The effects of phosphorus (P) deficiency on growth and nitrogen fixation of *Virgilia* trees from the Cape Floristic Region (CFR).

by

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Declaration

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Date: March 2013

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SUMMARY

The aim of this study was to determine how P nutrition affects biological nitrogen fixation (BNF) via effects on the N_2-fixing bacteria in the nodules of *Virgilia* species native to the Cape Floristic Region (CFR), South Africa. This was evaluated in 3 separate studies:

The first study aimed to determine how phosphorus deficiency affects N nutrition of two legume tree species from the Mediterranean Fynbos ecosystem. This study showed that during prolonged P deficiency, *V. divaricata* maintained a constant biomass, while *V. oroboides* showed a decreased biomass. *V. oroboides* showed a decrease in nutritional concentrations, which resulted in the decrease of symbiotic nitrogen fixation (SNF). Both plants utilized atmospheric N more efficiently per nodule under P deficiency. Maximum photosynthesis decreased in *V. oroboides*, while *V. divaricata* maintained its photosynthesis. Both species also had greater carbon construction costs during P deficiency. *V. divaricata* showed clear adaptive features during P-deficiency, as it maintained its growth respiration. The two legume species appear to have different adaptations to P deficiency, which may influence their performance and distribution in their naturally low P environment.

The second study aimed to evaluate if soil environmental conditions and mineral nutrient concentration play a major role in microbial communities in plant rhizosphere and nodulation during N_2 fixation in legumes. Therefore this study firstly aimed to determine the composition of the N_2 fixing bacterial population in the rhizosphere and nodules of *V. divaricata*. Secondly, it aimed to determine the contribution of these bacteria to N_2 fixation during conditions of P deficiency in the Fynbos environment. In the study, the effects of phosphate (P) nutrition on N_2 fixing bacterial community structures in *Virgilia divaricata* rhizosphere and nodules were examined in a pot experiment. *V. divaricata* were germinated in Fynbos soil as natural inoculum, transferred to clean sand cultures and supplied with 500 µM P and 5 µM P. The N_2 fixing bacterial communities in the rhizosphere and nodules were examined based on the PCR-DGGE banding patterns of 16S rDNA and sequencing methods. The GenBank blast results revealed that *V divaricata* was efficiently nodulated by a wide range of root-nodule bacterial strains, including *Burkholderia phytofirmans*, *Burkholderia sp*. and *Bradyrhizobium sp*. during low P supply. The ^{15}N

natural abundance data also confirmed that 40-50% of the N nutrition was acquired through symbiotic N_2 fixation. This is not only evidence of nodulation, but also an indication of the adaptation of a range of N_2 fixing bacterial strains / species to the nutrient poor, sandy, acidic soil of the Mediterranean-type ecosystems of the Fynbos.

The third study examined the physiological effects, such as N_2 fixation parameters, plant dependence on N_2 fixation, N preference, legume plant growth, carbon costs and amino acid biosynthesis during P deficiency and mineral N supply as NH_4NO_3 in a slow-growing, Fynbos legume tree, *Virgilia divaricata*. Continued application of NH_4NO_3 to the legume plant showed a greater increase in plant dry matter compared to plants with two nitrogen sources (mineral N and atmospheric N_2) or plants that relied on atmospheric N_2 fixation. Carbon construction costs were more pronounced in plants supplied with two N sources and during P deficiency. Maximum photosynthetic rates per leaf area increased during prolonged P deficiency, irrespective of the N sources. Though plants nodulated, plant dependence on N_2 fixation decreased with the addition of NH_4NO_3. Roots and nodules of the P deficient plants showed an increase in asparagine content, most strikingly so in plants treated with a single source of N.

These studies reveal that different legume species of the same genus, may employ contrasting adaptations in order to maintain N nutrition under P deficiency.

OPSOMMING

Die doel van hierdie studie was die bepaling van die wyse waarop fosfaat (P) voeding die biologiese stikstof binding (BNF) deur middel van die effek op N_2-bindingsbakterië in die wortelknoppies van *Virgilia* spesies wat inheems tot die Kaap floraryke area (CFR), Suid Afrika is, affekteer. Drie aparte eksperimente is uitgevoer om die doel te evalueer:

Die eerste studie het gepoog om te bepaal hoe ‘n fosfaat tekort N voeding van twee peulplant spesies van die Mediterreense Fynbos ekosisteem affekteer. Hierdie studie het getoon dat *V. divaricata* ’n konstante biomassa tydens verlengde P tekort behou, terwyl *V. oroboides* ’n verlaagde biomassa getoon het. *V. oroboides* het ’n verlaging in voedingskonsentrasies getoon, wat tot ’n verlaging in simbiotiese stikstof binding (SNF) gelei het. Beide plante benut atmosferiese N meer doeltreffend per nodule tydens P tekort. Die maksimum fotosintese in *V. oroboides* het afgeneem, terwyl *V. divaricata* sy fotosintese gehandhaaf het. Beide spesies het ook ’n groter koolstof konstruksie koste tydens P tekort gehad. *V. divaricata* toon duidelike aanpassingsmeganismes tydens P-tekort, aangesien hierdie species sy groei respirasie konhandhaaf. Dit wil voorkom asof die twee peulplant spesies verskillend aangepas is vir P tekort, wat hulle producksie en verspreiding in hulle natuurlike lae P omgewing mag beïnvloed.

Die doel van die tweede studie was om te bepaal of grond omgewingskondisies en minerale voedingskonsentrasie ’n belangrike rol speel in die mikrobiese gemeenskappe in die plant risofeer en wortelknoppie vorming tydens N_2 binding in peulgewasse. Eerstens het die studie dus gepoog om die samestelling van die N_2 bindende bakteriële populasie in die risosfeer en die wortelknoppies van *V. divaricata* te bepaal. Ten tweede, is die bydrae van die bakterië tot N_2-binding tydens P tekort kondisies in die Fynbos omgewing bepaal. In die studie is die effek van fosfaat (P) voeding op die N_2-bindende bakteriële gemeenskapstrukture in die *Virgilia divaricata* risofeer en wortelknoppies in ’n pot eksperiment ondersoek. *V. divaricata* sade is in fynbos grond as ’n natuurlike inokulum ontkiem, waarna dit na skoon sand kulture oorgedra is en van 500 µM P en 5 µM P voorsien is. Die N_2-bindende bakteriële gemeenskappe in die *risofeer* en wortelknoppies is op grond van die PCR-DGGE band patrone van die 16S rDNA en volgorde bepalingsmetodes

ondersoek. Die GenBank Blast resultate het getoon dat *V. divaricata* doeltreffend deur ‘n wye reeks wortel-wortelknoppie bakteriële stamme genoduleer is, insluitende insluitende *Burkholderia phytofirmans*, *Burkholderia sp*. en *Bradyrhizobium sp*. tydens lae P toediening. Die natuurlike ^{15}N voorkoms data het ook bevestig dat 40-50% van die N voeding deur simbiotiese N_2 binding bekom is. Dit dien nie net as bewys vir wortelknoppie vorming nie, maar ook ’n aanduiding van die aanpassing van ’n reeks N_2 bindende bakteriële stamme/ spesies tot die voedingsarme, sanderige, suur grond van die Mediterreanse ekosisteem van die Fynbos.

Die derde studie het die fisiologiese effekte soos bv. N_2 fikserings faktore, die afhanklikheid van plante op N_2 fiksering, N voorkeur, peulgewas groei, koolstof kostes en aminosuur biosintese tydens P tekort en minerale N toediening soos NH_4NO_3 in ’n stadig-groeiende, Fynbos peulgewasboom, *Virgilia divaricata* ondersoek. Volgehoue toediening van NH_4NO_3 aan die peulplant toon ’n groter toename in plant droë weefsel. Tydens P tekort is die koolstof *bou* koste meer verhoog in plante wat met twee N bronne voorsien is. Tydens verlengde P tekort het die maksimum fotosintese tempo per blaaroppervlakte toegeneem, ongeag die N bron. Alhoewel die plante wortelknoppies gevorm het, het die plant se afhanklikheid van N_2 binding tydens die toediening van NH_4NO_3 afgeneem. Wortels en wortelknoppies van die P *tekort* plante het ’n toename in asparagien inhoud getoon, veral in die plante wat met ’n enkele bron van N behandel is.

Hierdie studies toon dat peulplantspesies van dieselfde genus, teenstellende aanpassings gebruik om optimale N voeding te kan behou tydens P tekorte.

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By Anathi Magadlela[1], Aleysia Kleinert[1], Léanne L. Dreyer[1], Alexander J. Valentine[1*]

CHAPTER 5

Title: The role of P nutrition in N acquisition from different inorganic N sources in the legume tree species, *Virgilia divaricata* in native Cape Fynbos soils.

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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 (Mabberley, 1997; Cronquist, 1981) 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). 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). Caesalpinioids have been considered to comprise a number of unrelated lineages, which was 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).

