

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

Anathi Magadlela¹, Chrizelle Beukes², Fanus Venter², Emma Steenkamp², Alex Valentine^{1*}

¹ Botany and Zoology Department, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa.

² Forestry and Agricultural Biotechnology Institute (FABI), Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa.

*Corresponding author: e-mail: alexvalentine@mac.com

Tel: (+27+21) 808-3067 Fax: (+27+21) 808-2405

Highlights

- P-poor soils can affect biological nitrogen fixation (BNF) in several ways.
- Nodule bacterial composition was not affected by P deficiency.
- However, the nodule metabolic environment was affected.
- This caused an alteration in N metabolism the export of N compounds.
- Bacterial tolerance to soil P stress may account for host species success.

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 N₂ fixing (BNF) efficiencies in *V. divaricata* during P deficiency. Furthermore, the aim was to integrate plants C and N metabolism to the N product exported via xylem to the shoots. Plants were grown at high and low P levels, both the high and low P plants were then supplied with either 500 μM NH₄NO₃ as soil nitrogen (N) source or exclusively relied on BNF. Although the bacterial composition of nodules remained seemingly unchanged by P and N supply, the nodule function was greatly altered. In this regard, plants reliant on only N₂ 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 N₂ fixation. The declines 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 NH₄. 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, N₂ fixation, nodule bacteria, xylem export, metabolites

Introduction

Among the plant families indigenous to the fynbos, the 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. 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 complexation 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 micromolar (μM) 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 optimal nodulation, and an excessively acidic pH will severely reduce nodule formation. Soil acidity and nutritional disorders, including P deficiency have been reported to adversely affect not only legume plant growth but also the legume BNF-bacterial symbiosis as well as nitrogen-fixing efficiency (Lie 1981; Munns 1986; Graham 1992; Magadlela et al, 2014).

Various fynbos plants including legume species have been reported to implement different mechanisms so as to enhance P acquisition and legumes in order to improve their capacity to fix atmospheric nitrogen (N₂). 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). Fynbos legumes can form symbiotic nodules with a wide range of biological N₂ fixing (BNF) bacteria, upon whom the plants rely for a large proportion of their N acquisition (Muofhe and Dakora 1999; Elliot et al, 2007; Kanu and Dakora 2012; Beukes et al, 2013). The symbiosis appears to be well adapted to the low nutrient, acidic soils of the Cape Fynbos. Given their reliance on BNF, legumes are expected to dominate the nutrient-poor fynbos ecosystem, but 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 BNF bacterial species in the nodule, integrated with plants carbon (C) and nitrogen (N) metabolism. Symbiotic N₂ 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 N₂ via bacterial symbiosis located in the nodules, such N fixation is fueled 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 legumes 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, the ammonium (NH₄) is incorporated into amides such as, glutamine and asparagine (Shi et al, 1997; Hirel and Lea, 2001; 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 BNF bacteria acquire large amounts of ammonium for the host plants, great research efforts have been dedicated in order to understand the regulation and mechanisms of ammonium assimilation in model plants

(Desbrosses et al., 2005; Barsch et al., 2006; Molero et al., 2014). However, 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 ecosystems, where P deficiency might nodule formation and function.

Since P nutrition in nodules can greatly alter the metabolic environment within these organs, it remains unknown how nodule occupation by N₂ fixing bacteria and their consequent N₂ fixation, are influenced by this. It is therefore hypothesized that during P deficiency in nodules, the altered metabolic environment would not affect nodule occupation by N₂ fixing bacteria in an indigenous legume from a nutrient-poor ecosystem, but that BNF, N metabolism and export would be altered.

Material and Methods

Plant material and growth conditions

Seeds of *Virgilia divaricata* Adamson 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 (H₂SO₄) for 30 min and then rinsing them 10 times with distilled water. Thereafter seeds were treated overnight with diluted smoke water (also obtained from Kirstenbosch Botanical Gardens).

The seeds were germinated in natural fynbos soils, which were randomly collected from 9 plots in a range where *V. divaricata* grows and these soil samples were also used as a natural source of bacterial inoculum. These soils are typical fynbos soils, derived from quartzitic sandstone from the Table Mountain group. These soils are

nutrient-poor (especially N and P) and acidic ($\text{pH } 5.85 \pm 0.61$). The co-ordinates of these sites were:

- (a) At the forest margins at the southern parts of the Western and Eastern Cape province within the Garden Route National Park of the Cape Floristic Region, South Africa $33.834975^\circ \text{ S}$, $23.446300^\circ \text{ E}$ and,
- (b) The mature mountain fynbos at the western parts of the Western Cape province within the Stellenbosch mountain, South Africa $33.959566^\circ \text{ S}$, $18.902278^\circ \text{ E}$.

