of symbiotic partners in the Cape fynbos ecosystem has been widely studied by Elliot *et al*. (2007), Kanu and Dakora (2012), Beukes *et al*. (2013) but not integrated with metabolite and energy status in the plant underground organs (roots and nodules). Even more the different internal strategies that these legumes might employ to recycle and conserve energy. These recycling and energy conserving strategies were reported in this study, including internal recycling of P by possible replacement of membrane phospholipids with sulpholipids and galactolipids. Employing glycolytic bypasses that favour PPi-dependent reactions instead of ATP-requiring reactions, in order to conserve diminishing cellular ATP pools. Furthermore, exporting N dense products such as ureides for plant use, and employing glutamate dehydrogenase (GDH) for assimilating N and regulate *V. divaricata* C and N balances. Studies listed in Table 7.2 have reported some of the finding presented in this study but mostly in non-legume and model legume plants not Cape fynbos legume plants endemic to nutrient

poor soils. This study is the first to examine and report that a Cape fynbos indigenous legumes export different N products for plant use. In addition employs GDH to recycle nitrogen products such as amino acids to fuel the TCA cycle and produce N for internal use in indigenous legume species from a nutrient impoverished ecosystem. Earlier studies by Stock and Lewis (1984) looked at the uptake of different N forms such as nitrate and ammonium in an indigenous Cape fynbos non-legume plant, *Protea repens* L. lacking the knowledge of the enzymes involved during N assimilation and metabolism in these plants. Therefore the current study added knowledge to the gap by reporting on the enzymes involved during N assimilation and metabolism.

The application of these findings is important for understanding that *V. divaricata* employs different genetic mechanisms to overcome or adapt to P stress, this contributing to its success to growing in nutrient rich soils in the forest margins in the southern Cape of South Africa and ability to invade the nutrient poorer soils in the Cape fynbos.

This study was restricted however, as it focused largely on N metabolism and energy conserving strategies affecting the physiological responses of *V. divaricata* to P stress not exploring and integrating other mechanisms, P recycling enzymes and organic acid synthesizing enzymes, employed by *V. divaricata*. In this regard, a sequencing project should be undertaken, in order to assess the N metabolising, P recycling and organic acids associated genes and their regulatory networks for adaptation to P-deficiency in a legume species, which is native to a P-poor environment. Furthermore, the plant responses should also be subjected to a proteomic profile investigation, in

order to compliment the transcriptome of the P-deficient responses (Syntichaki *et al*., 1996; Turano *et al*., 1997; Ficarelli *et al*., 1999; Miyashita and Good, 2008; Lehman *et al*., 2011). This knowledge would provide insight on which proteins are involved in P efficient signaling and if there are different isoforms of the protein exists in different organs during P stress.

The potential for unique genes and proteins involved in the P-stress responses of these legumes may prove essential for applications in a bio-economy, involving agro-forestry biotechnology.

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Table 7.1 Mechanisms of P acquisition, utilization and energy conserving documented in the Cape fynbos non-legume and legume plants during P-stress.

Family/Species	Mechanism induced during limited P-supply	Effects	Reference
Hakae verrucosa (Proteaceae)	Mycorrhizal symbiosis, root clusters and release P solubilising compounds such as caboxylates and phosphates	Facilitates P-aquisition and liberates P	Boulet and Lambers *et al*., 2005
Leucadendron strictum (Proteaceae)	Root clusters and release P solubilising compounds such as phosphates	Fcilitates P-acquisition and liberates P	Maseko and Dakora, 2013
Protea repens (Proteaceae)	Absorb and assimilate small quantities of both nitrate and ammonium	Satisfy the N demand during growth	Stock and Lewis, 1984
Cyclopia (Fabaceae)	Mycorrhizal symbiosis, root clusters and release P solubilising compounds such as phosphates	Facilitates P-aquisition and liberates P	Spriggs 2004
Asphalathus (Fabaceae)	Mycorrhizal symbiosis, root cluster and release P solubilising compounds such as phosphates	Facilitates P-aquisition and liberates P	Allsopp and Stock, 1993, Maistry *et al*., 2014;2015
Psolaria (Fabaceae)	Mycorrhizal symbiosis	facilitates P-acquisition	Allsopp and Stock, 1993
Podalyria sp. (Fabacea)	Oragnic acid exudation, greater specific root length	facilitates P-acquisition	Maistry *et al*., 2015
Virgilia divaricata (Fabaceae)	Increased root to shoot ratio	Combined adaptations to acquire P,	Vardien *et al*., 2014
	Increased nodular Fe concentration	conserve energy, effifiently use	Vardien *et al*., 2014
	Increased extracellular root and nodule Apase activity	P internally and recycle N products	Vardien *et al*., 2014
	Increased intracellular root and nodule Apase activity		Vardien *et al*., 2014
	Increased phytase and Rnase activity		Vardien *et al*., 2014
	increased below ground arbon allocation		Magadlela *et al*., 2014
	Increased ureides export (N dense product) relative to amino acids		Chapter 4
	Switch N sources, favouring soil N assimilation than atm N fixation to conserve energy		Chapter 4
	Reduced adenylates levels, increased ADP to ATP ratio in nodules		Magadlela *et al*., 2015/Chapter 5
	Internal recycling of P (replacement of membrane phospholipids with sulpho/ galactolipids)		Magadlela *et al*., 2015/Chapter 5
	Reliance on Pyrophosphate (PPi) metabolising pathways		Magadlela *et al*., 2015/Chapter 5
	Increased aminating glutamate dehydrogense (GDH) activities (N assimilation)		Magadlela *et al*., 2015/Chapter 5
	Increased deaminating glutamate dehydrogense (GDH) activities (recycling amino acids)		Magadlela *et al*., 2015/Chapter 5
	Differential expression of GDH 1 and 2 transcripts		Chapter 6

Table 7.2 Nitrogen metabolising, utilization and energy conserving mechanisms documented in non-legume and model legume plants during environmental stress

Plant	Mechanism induced during stress	Effects	Reference
Arabidopsis thaliana **Transgenic tobacco plant** **Carrot cell suspension cells** **Potato**	NAD(H)-dependent GDH induced during carbon starvation	Amino acid breakdown fuel the TCA cycle and balance cellular C:N ratio	Miyashita and Good, 2008 Purnell and Botella, 2007 Robinson *et al*., 1991 Aubert *et al*., 2001
***Cucumis sativus* L.**	Higher NR activity during salt and osmotic stresses	Nitrogen metabolism	Sacala *et al*., 2008
Rice (*Oryza sativa*)	GDH gene family expressed during N and P stress	Aminating ammonium and deaminating amino acids	Qui *et al*., 2009
Arabidopsis thaliana	Higher aminating GDH activity during higher ammonium supply	Aminating ammoium	Sarasketa *et al*., 2014
***Pisum sativum* L.**	Aminating and deaminating GDH activity stimulated by short term ammonium supply	Aminating ammonium and deaminating amino acids	Frechilla *et al*., 2002
Soybean (Glycine max)	Export ureides as a N product during drought stress	Ureide strategy conserving of photosynthates than amide strategy	Alamillo *et al*., 2010
***Trifolium repens* L.**	Increased asparagine concentration in roots and nodules under low P supply	N feedback regulating atm N fixation	Almeida *et al*., 2000