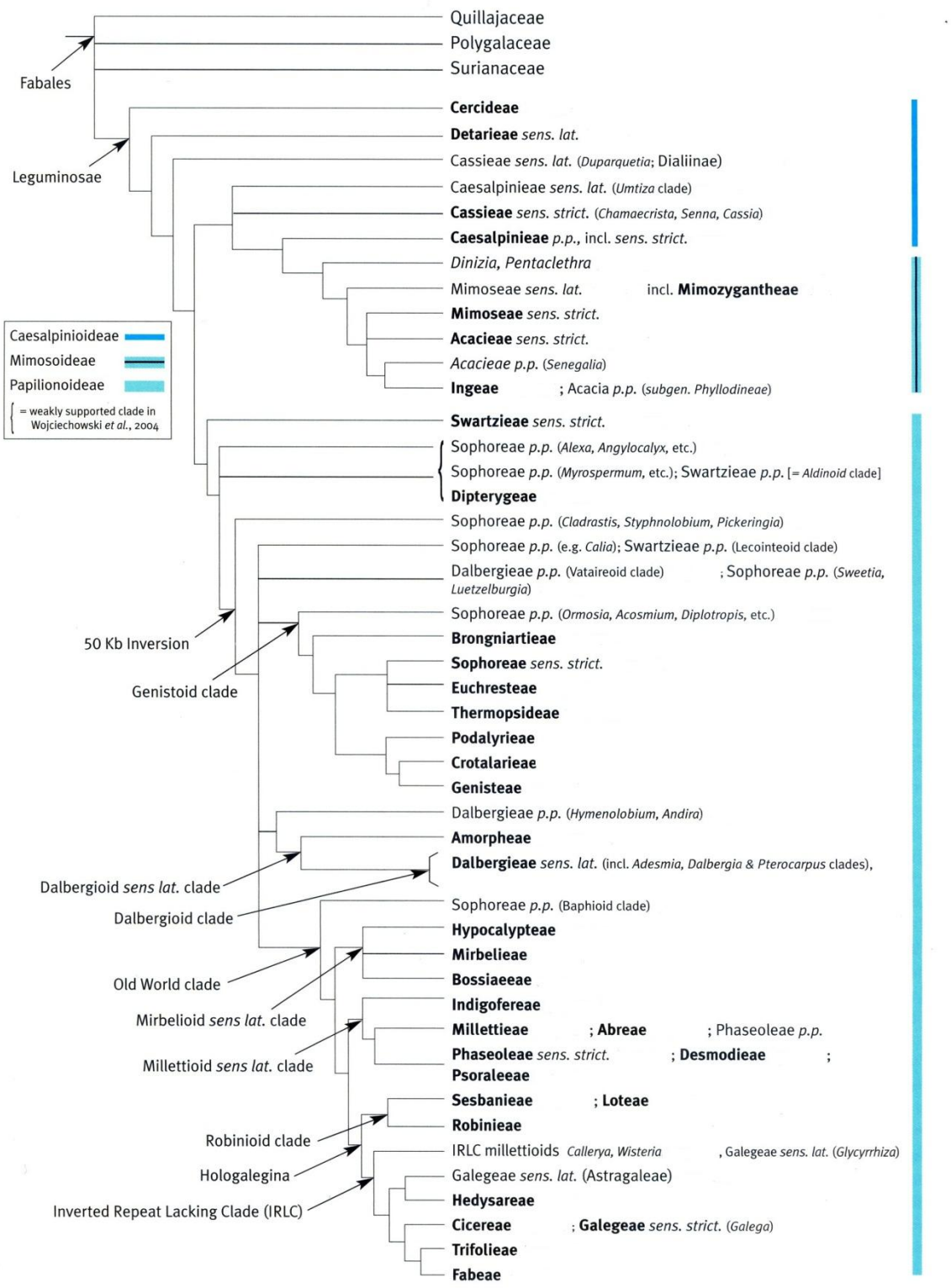

Figure 1.1 Phylogeny of Fabaceae compiled as a supertree based on genetic data derived from the matK gene, analysed by Doyle *et al*., (2000); Kajita *et al*. (2001); Wojciechowski, 2003. Redrawn from Lewis *et al*. (2005)

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). Fabaceae represents one of only a few plant families known to be able to undertake biological nitrogen fixation. By doing so, legumes form an important component of the nitrogen cycle on land, and for this reason agricultural systems have traditionally relied heavily on legumes for nitrogen 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 nitrogen. 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 nitrogen 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 protection of the environment is effective management of nitrogen 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 nitrogen 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, biological N_2 fixation and the growth of the legumes, as determined by climate and soil, and the management of residues. Grain legumes contribute less nitrogen than herbaceous legumes to subsequent crops in rotation, because most of the nitrogen 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 nitrogen 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 nitrogen (N_2) in the atmosphere is converted into ammonia. Most of the nitrogen available to plants is in the form of gaseous nitrogen (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). 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 (Raven *&* Johnson, 2008; Taiz & Zeiger, 2010; Valentine *et al*., 2011; Van Kessel & Hartley, 2000).

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

Nitrogen fixation is utilised by microorganisms, *Cyanobacteria, Azotobacteraceae, Rhizobia* and *Frankia,* collectively called diazotrophs. 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 especially for this purpose. The special plant organs that enclose these bacterial symbiosomes are called nodules. One of the most important sites of biological nitrogen fixation 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 determinate or indeterminate. This is characterised by the nitrogen fixation product (NH_3/NH_4^+) during N assimilation which involves enzymes, glutamine synthetase

(GS) and glutamate synthase (GOGAT), which can either export amides (mainly asparagine) or ureides (allantoin and allantoic acid). Both amides and ureides are exported from this cite of production to the shoot via the xylem of the host plant (Schubert *et al*., 1995; Walsh, 1995).

NH_3/NH_4^+ + GLUTAMATE + ATP → GLUTAMINE + ADP + P_i + H^+

This nitrogen fixation process is essential for life, because fixed nitrogen is required to biosynthesize the basic building blocks of life, e.g. nucleotides for DNA and RNA and amino acids for proteins (Raven & Johnson, 2008; Taiz & Zeiger, 2010). The family Fabaceae represent one of only a few plant families known to be able to form such root nodules. They are able to establish symbiosis with diazatrophic bacteria (for example, the genus *Rhizobia,* which has the unique ability to establish N_2-fixing symbiosis on legume roots) in exchange for metabolites and reduced carbon (Valentine *et al*., 2011; Van Kessel & Hartley, 2000).

1.3 Carbon and energy cost of nitrogen fixation

Nitrogen fixation is the most energetically expensive reaction known to occur in any plant cell (Raven & Johnson, 2008). In order to obtain reduced nitrogen as ammonia, the plant trades the symbiotic nitrogen fixation 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 nitrogen 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 nitrogen fixation is assumed to require significantly more energy per N fixed than NO_3^- uptake and reduction (Valentine *et al*., 2011). 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 legumes species is the fact that they can easily succeed in low N environments where other species struggle (Valentine *et al*., 2011; Bordeleau & Prevost, 1994). Symbiotic nitrogen 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 nitrogen 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 nitrogen fixation if exposed to limiting factors (Zahran, 1999).

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

After nitrogen, phosphorus (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 (Le Roux *et al*., 2006; Plaxton & Tran, 2011; Rychter *et al*., 1992). In the case of legumes, more P is required by symbiotic than non-symbiotic plants. Symbiotic nitrogen 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 (Jebara *et al*., 2004; Vadez *et al*., 1997) 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 nitrogen fixation (Sar & Israel, 1991; Valentine *et al*., 2011). This coincides with findings of Le Roux *et al*. (2009), who showed that the nodule construction cost and growth respiration of soybeans increased with P deficiency. Thus P deficiency can lead to a reduction of both nodulation and symbiotic nitrogen fixation (Drevon & Hartwig, 1997)

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. 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 of acidity. This legume also displayed the ability to modify its rhizosphere pH in order to promote symbiotic establishment. 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, P deficiency is commonly reported along with Al^{3+} toxicity. 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 (Le Roux *et al.,* 2007). The effect of the relationship between low phosphate supply and N_2 fixation on legumes remains unclear, primarily because of the indifference in responses to P deficiency by some legume species (Le Roux *et al*., 2007). 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. 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 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; Horst *et al.,* 2001; Vance, 2011; Raghothama, 1999). 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 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 servere P deficiency in white lupin proteoid roots. Moreover, the formation of cluster roots results in an increase in nodule number, increasing nodule 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 and Read, 1997; Mortimer *et al*., 2008, 2009). Apart from these adaptations of cluster roots, the indigenous legumes that occur naturally in low nutrient ecosystems, such as in the Cape Floristic Region (CFR), may have other unique adaptations to grow in such a low P environment.