For the natural soil phase of the experiments, 19 cm plastic pots were used. The germinated seeds were grown in 19 cm pots under the following glasshouse conditions: the range of midday irradiances was between $600\text{-}800 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and the average night/day temperatures were $15\text{-}25^\circ\text{C}$. For the quartz sand phase of the experiments, 19 cm plastic pots were used as well. After the seedlings were nodulated at 60 days of soil culture, they were transferred to 19 cm pots containing sterile quartz sand and initially watered with distilled water for a week, in order to acclimatize. At this stage most of the nodules were newly-matured and contained pink zones as an indication of being functionally active.

Hereafter seedlings were supplied with quarter strength Long Ashton nutrient solution ($\text{pH } 5.8$), modified with either high P ($500 \mu\text{M}$) or low P ($5 \mu\text{M}$) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and low P seedlings were then supplied with either $500 \mu\text{M}$ 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 ($500 \mu\text{M}$ NH_4NO_3 as soil

nitrogen (N) source or exclusively reliant on atmospheric N₂ fixation). The combination of the P concentrations and N sources resulted in four treatments, with 35 replicates of each treatment.

Harvesting and nutrient analysis

Harvesting (of xylem sap) occurred at 180 days at the sapling stage of plant growth, during which the sap was extracted between 06:00 and 09:00 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.

The nodules to be used for bacterial identification were harvested with the root system, separated and sterilized immediately. This involved the removal of fresh nodules from the roots of 10 plants per treatment, being surface sterilized by immersion in 70% ethanol for 30s, and in a 3% sodium hypochlorite solution for 3 minutes. These nodules were subsequently washed at least five times with sterile water and thereafter stored on ice until the bacterial extraction step. The remaining nodules were stored in airtight, closed vials containing silica and cotton wool, and were stored at -80° C for later assays.

Ten plants were harvested and 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 $\delta^{15}\text{N}$ 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 analyses

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

Bacterial diversity of soil inoculum

To assess the diversity of potential BNF bacterial taxa present in the soils used for inducing nodulation in *V. divaricata*, we used denaturing gradient gel electrophoresis (DGGE) analyses. For this purpose, 9 subsamples of the fynbos soils used as the natural inoculum were subjected to DGGE at a commercial laboratory (MicroSci, University of Pretoria, Pretoria, South Africa). At MicroSci 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), after which the 9 DNA samples were pooled into 3 and analyzed separately (see below). DNA concentration was determined by agarose gel electrophoresis.

To evaluate the bacterial diversity represented in each of the pooled DNA samples, 16S rRNA gene-based DGGE analyses was used. For this purpose, a 510 base pair (bp) portion of the 16S rRNA gene was amplified as described by Magadlela et al. (2015). The PCR products were subjected to DGGE analyses following the methods

described by Muyzer *et al.* (1993). Twelve microlitres (ca. 250 ng) of 16S rRNA gene PCR product was loaded per lane using 40-55% denaturing gradient gels. Gels were run at 70V 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).

To obtain tentative species identifications, individual bands on the DGGE gels were sequenced at Inqaba Biotec (Pretoria, South Africa). The resulting 16S rRNA gene sequences were subjected to BLAST analysis (Altschul *et al.*, 1990) against the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>).

Bacterial isolation, sequencing and phylogenetic analysis

Presumptive root nodule forming and N₂-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 (~ 1500 bp) of the 16S rRNA locus 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 MgCl₂, 250 µM Super-Therm Taq DNA polymerase (Inqaba Biotech, Pretoria), 10 mg/ml BSA and diluted pure bacterial colony. Prokaryotic DNA amplification was performed on a 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 3 amplicons from each treatment (low and high P with either NH₄NO₃ supplemented or reliantly solely on the fixation of atmospheric N₂) 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/>). Upon inspection of the 12 consensus sequences we found no difference in bacterial identity and therefore decided to group them based upon their P treatment (i.e., sequence 1 to 6 for low P and 7 to 12 for high P). We repeated the process and found no differences (i.e., sequences marked with 'A' correspond to the first round of sequencing while those marked with 'E' are from the second round).

The original sequence trace files for these 24 sequences were manually edited with the software ChromasLite v2.01 (Technelysium, Queensland, Australia) and BioEdit v7.0.5 (Hall, 1999). To verify the genus placement of the isolates, *blastn* searches were performed against the NCBI's GenBank database (Altschul et al., 1990; Benson et al., 2004). Decisions regarding taxon selection for the maximum-likelihood 16S rRNA phylogeny depended upon the *blastn* results; which is why all available type strain sequences for the environmental *Burkholderia* species was 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 was not yet available on the list as of 14 March 2016, 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) and *B. dipogonis* (Sheu et al., 2015).