Most legumes species in the CFR (Goldblatt & Manning, 2000) of South Africa seem to have developed these adaptations, 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 9 000 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 nitrogen and phosphate (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). P availability has been shown to vary in these different soil types. For instance, Witkowski & Mitchell (1987) demonstrated that strandveld 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 dependant 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 nitrogen and phosphorus in the soils of this ecosystem has been well-studied, as they seem to have different nutrient patterns and they are the two elements that are suggested to be most likely to limit primary production of legumes endemic to the Fynbos (Cowling, 1992). Coupled with this low nutrient status, Fynbos vegetation experiences extensive periods of summer drought alternating with, wet winters associated with low temperatures. Plants in the Fynbos possess specialized nutrient uptake strategies (Lamont, 1983) and internal nutrient cycling pathways (Mitchell *et al*., 1986). Nutrient availability in Fynbos communities plays an important ecological role in determining species distributions and community composition (Kruger *et al*., 1983). Certain Fynbos areas, such as the Agulhas Plain, for example, have very high levels of edaphic endemism (Cowling, 1992).

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 (Lewis, *et al*., 2005; Van Wyk, 1986), 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).

This decrease in legumes numbers with increasing veld age may be ascribed to the intense energetic costs of nitrogen fixation, which potentially limits the ability of competition in low light or low macro-nutrient (e.g. P) availability (Power *et al*., 2010). 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, indigenous CFR legumes are adapted for growth in these soils (Maistry, 2010) and many display high phosphorus-use efficiency and specialized phosphorus uptake strategies (Maistry, 2010). Therefore these adaptations enable Fynbos legumes to fix atmospheric N_2 under these low macro-nutrient soil conditions of the Fynbos (Maistry, 2010; Power *et al*., 2010).

Clearly there is a need for information on the dynamics of N and P 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. A better understanding of metabolic and ecological costs associated with phosphorus acquisition strategies is needed for the management and future conservation of Fynbos legume species (Lynch & Ho, 2005; Maistry, 2010; Power *et al*., 2010). We need to understand the mechanisms of legume adaptations to fix N_2 in a low P environment. This research may also provide genetic insights into these physiological adaptations that 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, 2002). These genes can also be used in non-genetic modification approaches, where they can be used to identify molecular markers, which breeders can use to breed crop plants with these genetic traits.

1.5 Research problem

For legume in nutrient-poor ecosystems, the understanding of the role of phosphorus in the carbon costs, nodulation and efficiencies of N acquisition from the soil and atmosphere, remains incomplete. This is important for legume species indigenous to the low phosphorus soil ecosystems associated with Fynbos vegetation in the Cape Floristic Regions (CFR).

1.6 Aim

To determine the effects of P deficiency on the carbon economy of *Virgilia oroboides* and *V. divaricata* plants acquisition N from soil and atmospheric sources.

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CHAPTER 2

General Introduction

2.1 General introduction

The Cape Floristic region 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 Fynbos near forest margins, besides streams or on river banks. They have also been documented from hillside thickets on the narrow strip along the southeastern coast of South Africa (Greinwald *et al*., 1989; Van der Bank *et al*., 1996). 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). These legume plants grow well on these phosphate deficient characterised soils of the Fynbos in the Cape Floristic Region (CFR), South Africa, where they are able to fix atmospheric N_2 in these environmental conditions (Goldblatt & Manning, 2000; Maistry, 2010).

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). Phosphate 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. The nodules of legume host plants require comparatively high amounts of P and energy during N fixation (Sar & Israel, 1991; Drevon, 1997; Olivera *et al*., 2004; Valentine *et al*., 2011). Most studies related to phosphate (P) deficiency during symbiotic nitrogen (N) 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 Fynbos ecosystems. Given the

nutrient poor state of these soils, Fynbos legumes may display specific adaptive strategies to function under these challenging conditions (Maistry, 2010).

Evaluating the physiological and biochemical reactions of the two *Virgilia* species during prolonged periods of P deficiency will aid our understanding of the carbon economy costs and efficiency of these plants to fix atmospheric N_2. 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 host carbon reserves. Such carbon economy costs need to be determined in order to enable better management of these trees with or without their symbiotic partners.

2.2 Research Objectives

1) To determine the carbon costs and efficiency of symbiotic nitrogen fixation (SNF) by the specific species of N_2-fixing bacteria in the nodules of *Virgilia* during P deficiency.

2) To determine the composition of the N_2-fixing bacterial population in the nodules of *Virgilia* during P deficiency.

3) To determine the costs of inorganic N acquisition and assimilation in the host nodules and roots during P deficiency in *Virgilia* species.

2.3 Research Methods

The research methods will be a suite of plant physiological and molecular biological methods in order to address these objectives. Briefly, these methods as follows:

2.3.1 Symbiotic N_2 fixation (SNF)

The method that will be used to quantify SNF during this study will be the stable isotope, ^{15}N natural abundance method and the carbon costs method. The stable isotope, ^{15}N natural abundance technique is based on the differences in the natural abundance of the stable isotope of N, (^{14}N and^{15}N) between atmospheric N_2 and other sources of N (Herridge *et al*., 1995). The heavier ^{15}N isotope occurs less in the atmosphere as compare to ^{14}N isotope (Mariotti, 1983).

The corrected $\delta^{15}N$ values will be used to determine the percentage N derived from the atmosphere (NDFA). %NDFA will be 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 the reference plant will be grown under the same glasshouse conditions. The B-value will be the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue grown in a N-free solution.

2.3.2 Carbon costs

The allocation of carbohydrates to various plant parts and functions is a governing parameter for plant survival (Nielsen *et al*., 2001). Therefore the carbon costs technique measures the costs of symbiotic N_2 fixation in terms of respired simple carbohydrates, as the success of plant under stress is determined by the ability to control its carbohydrate utilization for metabolic energy (Nielsen *et al*., 2001).

Carbon construction costs, C_W (mmol C g^{-1} DW), will be calculated according to the methods of 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 (mmol C g^{-1} DW), C is the carbon concentration (mmol C/g), *k* is the reduction state of the N substrate (*k*=-3 for NH_3) and N is the organic nitrogen content of the tissue (g/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 to mmol C/ gDW.

2.3.3 Gas exchange

Infra-red gas analyses was conducted to measure photosynthetic gas exchange on light and CO_2 response curves. The data will be used to calculate the Rubisco activity and electron transport capacity. Measurements will be performed on the using an open gas exchange system Li-6400 (LI-COR Inc., IRGA, Lincolin, NE, USA).

2.3.4 PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

DGGE will be used to identify the bacterial population within root nodules. DGGE is the most appropriate molecular method for monitoring microbial community ecology (Banks and Alleman, 2002; Gillan, 2004). The method involves a separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often 16S rRNA gene. The PCR amplicons are then electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The resulting band pattern on the gel forms a genetic “fingerprint” of the entire community being examined (Banks and Alleman, 2002; Gillan, 2004).

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CHAPTER 3

Phosphorus deficiency affects N nutrition of two legume tree species in the Mediterranean Fynbos ecosystem

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Phosphorus deficiency affects N nutrition of two legume tree species in the Mediterranean Fynbos ecosystem

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Abstract

Nodulated *Virgilia divaricata* and *V. oroboides* were grown in sand and supplemented with N-free, quarter strength Long Ashton nutrient solution modified with either high phosphate (500 µM) or low phosphate (5 µM) nutrient solution for 160 days. The physiological parameters of the saplings, including biomass, gaseous exchange, respiratory carbon metabolism and biological nitrogen fixation, under prolonged P deficiency, were assessed during this experiment. During prolonged P deficiency, *V. divaricata* maintained a constant biomass, while *V. oroboides* showed a decreased biomass. *V. oroboides* showed a decrease in nutritional concentrations, which resulted in the decrease of Biological Nitrogen Fixation (BNF). Both plants utilized atmospheric N more efficiently per nodule under P deficiency. Maximum photosynthesis decreased in *V. oroboides*, while *V. divaricata* maintained its photosynthesis. Both species also had greater carbon construction costs during P deficiency. *V. divaricata* showed clear adaptive features during P-deficiency, as, unlike *V. oroboides*, it maintained its growth respiration rate.

These two legume species appear to have different adaptations to P deficiency, which may influence their performance and distribution in their naturally low P environments.

Keywords: *Virgilia* species, legume, P deficiency, N nutrition, C costs

3.1 INTRODUCTION

The Mediterranean-type ecosystem of the Cape Floristic Region (CFR) in South Africa mainly grows on sandstone-derived soils (Goldblatt & Manning, 2000), which are typically very acidic and nutrient poor (Kruger *et al*., 1983). This region bears a resemblance to the soils of the Western Australian heathlands rather than other Mediterranean-climate regions (Groves, 1983; Mitchell *et al*., 1984). *Virgilia* is one of legume genera that are endemic to these CFR (Fynbos) acidic soils. Mediterranean acidic soils usually have different concentrations of elements, coupled with related nutrient deficiencies, associated with nitrogen (N) and phosphate (P) deficiencies (Bordeleau & Prevost, 1994; Von Uexkull & Murtet, 1998; Grigg, Veneklass & Lambers, 2008). Soil acidity is a significant problem facing legume and agricultural production in many areas of the world, including southern Africa (Graham, 1992; Bordeleau & Prevest, 1994; Correa & Barneix, 1997; Marschner, 1995). Most legume plants require neutral to slightly acidic soils for growth, but experience problems with nodulation if the pH drops to a very acidic state. Soil acidity and nutritional disodrder adversely affects legume plant survival, growth and legume-*Rhizobium* symbiosis affecting nitrogen fixation of micro-organisms (Lie, 1981; Munns, 1986; Graham, 1992). P remains mostly unavailable for plant uptake, specifically on acid-weathered soils in both tropical and subtropical regions (Bieleski, 1973; Schactman *et al*., 1998; Vance, 2011; von Uexkull & Mutert, 1998).