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 (Kato and Standley, 2013). The best-fit evolutionary model (and parameters therefore) was determined with the programme jModelTest v2.1.7 (Dariba et al., 2012). A maximum-likelihood phylogenetic analysis was performed with PHYML v3.1 (Guindon, 2010; Guindon and Gascuel, 2003) using the best-fit model parameters and branch support was estimated using 1,000 pseudoreplicates (Felsenstein, 1985).

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

The $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ was calculated as $\delta = 1000\% (R_{\text{sample}}/R_{\text{standard}})$, where R is the molar ratio of the heavier to the lighter isotope of the samples and standards are as 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 $\delta^{15}\text{N}$ values for the nitrogen gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift: two in-house standards (Merck Gel and *Nasturtium*) and the IAEA (International Atomic Energy Agency) standard- $(\text{NH}_4)_2\text{SO}_4$. The %NDFA was calculated according to Shearer and Kohl (1986): $\% \text{NDFA} = 100((\delta^{15}\text{N}_{\text{reference plant}} - \delta^{15}\text{N}_{\text{legume}}) / (\delta^{15}\text{N}_{\text{reference plant}} - \text{B}))$. Where the reference plant was *Virgilia divaricata* grown under the same glasshouse conditions. The B value is the $\delta^{15}\text{N}$ 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 30°C in 40 μL 30 $\text{mg}\cdot\text{ml}^{-1}$ methoxyamine hydrochloride (in pyridine). Subsequently samples were trimethylsilylated by a 30 min treatment at 37°C 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

Construction costs, C_w (mmol C g⁻¹ DW), were calculated according to the methods proposed by Mortimer et al. (2005), modified from the equation used by Peng et al. (1993):

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

Where C_w is the construction cost of the tissue (mmol C g⁻¹ DW), C is the carbon concentration (mmol C g⁻¹), k is the reduction state of the N substrate ($k=-3$ for NH₃) 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 mmol C g⁻¹ DW.

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):

$$SNAR = [(M_2 - M_1/t_2 - t_1)] \times [(\log_e R_2 - \log_e R_1)/(R_2 - R_1)] \quad (2)$$

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

Specific Nitrogen utilization rate (SNUR) (g DW mg⁻¹ N d⁻¹) is a measure of the DW gained for the N taken up by the plant (Nielson et al. 2001):

$$\text{SNUR} = [(W_2 - W_1/t_2 - t_1)] \times [(\log_e M_2 - \log_e M_1)/(M_2 - M_1)] \quad (3)$$

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

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):

$$df/dt = \text{RGR} (\partial - B_r/B_t) \quad (4)$$

Where RGR is the relative growth rate ($\text{mg g}^{-1} \text{ day}^{-1}$) 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) ($P \leq 0.05$).

Results

Bacterial species identity and diversity

Sequence analysis of the 15 dominant DGGE bands obtained from the pooled soil samples showed that only three had similarity to bacteria that could potentially form effective nodules (i.e. *Bradyrhizobium* sp. (GenBank accession AB121773; similarity 89%), *Bradyrhizobium* sp. (GenBank accession AY547290; similarity 94%) and *Bradyrhizobium* sp. (GenBank accession GQ342569; similarity 90%). From the remaining 12 sequences, seven were highly similarity to various uncultured bacteria

while five were similar to those of bacteria in genera known to be capable of nitrogen fixation (i.e. *Sphingomonas*, *Frankia* and *Methylosinus*).

Sequence analysis of the nearly complete 16S rRNA gene amplified from the pure cultures isolated from the various *V. divaricata* root nodules, revealed that the bacteria represent members of the beta-rhizobium genus *Burkholderia*. The results included several sequences with high sequence similarity (99%) to *B. phytofirmans* isolate PSB48, while 19 of the isolates shared high similarity (99-100%) with the undefined species, *Burkholderia* sp. N362.

The sequences for all of the root nodule isolates formed part of a monophyletic and highly supported (93%) group in which there was very little sequence diversity. These isolates grouped with *Burkholderia* spp. N362, FM-A and CYEB-3, as well as an isolate labelled *B. phytofirmans* isolate PSB48 (Fig. 1). *Burkholderia dipogonis* LMG28415^T and *B. phytofirmans* PsJN^T (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* WSM3556^T and *B. rhynchosiae* WSM3937^T (Fig. 1).