Phosphate is quite abundant in many soils, but because it forms insoluble complexes with cations and is bound to organic compounds by microbial action, it is often unavailable for plant uptake (Vance, 2011; Richardson, 1994; Jungk *et al*., 1993). Low P soils will limit legume growth to a greater extent than low nitrogen soils, since during symbiosis with *Rhizobia* legumes can utilise both atmospheric nitrogen and soil nitrogen acquired through *Rhizobia* in their nodules (Mortimer *et al*., 2008). It has been reported that host legume plants require comparatively high amounts of P and energy, thus P deficiency can impair both nodulation and symbiotic nitrogen fixation, consequently reducing N nutrition of the plant affecting photosynthesis, respiration, growth, organic acid supply and production of the host plant (Vadez *et al*., 1997; Drevon & Hartwig, 1997; Almeida *et al*., 2000; Lynch & Ho, 2005; Olivera *et al*., 2004; Harrison *et al*. 2009). Despite these complications, legume species endemic to

Fynbos grow specifically in such acidic soils. They must therefore have evolved adaptations to obtain adequate P under these conditions (Vance *et al*., 2003).

Such adaptations may follow one of two possible strategies. Some are aimed at conserving the use of P, while others are 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,b). The enhances root nodule efficiency for P utilization (Le Roux *et al*., 2009), includes root exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters (Gilbert *et al*., 1999; Gilroy & Jones, 2000; Lynch & Brown, 2001; Neumann & Martinoia, 2002; Uhde Stone *et al*., 2003a; Lamont, 2003; Vance *et al*., 2003). The exudation of organic acids such as malate and citrate stimulated by P stress has mostly been reported in non-mycorrhizal species such as Lupin (Dinkelaker *et al*., 1995; Hinsinger, 2001; Jones, 1998; Le Roux *et al*., 2008; Ryan *et al*., 2001; Le Roux *et al*., 2008).

The high sensitivity of legume plants, and indeed the N_2 fixation process, to environmental conditions, acidic soils associated with P deficiency may result in higher carbon costs (Mengel, 1994). This concurs with Le Roux *et al.* (2009), who showed that lupin nodules under P stress acted as stronger carbon sinks during plant growth. Nodules are known to have a strong carbon sink capacity for P assimilation during P starvation to maintain nitrogen fixation (Hogh-Jensen *et al.,* 2002). The enhanced nodule efficiency for P utilization is considered to be an essential coping strategy during P stress (Le Roux *et al*., 2009). Furthermore, carbon sink was found to be more pronounced in plants during double symbiosis under low P conditions (Mortimer *et al*., 2008). This was shown by a greater growth respiration of low P plants compared to high P plants (Mortimer *et al*., 2008). The sink effect was also evidenced by the higher photosynthetic rates of host plant (Mortimer *et al*., 2008). In the case of P stress, the most direct currency is P itself, or growth parameters related to P accumulation (Koide *et al*., 2000).

Virgilia is a small tree genus that includes two species (*V. divaricata* Adamson and *V. oroboides* (P. J. Bergius) Salter) and 2 subspecies. It is confined to the southwestern and southern coastal regions of the CFR (Greinwald *et al.,* 1989). A few studies have been done on growth and adaptations of legumes species native to Mediterranean-type ecosystems that occur on naturally acidic soils. However, published information on the dynamics of N and P uptake and utilisation in legume plants in Fynbos soils remains limited.

The aim of this study was to determine how P deficiency affects nitrogen nutrition (BNF) via soil and atmospheric sources in the two Fynbos legume species *V. divaricata* and *V. oroboides*. Results should enhance our understanding of the metabolic and ecological costs associated with phosphorus acquisition strategies on acidic Fynbos soils. This, in turn, will aid the future management and conservation of legume species native to the acid soil environments of the CFR.

3.2 MATERIALS AND METHODS

Plant material and growth conditions

Seeds of both *V. oroboides* and *V*. *divaricata* were obtained from Kirstenbosch Botanical Gardens, Cape Town, and 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. Hereafter seeds were soaked overnight with 250 ml smoke water dilution, also obtained from Kirstenbosch National Botanical Garden, Private Bag X7, Claremont 7735, Cape Town, South Africa. The seeds were germinated in natural Fynbos soil (natural inoculation), obtained from Stellenbosch Mountain, Stellenbosch, South Africa. Plants were grown under the ambient conditions in the glasshouse of the Department of Botany and Zoology, University of Stellenbosch (the maximum daily photosynthetically active irradiance was between 600 and 700 µmol m^{-2} s^{-1} and the average day and night temperatures and humidities were 25/15 $^{\circ}$C and 35/75% respectively). They were watered daily, with 100 ml of distilled water (H_2O). After seedling emergence, they were transferred to sterilized sand (autoclaved and rinsed with distilled H_2O) and initially watered with 100ml distilled water for a week to acclimatize. Hereafter seedlings were supplied

with low N (500 µM NH_4NO_3), quarter strength Long Ashton nutrient solution. Fynbos plants are adapted to low nutrients, but can experience P stress under very low P supply levels. Extremely low P levels were imposed to stretch limits of P requirements, where the nutrient solutionwas modified to high P (500 µM) and low P (5 µM) (pH 5.8). Plants were supplied with nutrient solution once a week and watered with distilled H_2O in between nutrient solution supply. The split design experiment was replicated eight times per species and per treatment.

Harvesting and nutrient analysis

Harvesting intervals occurred at 42 days when seedlings emerged and 90 days after seedling emergence based on plant size. Upon harvesting, the plants were separated into nodules, roots, stems and leaves. The harvested plant material was placed in a drying oven, at 40˚C for 3 days and their dry weights (DW) were recorded. The dried material was milled with a ball mill. The milled samples were analysed for their respective C, N and P concentrations by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (BemLab, De Beers Road, Somerset West, South Africa).

Gas exchange measurements

The photosynthesis response to varying intercellular CO_2 concentrations was done to measure maximum photosynthesis (Pmax), Rubisco activity and electron transport. Measurements were performed on the youngest fully expanded leaves (5 replicates in each treatment per species), using an open gas exchange system Li-6400 (LI-COR Inc., IRGA, Lincolin, NE, USA). Measurements were taken from 9:00 to 16:00h. A full response curve took 45 minutes to 1 hour to complete. The leaves were enclosed in a leaf chamber (6 cm^2), which received a steady light of 1800 $\mu mol.m^{-2}.s^{-1}$ at leaf temperature of 24 ˚C. The carbon varied from 50, 100, 150, 200, 250, 350, 500, 650, 800, 900, 1000, 1500 to 2000 ppm.

Carbon and nutrition cost calculations

Construction costs, C_W (mmol C g^{-1} DW), were calculated according to the methods of 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 (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 NH_3) and N is the organic nitrogen content of the tissue (g.DW^{-1}) (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 DW^{-1}.

Specific N absorption rate (SNAR) (mgNg^{-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.

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):

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

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

Growth respiration R_g (t) (µmol CO_2 d^{-1}) is the daily growth respiration of the plant (Peng *et al.*, 1993)

Growth respiration R_g (t) = $C_t - \Delta W_c$,

C_t (µmol CO_2 day^{-1}) is the C required for daily construction of new tissue. C_t was calculated by multiplying the root growth rate (g DW day^{-1}) by tissue construction cost (C_w). ΔW_c (µmol C day^{-1}) is the change in root C content and was calculated by multiplying the root C content and root growth rate.

Calculations of % NDFA

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of $\delta^{15}N$ was calculated as δ=1000‰ ($R_{sample}/R_{standard}$), where *R* is the molar ratio of the heavier to the lighter isotope of the samples and standards is as defined by Farquhar *et al*. (1989). Between 2.100 and 2.200 mg of each milled sample were weighed into 8mm x 5mm 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 the reference plant was wheat (*Triticum aestivum*) 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 *Virgilia divaricata*, grown in a N-free solution. The B value of was determined as -0.71‰.

Statistical analysis

The effects of the factors (P and the different *Virgilia* spicies) and their interactions were tested with an analysis of variance (ANOVA) (Super-Anova). Where the ANOVA revealed significant differences between treatments, the means (6-8) were separated using post-hoc Student Newman Kuehl's (SNK) multiple-range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

3.3 RESULTS

Biomass

Under high P conditions (500µM P), *Virgilia oroboides* is evidently better adapted than *V. divaricata*, as it had greater total biomass for the duration of the experiment (Table. 3.1). The greater biomass resulted from the shoot dry weight, while root and nodule dry weight were consistently lower for the duration of the experiment for both species. There were no significant differences between the leaf areas (Table 3.1) of the two species. Under low P conditions (LP), *V. divaricata* maintained a constant

plant biomass, while *V. oroboides* showed a decline in plant dry weight (Table 3.1). This decline resulted from an 80% decline in nodule dry weight, and a 63% decline in shoot dry weight, respectively. Low P conditions might have inhibited the growth of nodules, resulting in a decline in biological nitrogen fixation, which might have affected shoot growth.

Nutrition

The P concentration (Fig. 3.1a) of plants exposed to high P concentrations followed a similar pattern as the plant biomass. *V. oroboides* accumulated more P than *V. divaricata*. The increased nodule dry weight of *Virgilia oroboides* and the high plant P concentration of *V. oroboides* appear to favour BNF, as shown by the increase in % NDFA (Fig. 3.2a). There was a significant difference in N uptake between the species during adequate P conditions. *V. oroboides* showed a higher SNAR than *V. divaricata* (Table 3.1). This was also reflected by the higher plant N concentration (Fig. 3.1b). Specific N utilization rate was lower in *V. oroboides* compared to *V. divaricata* under adequate P conditions (Table 3.1), suggesting that *V. divaricata* is more efficient at utilizing N resources.

P stressed *V. oroboides* plants showed a decrease in P concentration, following a similar pattern of development of nodules, while *V. divaricata* maintained a constant P concentration (Fig. 3.1a). The decrease in P concentration of *V. oroboides* concurs with the decrease in % NDFA (Fig. 3.2a), which is an indicative decrease in BNF. Though *V. divaricata* maintained its P concentration during P stress, there was a decrease in % NDFA, while there was an increase in N concentration. This suggests that *V. divaricata* may be dependent on other N sources, e.g. soil N. In addition *V. oroboides* had a lower specific N uptake (SNAR) under P stress compared to adequate P conditions, but showed greater efficiency in N utilizing (SNUR) (Table 3.1). Though plants under P-stress had a decreased BNF, they were more efficient in utilizing atmospheric N per nodule, as indicated by the % NDFA/nodule (Fig. 3.2b).

Photosynthetic and respiratory C costs

There was a significant difference in photosynthetic rate between the two species under high P conditions. *V. oroboides* showed a higher photosynthesis rate (Fig. 3.4a) to maintain its increased growth. The two species did, however, not differ

significantly in either Rubisco activity (Fig. 3.4b) or electron transport (Fig. 3.4c). The higher photosynthetic rate of *V. oroboides* was complemented by an increase in plant growth respiration (Fig. 3.3a).

During P stress, *V. oroboides* had a decreased photosynthetic rate, shown by the significant difference between treatments (Fig. 3.4 a), while *V. divaricata* maintained its photosynthetic rate. Both species showed greater construction costs (Fig. 3.3a) during P stress. There was no significant difference in growth respiration (Fig. 3.3b) in *V. divaricata* during the two treatments. This may be because *V. divaricata* was able to maintain its dry weight and P nutrition during the experiment. Contrary *V. oroboides* decreased growth respiration by 60% under P stress. During P stress both plant species showed both decreased Rubisco and electron transport activities (Fig. 3.4b, c) during photosynthesis.

3.4 DISCUSSION

The two CFR legumes appeared to have different adaptative strategies to P starvation, which may influence their performance in their naturally low P environment. *V. divaricata* maintained its biomass during low P supply by altering its N economy, relative to *V. oroboides.*

The limited P supply on *V. oroboides* reduced plant growth and thereby may have limited the N demand for the plant and nitrogen fixation. This is consistent with results from previous studies where an increase in P nutrition improved nodule growth, metabolism and N_2 fixation, thereby improving plant growth (Sa & Israel, 1991; Al-Niemi *et al*., 1998; Muofhe & Dakora, 1999; Almeida *et al*., 2000; Olivera *et al*., 2004; Hernández *et al*., 2007; Le Roux *et al*., 2008; Mortimer *et al*., 2009; Le Roux *et al*., 2009). Under high P supply levels, *V. oroboides* accumulated more P, which promoted nodular growth, BNF and SNAR during the experiment. Israel (1987) similarly demonstrated that in soybean symbiotic N_2 fixation required more P for optimal functioning than plant growth. These benefits to plant N nutrition supported the increased biomass accumulation of *V. oroboides*, although *V. divaricata* was more efficient in utilizing N for growth. During P deficiency, it is

evident that *V. divaricata* was physiologically better adapted for maintaining biomass and nutrient concentration than *V. oroboides*.

During P deficiency *V. oroboides* showed a 37% decline in plant dry weight compared to under high P conditions, coupled by a 64% decline in plant P concentration. These results agree with those of Hernández *et al*. (2007), who observed a drastic reduction (2-23-fold lower) in P_i content in plants grown under P-deficient conditions. This might have caused a decline in the nodule dry weight, which would support findings by Gordon *et al*., 1990; Almeida *et al*., 2000; Tang *et al*., 2001; Høgh-Jensen *et al*., 2002). In this study it was shown that the increase in nodule mass stopped after P deprivation, while it was maintained in control plants. The decrease in plant dry weight in *V. oroboides* may have affected BNF, shown by the decline in % NDFA of the host plant. This interpretation is supported by interpretations of Sa & Israel (1991), Gordon *et al*. (1997) and Almeida *et al*. (2000), all of whom showed that where a low or limiting P supply reduced host plant growth and nodule biomass, there was a reduction in N demand and N_2 fixation.

Even though the nodules did not grow under P deficiency, relatively 50% of the total N_2 assimilated by the host plants was due to BNF. This agrees with findings by Almeida *et al*. (2000), who showed that even though clover plant nodules did not grow under severe P deficiency, approximately 30% of the N assimilated was still acquired by symbiotic N_2 fixation. The fact that *V. divaricata* had higher N concentrations under conditions of P deficiency suggests that it may rely on both BNF and other external N sources (e.g. soil N_2) during P deficiency. This is further supported by the specific N absorption rate. Though P deficient plants had lower nodule biomass, they had greater efficiency in BNF and N_2 nutrition, shown by the increase in the % BNF per nodule. The enhanced nodule efficiency for nutrient utilization is considered a pivotal coping strategy during P deficiency (Israel, 1987; Vadez *et al*., 1997; Raghothama, 1999; Høgh- Jensen *et al*., 2002; Vance *et al*., 2003; Lynch *et al*., 2005; Le Roux *et al*., 2009; Mortimer *et al*., 2008, 2009). Phosphorus status of the nutrient media supplied to the plants during the experiment may have affected photosynthesis due to C partitioning of the host plant.

The cumulative sink effect by the host plant nodules imposes a drain of the host C reserves to maintain N nutrition. This may increase the photosynthetic rate of the

host plant (Ainsworth *et al*., 2004; Jia *et al*., 2004; Kaschuk *et al*., 2009, 2010a, 2010b). The C allocation to the below-ground sink was more pronounced in *V. oroboides*, as it showed higher photosynthetic rates, attributed by the higher growth respiration rate. This concurs with findings of Jia *et al*. (2004) in *Vicia faba* L. and Mortimer *et al*. (2008) in *Phaseolus vulgaris* L.

Under low P conditions, *V. divaricata* maintained a constant photosynthetic rate, while there was a 60% decrease in *V. oroboides* compared to the same species under high P conditions. This agrees with the results of Hernández *et al*. (2007), who documented a significant inhibition of net photosynthesis in P deficient plants. The lower photosynthesis rate may be explained by the 70% reduction in their leaf area. This is supported by results of Almeida *et al.* (2000), Tang *et al.* (2001) and Høgh-Jensen *et al*. (2002), all of whom found changes in photosynthetic activity of host plants after P withdrawal, which coincided with reduced specific leaf area. Leaf photosynthesis per leaf area declined, resulting in the decrease in photosynthesis of the total plant during P deficiency. Hernández *et al*. (2007) reported a 4-fold reduction of leaf area in P deficient plants compared to high P control plants. This is supported by results of Bloom *et al*. (1985) and Hunt and Nicholls (1986). They found that during continuous low P supply, the morphology of white clover plants changed, resulting in a reduction of shoot mass and in specific leaf area. The reduction in Rubisco and Electron transport activities may further explain the reduction in photosynthesis rate.