Nodulating South African Burkholderia
Other nodulating Burkholderia
Nitrogen-fixing Burkholderia
BLAST results

Model selected:
 Model = TIM2+I+G
 Partition = 010232
 -lnL = 7989.2771
 K = 206
 freqA = 0.2354
 freqC = 0.2464
 freqG = 0.3282
 freqT = 0.1900
 R(a) [AC] = 0.7007
 R(b) [AG] = 1.4692
 R(c) [AT] = 0.7007
 R(d) [CG] = 1.0000
 R(e) [CT] = 2.9930
 R(f) [GT] = 1.0000
 p-inv = 0.7470
 gamma shape = 0.4610

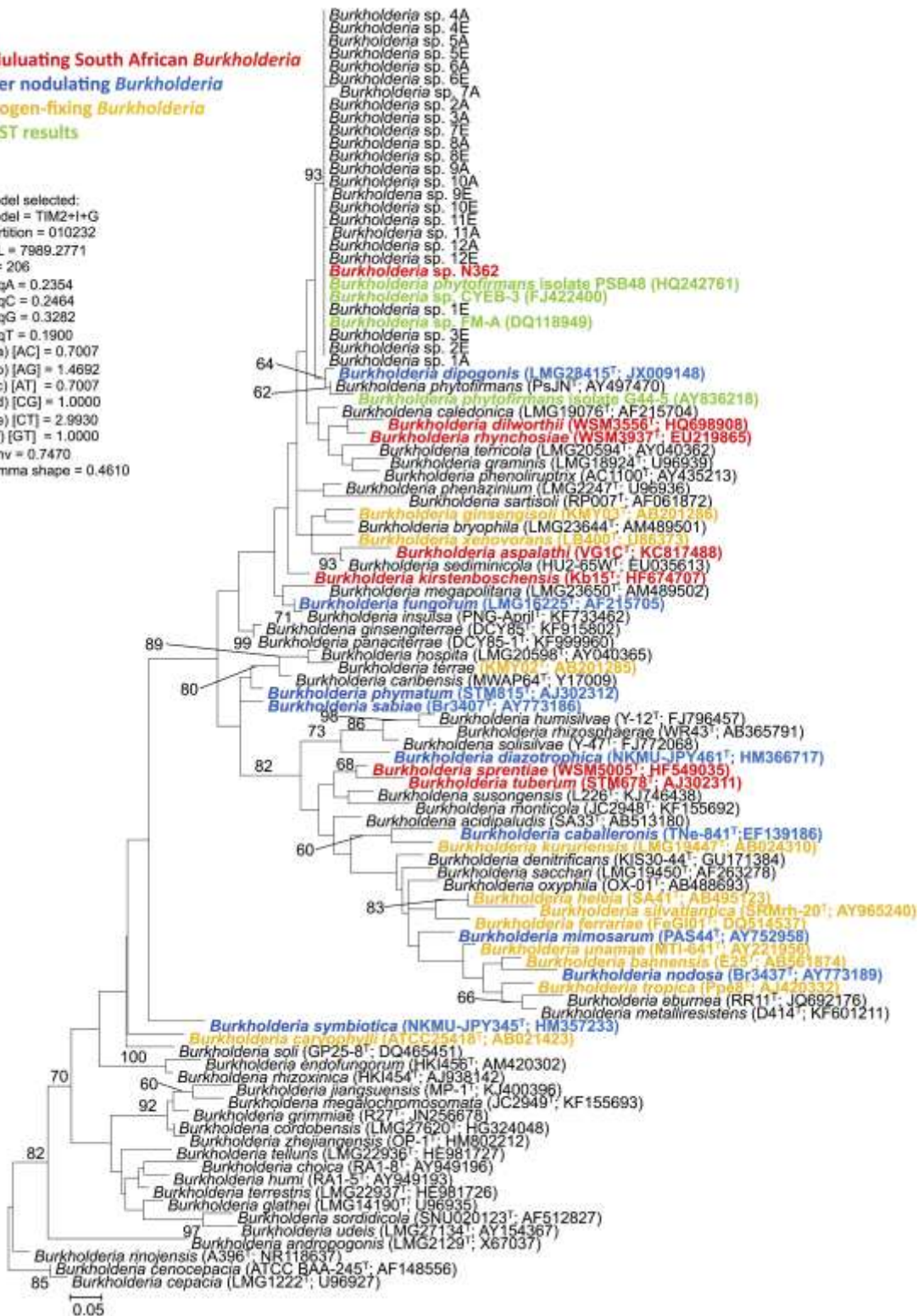


Figure 1. A 16S rRNA gene maximum-likelihood phylogeny for the *Burkholderia* sequences examined in this study. Only bootstrap support of $\geq 60\%$ are indicated. The sequence information for the type strains of *B. cepacia* and *B. cenocepacia* were used as for outgroup purposes. N_2 -fixing, nodulating and nodulating South African species are all indicated in different colours. For each taxon, type strain number and GenBank accession numbers are provided in parentheses.

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, while 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 N₂, which is presented by the reduction of % N derived from atmospheric N₂ irrespective of P supply (Table 2). This reduced plant reliance to atmospheric N₂ 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 N₂ fixing efficiency, low P plant exclusively reliant on atmospheric N₂ had a higher N₂ 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 N₂ (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 N₂, 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.