The effects of P_i deficiency on electron transport during growth on the contents of adenylates and pyridine nucleotides and the *in vivo* photochemical activity of photosystem II (PSII) were determined in leaves of *Helianthus annuus* and *Zea mays* grown under controlled environmental conditions (Jacob & Lawlor, 1993). Phosphate deficiency decreased the amounts of ATP and ADP per unit leaf area and the adenylate energy charge of leaves. The amounts of oxidized pyridine nucleotides per unit leaf area were also decreased. This resulted in an increase in the ratio of reduced to oxidized pyridine nucleotides in leaves. This decreases the efficiency of excitation capture by open PSII reaction centres, indicating possible photo inhibitory damage to PSII. They concluded though that *in vivo* photosynthetic electron

transport through PSII did not limit photosynthesis in Pi-deficient leaves. The decreased CO_2 assimilation was a consequence of a smaller ATP content and lower energy charge, which restricted production of ribulose, 1-5, bisphosphate, the acceptor for CO_2 (Jacob & Lawlor, 1993). Limitation in the regeneration of Rubisco and ribulose 1, 5-bisphosphate (*RubP*) as leaf P_i declines may cause a simultaneous decline in photosynthetic rate (Almeida *et al*., 1999; Brooks, 1986; Rao, 1997; Hernández *et al*., 2007). This agrees with our results in which there was a decrease in electron transport and Rubisco activities in both species during P deficiency. Expression of photosynthesis genes responds to N nutrition (Paul & Stitt, 1993), therefore the reduction of plant N concentration may also reduce the photosynthetic rate of the host plant. The increase in carbon construction costs in both plants under low P may be related to maintenance of their below ground symbiosis. Thought photosynthesis was decreased, 50% of their total N was acquired by symbiosis. This might have increased C allocation to the combined activities of growth and biological nitrogen fixation during the experiment. The increase in C costs coupled with the decrease in photosynthesis could significantly reduce plant growth during low P as observed. This was confirmed in studies on bean plant, where there was an increase in C budget during low P (Lynch & Beebe, 1995; Nielsen *et al*., 1998; Mortimer *et al*., 2008).

3.5 CONCLUSION

Nutrient availability in the CFR communities plays an important role in determining legume species distribution and community composition. During P starvation, *V. divaricata* saplings were functionally better adapted to maintaining their biomass, nutrition and biological N_2-fixation than those of *V. oroboides*. The results of this study suggest that *Virgilia divaricata* is more resilient, and would do well both in habitats with high or low P, as it was able to maintain its physiological functions during P stress. This is interesting, as *V. oroboides* is more specifically associated with nutrient poor Fynbos soils than *V. divaricata*, the latter species often growing in richer P nutrient soils.

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Table 3.1 Biomass parameters and growth nutrition of 90 days old *Virgilia divaricata* and *V. oroboides* saplings, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6-8). The different letters indicate significant differences among the treatments. (P≤0.05).

Parameters	500 uM P						5 uM P					
	V oroboides			*V divaricata*			*V oroboides*			*V divaricata*		
Biomass												
Plant dry weight (g)	1.27	±0.188	b	0.49	±0.024	a	0.61	±0.057	a	0.39	±0.051	a
Shoot dry weight (g)	0.82	±0.127	b	0.27	±0.015	a	0.36	±0.047	a	0.25	±0.033	a
Root dry weight (g)	0.35	±0.064	b	0.15	±0.015	a	0.22	±0.023	a	0.12	±0.019	a
Nodule dry weight (g)	0.10	±0.029	b	0.07	±0.028	ab	0.03	±0.003	a	0.02	±0.005	a
Leaf area (m^2)	0.01	±0.002	b	0.004	±0.001	ab	0.003	±0.0004	a	0.004	±0.001	ab
Growth Nutrition												
Specific N Absoption Rate ($mmol\ N.g^{-1}.d^{-1}$)	0.06	±0.014	b	0.02	±0.005	a	0.01	±0.003	a	0.05	±0.025	ab
Specific N Utilization Rate ($g\ dw.g^{-1}.N\text{-}1.d^{-1}$)	0.01	±0.001	a	0.04	±0.011	b	0.03	±0.004	ab	0.01	±0.001	a

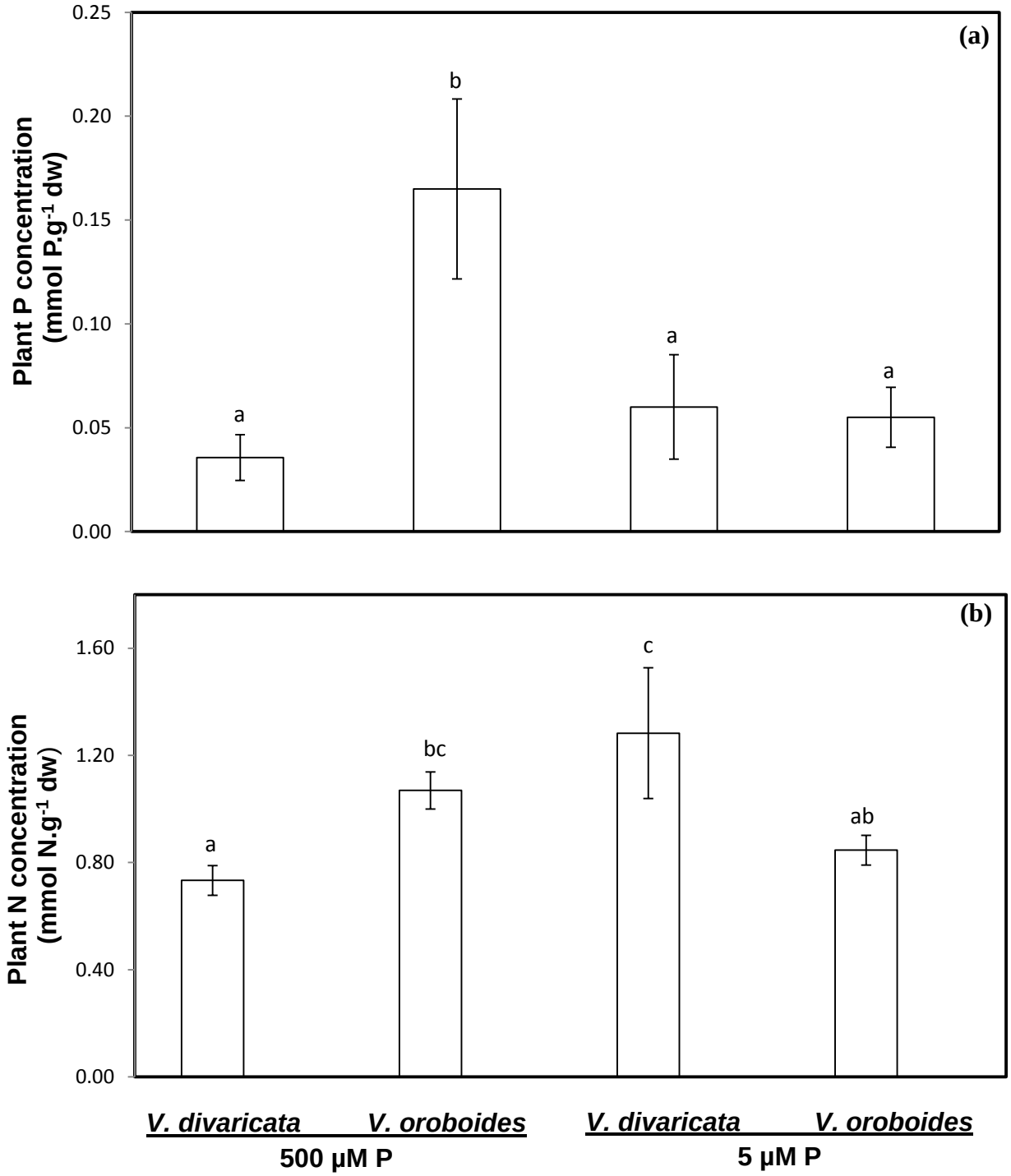

Figure 3.1 (a) Plant P concentration and (b) Plant N concentration of 90 days old *Virgilia divaricata* and *V. oroboides* saplings, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

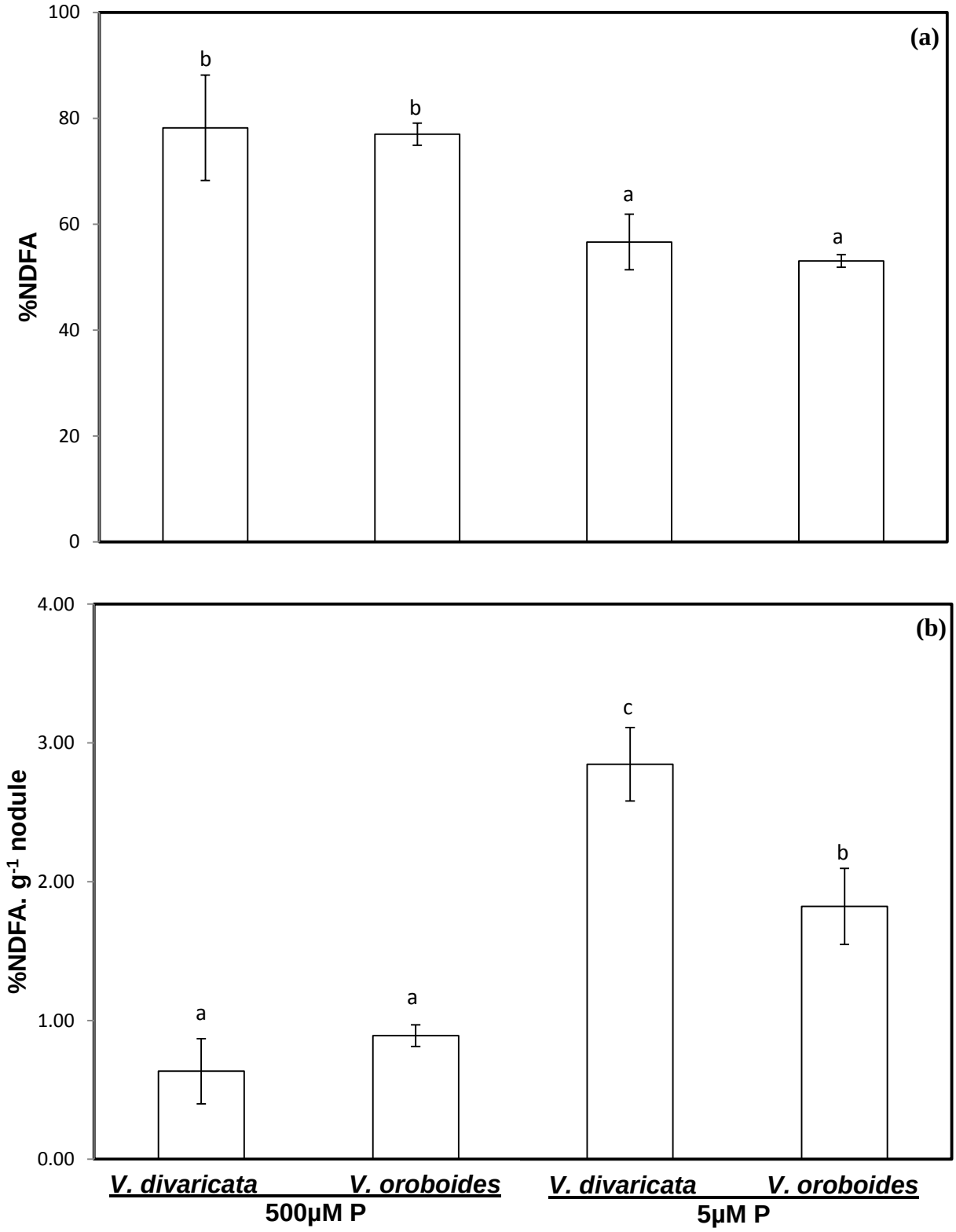

Figure 3.2 (a) Percentage N derived from the atmosphere (%NDFA) and (b) %NDFA per nodule of 90 days old *Virgilia divaricata* and *V. oroboides* saplings, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

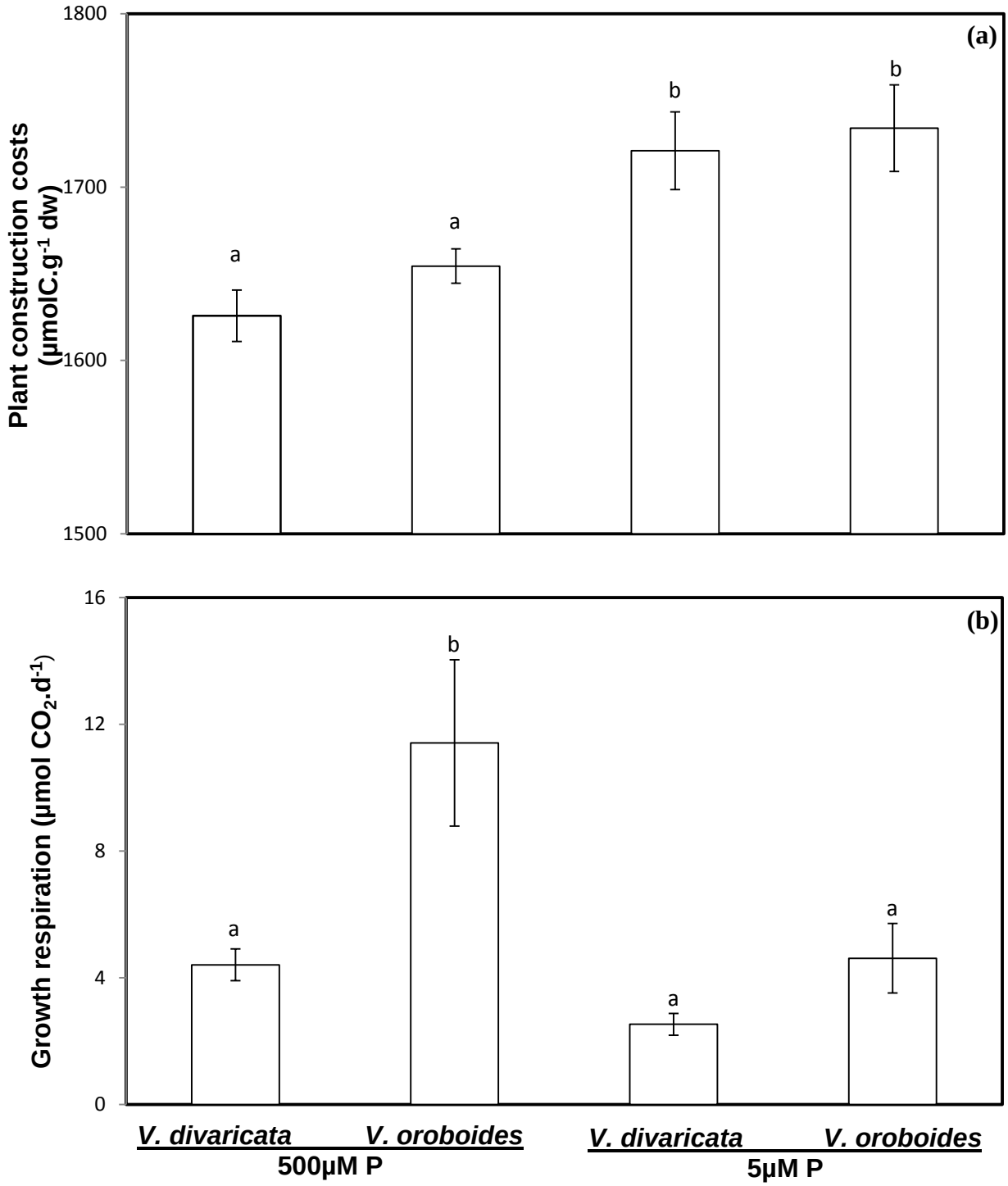

Figure 3.3 (a)Plant construction costs Plant growth respiration of 90 days old *Virgilia divaricata* and *V. oroboides* saplings, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