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 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and Low P plants were either supplied with either 500 μM NH_4NO_3 as soil nitrogen (N) source (N + Bacteria) or exclusively reliant on atmospheric N_2 (Bacteria). Values are presented as means (n = 10). The different letters indicate significant differences among the treatments. (*P < 0.05).

Parameters	Treatments			
	500 μM		5 μM	
	N + Bacteria	Bacteria	N + Bacteria	Bacteria
Dry weights				
Plant (g)	7.67 \pm 0.86a	6.72 \pm 0.90a	8.97 \pm 0.483a	6.82 \pm 0.535a
Shoot (g)	4.40 \pm 0.04a	5.21 \pm 0.58a	5.08 \pm 0.172a	4.68 \pm 0.319a
Roots (g)	2.42 \pm 0.884ab	1.07 \pm 0.28a	3.00 \pm 0.508b	1.65 \pm 0.239ab
Nodules (g)	0.85 \pm 0.135ab	0.44 \pm 0.04a	0.90 \pm 0.213b	0.49 \pm 0.100ab
Growth kinetics				
Relative growth rate ($\text{mg g}^{-1} \text{day}^{-1}$)	7.66 \pm 0.860a	6.71 \pm 0.90a	8.96 \pm 0.48a	6.81 \pm 0.53a
Root allocation ($\text{mg g}^{-1} \text{day}^{-1}$)	4.34 \pm 0.050a	5.09 \pm 0.59a	5.02 \pm 0.170a	4.60 \pm 0.32a
Nodule allocation ($\text{mg g}^{-1} \text{day}^{-1}$)	0.09 \pm 0.030b	0.02 \pm 0.001a	0.08 \pm 0.040b	0.03 \pm 0.01a
C construction costs ($\text{mmolC g}^{-1} \text{DW}$)	0.13 \pm 0.001c	0.124 \pm 0.001b	0.108 \pm 0.001a	0.13 \pm 0.001b
Growth respiration ($\mu\text{mol CO}_2 \text{day}^{-1}$)	140.6 \pm 35.0b	58.5 \pm 21.2a	51.0 \pm 9.00a	54.1 \pm 9.36a
Root:shoot	0.55 \pm 0.04bc	0.20 \pm 0.03a	0.60 \pm 0.12c	0.35 \pm 0.04b
Plant Mineral Concentrations				
P conc ($\mu\text{mol g}^{-1} \text{DW}$)	88.9 \pm 0.62c	101.2 \pm 2.27d	55.0 \pm 1.42b	34.9 \pm 0.82a
N conc fixed from atm ($\text{mmolN g}^{-1} \text{DW}$)	1.21 \pm 0.15ab	1.33 \pm 0.36c	1.05 \pm 0.14a	1.18 \pm 0.31bc
N conc assimilated by roots ($\text{mmolN g}^{-1} \text{DW}$)	0.83 \pm 0.13ab	1.35 \pm 0.35b	0.66 \pm 0.11ab	0.83 \pm 0.31a

Table 2
Nitrogen (N) data of *Virgilia divaricata* plants, grown in sand culture under high P (500 μM) or low P (5 μM) as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and Low P plants were either supplied with either 500 μM NH_4NO_3 as soil nitrogen (N) source (N + Bacteria) or exclusively reliant on atmospheric N_2 (Bacteria). Values are presented as means (n = 10). The different letters indicate significant differences among the treatments. (*P < 0.05).

Parameters	Treatments			
	500 μM		5 μM	
	N + Bacteria	Bacteria	N + Bacteria	Bacteria
percentage N. derived from the atm (%NDFA)	52.9 \pm 1.004a	82.6 \pm 0.465c	50.6 \pm 3.697a	73.7 \pm 2.049b
N. fixing efficiency ($\text{mmolN g}^{-1} \text{g}^{-1} \text{nodule}$)	1.47 \pm 0.193a	3.13 \pm 0.840b	1.22 \pm 0.176a	2.63 \pm 0.833b
N. fixing efficiency ($\text{mmolN } \mu\text{mol}^{-1} \text{P g}^{-1}$)	0.60 \pm 0.012a	0.81 \pm 0.021a	0.93 \pm 0.084b	2.17 \pm 0.102c
Total plant N conc. ($\text{mmol N g}^{-1} \text{DW}$)	2.05 \pm 0.071b	2.68 \pm 0.021c	1.71 \pm 0.045a	2.01 \pm 0.074b
Specific N. absorption rate (atm) ($\text{mmolN g}^{-1} \text{nodule d}^{-1}$)	0.037 \pm 0.01a	0.194 \pm 0.03c	0.025 \pm 0.01a	0.112 \pm 0.02b
Specific N. absorption rate (soil) ($\text{mmolN g}^{-1} \text{roots d}^{-1}$)	0.010 \pm 0.004a	0.031 \pm 0.009b	0.004 \pm 0.002a	0.007 \pm 0.002a
Specific N. utilization rate ($\text{g DW mg}^{-1} \text{N d}^{-1}$)	1.369 \pm 0.27a	0.583 \pm 0.11a	3.632 \pm 0.49b	1.031 \pm 0.24a
Amino acids concentration ($\text{mmol } \mu\text{L}^{-1}$)	0.048 \pm 0.002c	0.068 \pm 0.004d	0.031 \pm 0.00078b	0.019 \pm 0.001a
Ureides concentration ($\text{mmol } \mu\text{L}^{-1}$)	0.0006 \pm 0.0003b	0.0004 \pm 0.00004a	0.0006 \pm 0.00003b	0.0004 \pm 0.00002a
Ammonium concentration ($\text{mmol } \mu\text{L}^{-1}$)	0.005 \pm 0.00004c	0.0032 \pm 0.00004b	0.0031 \pm 0.0001b	0.003 \pm 0.00004a
Ureide:Amino acids	0.013 \pm 0.001b	0.006 \pm 0.0004a	0.021 \pm 0.001c	0.023 \pm 0.003c
Amino acids:NH4	9.65 \pm 0.3b	21.09 \pm 0.00001c	10.2 \pm 0.25b	6.89 \pm 0.34a
Ureide:NH4	0.125 \pm 0.006b	0.004 \pm 0.00003a	0.207 \pm 0.009d	0.156 \pm 0.009c

Nodule metabolites

The variation in P and N supply affected nodule metabolites. During low P there was reduction in the sucrose concentration and glucose concentration in the plants that are exclusively reliant on atmospheric N₂, where it was maintained in 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 N₂ than the other treatments. Glutamic acid concentration in the low P nitrogen amended plants showed an increase compared to high P nitrogen amended plants and low P atmospheric N₂ reliant plants (Table 3).

In the roots the major variation are seen on the principal sugar, sucrose and organic acids, malic acid. Where there is a significant difference between the low P plants, with the low P supplied plants exclusively reliant on atmospheric N₂ 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 N₂. 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).

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 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Both the high and Low P plants were either supplied with either 500 μM NH_4NO_3 as soil nitrogen (N) source (N + Bacteria) or exclusively reliant on atmospheric N_2 (Bacteria). Values are presented as means (n = 10). The different letters indicate significant differences among the treatments. (*P < 0.05).

Plant organ	Parameters	Treatments			
		500 μM		5 μM	
		N + Bacteria	Bacteria	N + Bacteria	Bacteria
Roots	Sugars (umol g⁻¹ DW)				
	Sucrose	109.3 ± 9.00ab	89.03 ± 12.5ab	30.71 ± 4.20a	151.2 ± 10.20b
	Glucose	1.40 ± 0.20a	1.59 ± 0.60a	1.08 ± 0.10a	2.82 ± 1.40ab
	Fructose	5.86 ± 0.60ab	5.01 ± 1.30ab	6.15 ± 0.20b	6.69 ± 0.80b
	Xylose	0.19 ± 0.10a	0.04 ± 0.01a	0.01 ± 0.01a	0.06 ± 0.01a
Nodules	Ribose	0.19 ± 0.01a	0.19 ± 0.10a	0.16 ± 0.01a	0.43 ± 0.20ab
	Sucrose	174.3 ± 51.5b	307 ± 59.8c	138 ± 22.7b	103 ± 0.50ab
	Glucose	8.50 ± 4.20c	9.75 ± 1.80c	7.90 ± 0.80bc	1.42 ± 0.10a
	Fructose	3.13 ± 0.70ab	6.94 ± 2.50b	4.46 ± 0.70ab	2.13 ± 0.20a
	Xylose	0.18 ± 0.10a	0.08 ± 0.01a	0.07 ± 0.01a	0.03 ± 0.01a
Roots	Amino acids (umol g⁻¹ DW)				
	Ribose	1.24 ± 0.70bc	1.38 ± 0.20c	0.57 ± 0.10abc	0.19 ± 0.01a
	Glutamic acid	0.01 ± 0.01a	0.02 ± 0.01a	0.03 ± 0.001a	0.13 ± 0.09ab
	Aspartic acid	0.01 ± 0.001a	0.06 ± 0.03a	0.02 ± 0.01a	0.11 ± 0.06ab
	Alanine	2.54 ± 1.38b	0.64 ± 0.29a	1.43 ± 1.26ab	1.92 ± 1.04ab
Nodules	Lysine	0.00 ± 0.00	0.001 ± 0.001a	0.002 ± 0.001a	0.02 ± 0.001a
	Threonine	0.00 ± 0.00	0.01 ± 0.01a	0.01 ± 0.001a	0.12 ± 0.001b
	Cystine	0.01 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a	0.02 ± 0.001a
	Glycine	0.43 ± 0.03ab	0.46 ± 0.06ab	0.48 ± 0.05ab	0.57 ± 0.14ab
	Proline	0.08 ± 0.01ab	0.08 ± 0.03ab	0.08 ± 0.01ab	0.13 ± 0.05ab
	Lucine	0.02 ± 0.001a	0.08 ± 0.03a	0.06 ± 0.01a	0.10 ± 0.06a
	Valine	0.004 ± 0.001a	0.02 ± 0.01ab	0.02 ± 0.001ab	0.01 ± 0.001ab
	Isoleucine	0.003 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a
	Glutamic acid	0.03 ± 0.02a	0.45 ± 0.01c	0.19 ± 0.06b	0.08 ± 0.02ab
	Aspartic acid	0.01 ± 0.001a	0.19 ± 0.03b	0.05 ± 0.01a	0.02 ± 0.01a
	Alanine	3.08 ± 2.94b	0.25 ± 0.10a	0.11 ± 0.05a	0.06 ± 0.02a
	Lysine	0.03 ± 0.001a	0.08 ± 0.001b	0.02 ± 0.001a	0.01 ± 0.001a
	Threonine	0.04 ± 0.02a	0.06 ± 0.01a	0.03 ± 0.001a	0.01 ± 0.001a
	Cystine	0.04 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a
Glycine	0.81 ± 0.26bc	1.15 ± 0.06c	0.64 ± 0.11ab	0.40 ± 0.03a	
Proline	0.19 ± 0.08b	0.34 ± 0.03c	0.19 ± 0.04b	0.05 ± 0.001a	
Lucine	0.15 ± 0.07a	0.32 ± 0.03b	0.11 ± 0.02a	0.05 ± 0.01a	
Valine	0.06 ± 0.03b	0.04 ± 0.01ab	0.03 ± 0.001ab	0.01 ± 0.001a	
Isoleucine	0.03 ± 0.01a	0.03 ± 0.001a	0.01 ± 0.001a	0.01 ± 0.001a	
Roots	Organic acids (umol g⁻¹ DW)				
	Malic acid	4.27 ± 0.39bc	1.49 ± 0.49ab	0.85 ± 0.09a	5.27 ± 1.30c
Nodules	Succinate	0.20 ± 0.04a	0.09 ± 0.01a	0.09 ± 0.02a	0.30 ± 0.11ab
	Malic acid	11.1 ± 1.82c	11.4 ± 0.49c	4.29 ± 0.77ab	2.53 ± 0.31a
	Succinate	0.68 ± 0.33bc	0.70 ± 0.01c	0.35 ± 0.08abc	0.10 ± 0.01a

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 N₂ 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 N₂ (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, with low P plants exclusively reliant on atmospheric N₂ with 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 N₂. 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, while there is no significant difference in plants solely reliant on atmospheric N₂, 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 N₂ with no significant difference in the plants amended with external N.

Discussion

It appears as though isolates related to the undefined *Burkholderia* sp. N362 are able to form functional nodules with the legume host *Virgilia divaricata* (Fig. 1). These isolates seem to remain the preferred symbiont of *V. divaricata* even during P deficiency and in spite of the altered nodular sugar and organic acid concentrations. Our results do indicate that nodule function was greatly influenced by P deficiency, as evidenced by the reduced reliance on biological N₂ 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 root nodule-bacteria) and plant growth promotion (to name but a few characteristics). The genus *Burkholderia* was not represented in the soil sample sequences although it was the only genus identified from the pure cultures of the resulting root nodules. This difference could be explained by the fact that *Burkholderia* might not be one of the

more dominant occupants of the soil microbiota, which might be why it was not detected by DGGE (van Elsas and Boersma, 2011). In turn, this would suggest that the symbiotic interaction with *V. divaricata* is associated with high levels of specificity to allow for the consistent isolation of *Burkholderia* from the *V. divaricata* root 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 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 a well-known endophyte (a bacterium present in plant tissue without causing harm; Bacon and Hinton, 2006) with plant growth-promoting properties (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). In fact, one of the other *Burkholderia* strains to which our *V. divaricata* root nodule isolates showed high similarity (*Burkholderia* sp. N362) does in fact possess the full complement of *nod* and *nif* genes (unpublished data).

The use of 16S rRNA sequence analyses allowed efficient identification of the genus to which the *V. divaricata* root nodule isolates belong, but were not suitable for their diagnoses to the species level. This is a common limitation for 16S rRNA-based studies and has also been demonstrated for *Burkholderia* species; i.e. multiple closely related *Burkholderia* species may have identical or highly similar 16S rRNA gene sequences (Steenkamp et al., 2015). Our future research will therefore seek to identify the *V. divaricata* symbiont(s) to species level using other, more suitable, approaches like Multi-Locus Sequence Analysis (MLSA) (Gevers et al., 2005). Such data would undoubtedly allow for more detailed examination of the possible nodule occupancy changes that might have occurred during the various nutrient treatments evaluated in this study. However, our data suggest that, should such changes indeed have occurred, the bacteria involved must have represented closely related members of the genus *Burkholderia*.

Burkholderia remained persistent in the nodules, notwithstanding the diversity of BNF-bacteria available in the soil and despite the P-deficient changes in the 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; Ludwig 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 BNF bacterial partner was able to fulfill its function despite drastic changes in external nutrient supply or internal nodule metabolic changes.

Our data thus suggest that the bacterial composition of the resulting nodules remained largely unchanged, in spite of the initial soil inoculum containing a variety of possible N₂-fixing and nodulating partners and the change in soil dynamics during growth. The constant host preference for *Burkholderia* (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 N₂ fixation and the consequent N metabolism and export.

In this regard, the higher N acquisition in P-stressed legumes exclusively dependent on N₂, 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 N₂ and not amended with soil N. However there was a reduction in the atmospheric N₂ 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 in 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 NH₄ (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, showed decreased plant biomass during P stress (Vardien et al.,

2014). This difference might be related to N₂ 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: NH₄ 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. It is also possible that this legume could have been assisted by other soil microbial partners during low P supply. In this regard, the role of arbuscular mycorrhizal fungi (AMF) in the nutritional physiology of legumes in nutrient-poor ecosystems is known to be beneficial to the host (Mortimer et al, 2013). Even though the AMF component was not investigated this this study, it does remain a possible adaptation to P stress by *V. divaricata*.

Conclusion

The variability in P supply to *V. divaricata* did not affect the bacterial composition of nodules, which indicates that these BNF bacteria are extremely well-adapted to maintain a functional symbiosis with the host legume in the P-poor soils of the CFR's fynbos ecosystem. In spite of the nodule occupancy being unaffected, the acquisition and metabolism of inorganic N was greatly affected by P supply. 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, the apparent *Burkholderia*-specific preference of *V. divaricata* may imply a very specific functional co-existence in a variable nutrient environment.

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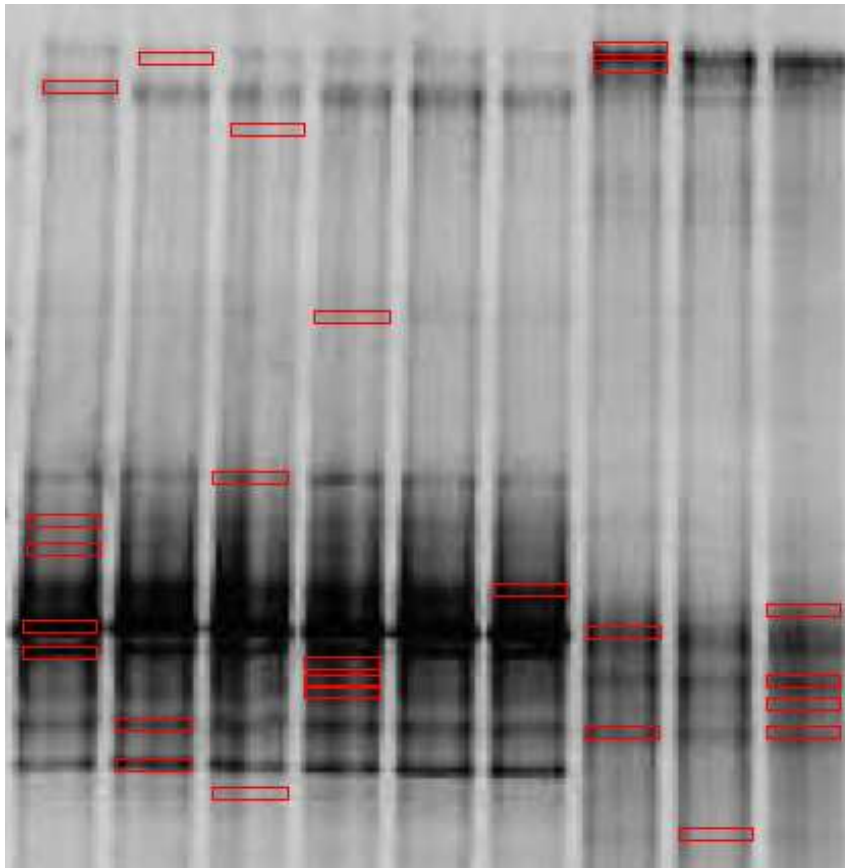
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Appendix A. Supplementary data



Supplementary figure 1. DGGE gel depicting the band pattern, indicating species diversity of bacteria from the rhizosphere soil subsample, run at 40-45% denaturants and dominant bands (highlighted in red) that were excised for sequencing and tentative bacterial identification.