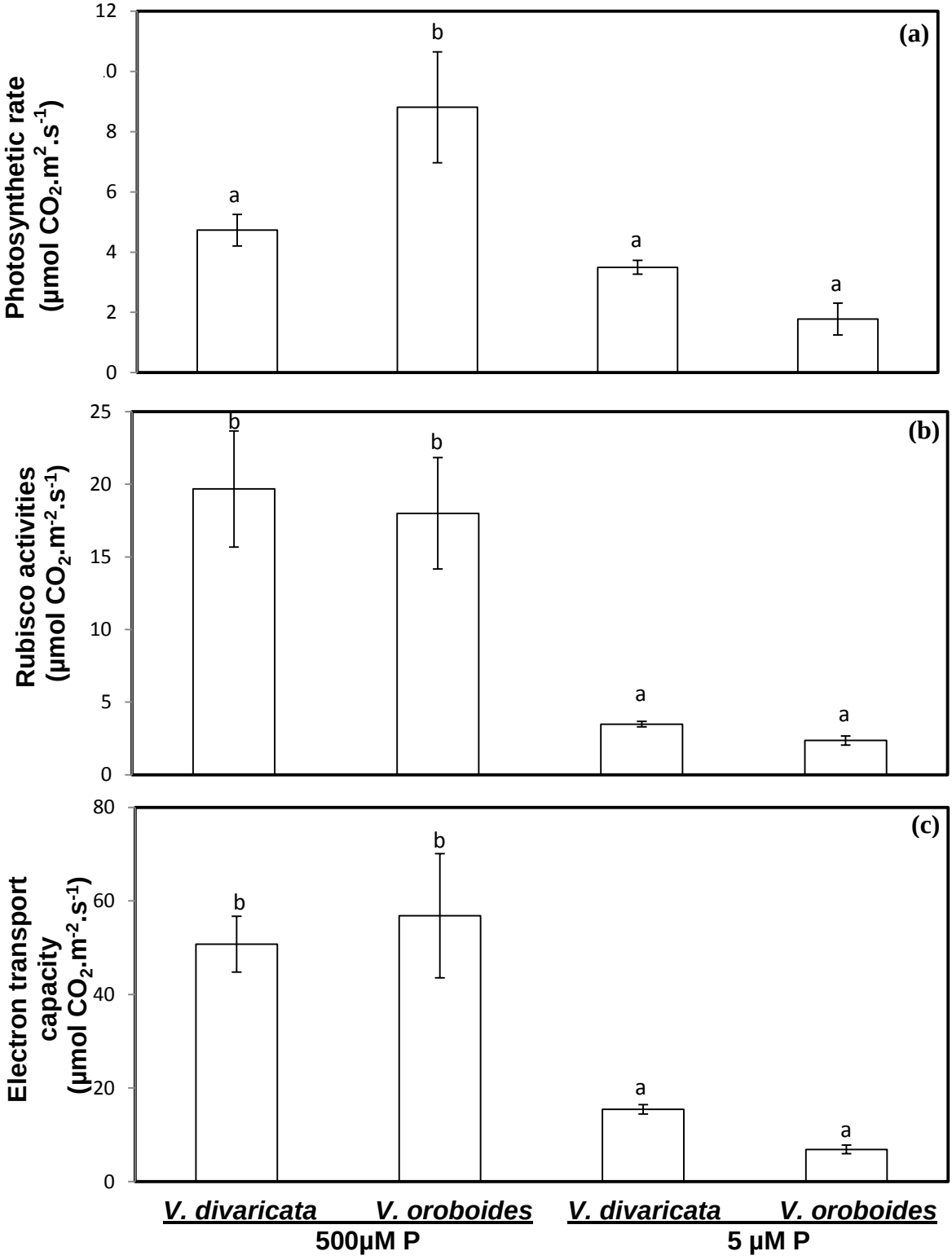

Figure 3.4 (a) Photosynthetic rate, (b) Rubisco activities (Vc) and (c) Electron transport capacity (Jmax) of 90 days old *Virgilia divaricata* and V. *oroboides* saplings, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6-8) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

CHAPTER 4

Phosphorus deficiency affects nodule bacterial association and N-nutrition of legume tree species, *Virgilia divaricata* in the Cape Fynbos ecosystem.

(Format of Soil Biology and Biochemistry Journal)

Phosphorus deficiency affects nodule bacterial association and N-nutrition of legume tree species, *Virgilia divaricata* in the Cape Fynbos ecosystem.

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Abstract

In nutrient poor ecosystems mineral nutrition plays a major role in microbial communities in plant rhizosphere and nodules during N_2 fixation in legumes. In this study, the effects of phosphate (P) nutrition on N_2 fixing bacterial community composition in *Virgilia divaricata* rhizosphere and nodules were examined in a potted plant experiment. *V. divaricata* were germinated in Fynbos soil as natural inoculum, transferred to sterile quartz sand cultures and supplied with 500 µM P and 5 µM P respectively. The N_2 fixing bacterial communities in the rhizosphere and nodules were examined based on the PCR-DGGE banding patterns of 16S rDNA and sequencing methods. The GenBank blast results revealed that *V. divaricata* was efficiently nodulated by a wide range of root-nodule bacterial strains, including *Burkholderia phytofirmans*, *Burkholderia sp.* and *Bradyrhizobium sp.* during low P supply. The ^{15}N natural abundance data also confirmed that 40-50% of the N nutrition was from symbiotic N_2 fixation. This is not only evidence of nodulation, but an indication of the adaptation of a range of N_2 fixing bacterial strain species to the nutrient poor, sandy, acidic soil of the Mediterranean-type ecosystems of the Fynbos vegetation in the CFR.

Legume species *V. divaricata* is highly adapted to the low nutrient soils of its native range by its associated with the symbiotic nitrogen fixation bacteria.

Keywords: Fynbos, Legume plants, *Virgilia divaricata*, P-deficiency, Bacterial strains

4.1 Introduction

The reduction of atmospheric dinitrogen (N_2) to ammonia during symbiotic nitrogen fixation is an exclusively prokaryotic process that contributes the largest single input of nitrogen to the biosphere, contributing to the global nitrogen cycle (Lodwig & Poole, 2003). Symbiotic N_2 fixation is a regulated process that involves different legume hosts and bacteria microsymbionts. The microsymbionts, collectively known as rhizobia, belong to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Bordeleau & Prévost, 1994). Symbiotic N_2 fixation is dependant on host cultivar and rhizobium species, but may also be limited by pedoclimatic and environmental factors associated with the acidic soil complex and its interaction with the rhizobia strains (Bordeleau & Prévost, 1994).

Since 40% of the world's arable soil is considered acidic, phosphate (P) deficiency is commonly reported globally. Such soils limit N_2 fixation and thus the productivity of legumes (Ward *et al*., 2011). Most legume plants require neutral to slightly acidic soils for growth, but experience problems with nodulation if the pH drops to a very acidic state. Soil acidity and nutritional disorder adversely affects the survival, growth and nitrogen fixation of micro-organisms by affecting the legume-rhizobia symbiosis (Graham, 1992; Lie, 1981; Munns, 1986). Phosphate is quite abundant in many soils, but because it forms insoluble complexes with cations and is bound to organic compounds by microbial action, it is often unavailable for plant uptake (Jungk *et al*., 1993; Richardson, 1994; Vance, 2011). It has been reported that host legume nodules require comparatively high amounts of P and energy during the symbiosis process. P deficiency can thus impair both nodulation and symbiotic N_2 fixation, negatively affecting growth, organic acid supply and production of the host plant (Almeida *et al*., 2000; Drevon & Hartwig, 1997; Lynch & Ho, 2005; Mortimer *et al*., 2008; Olivera *et al*., 2004; Vadez *et al*., 1997).

Many experiments have been done in the glasshouse using specific rhizobial strains under controlled conditions (Leung & Bottomley, 1987; Al-Niemi *et al*., 1997; Hernandez *et al*., 2007). In the natural environment, however, conditions are very different, as the rhizobial strains associated with N_2 fixation in legumes vary in their sensitivity to differences in soil pH and different nutrient deficiencies. This will result in differences in the success of nodulation and N_2 fixation (Muofhe & Dakora, 1999;

O'Hara, 2001). Results presented have indicated that different bacterial strains can tolerate acidity better than others, and tolerance may vary amongst strains within a species (Bordeleau & Prevost, 1994; Lowendorff, 1981; Vargas & Graham, 1988; Zahran, 1999). Strains of rhizobia differ significantly in their tolerance to P deficiency. Both Graham *et al*. (1994) and Zahran (1999) reported that slow growing *Rhizobium meliloti* (Dangeard) appear to be more tolerant to low P than fast growing strains. It appears that acid tolerance in rhizobia depends on the ability to maintain an intercellular pH even at an acidic external pH to maintain symbiotic nitrogen fixation (Graham *et al*., 1994). It was also reported by Muofe & Dakora (1999) that indigenous *Bradyrhizobia* in *Aspalathus linearis* (Brum.f) (Dahlg) is naturally tolerant of acidity as low as pH 3. *A. linearis* also displayed the ability to modify the pH of its rhizosphere in order to promote symbiotic establishment (Muofhe & Dakora, 1999). Other legumes indigenous to the acidic soils of the Fynbos of the Cape Floristic Region (CFR), South Africa, may follow the same strategy.

The Fynbos is rich in indigenous legume species that belong to many genera, including *Virgilia*. *Virgilia* is a taxonomically isolated genus consisting of 2 species (*V. divaricata* (Adamson) and *V. oroboides* (P. J. Bergius) Salter within the Fabaceae subfamily Papilionoideae (Lewis *et al*., 2005; Van Wyk, 1986). It is distributed along the southwestern and southern coastal regions of the CFR (Greinwald *et al*., 1989). Though some work has been done on the symbioses of Fynbos legumes, little is known about the bacterial symbionts nodulating them. This is also true for *V. divaricata*. The effect that the relationship between low phosphate supply and N_2 fixation has on Fynbos legumes remains unclear. There are differences in responses to P deficiency by different legume species associated with different rhizobia strains.

This study firstly aims to determine the composition of the N_2 fixing bacterial population in the rhizosphere and nodules of *V. divaricata*. Secondly, it aims to determine the contribution of these bacteria to N_2 fixation during conditions of P deficiency in the Fynbos environment. The microbial community will be determined by using PCR-DGGE, to identify rhizosphere effects with respect to bacterial diversity. Furthermore to identify the predominantly active groups of bacteria in the nodules and will be integrated with the nutritional physiology of the host plant during P deficiency.

4.2 Materials and methods

Plant material and growth conditions

Seeds of *V. divaricata* were obtained from Kirstenbosch Botanical Gardens, Private Bag X7, Claremont 7735, Cape Town, South Africa and scarified using an acid scarification method. It entailed soaking the seeds in 95-99% Sulphuric acid (H_2SO_4) for 30 minutes and then rinsing them 10 times in distilled water. Hereafter seeds were treated overnight with smoke water, also obtained from Kirstenbosch. The seeds were germinated in natural Fynbos soil (natural inoculation), obtained from Stellenbosch Mountain, Stellenbosch, South Africa. Plants were grown under the ambient conditions in the glasshouse of the Department of Botany and Zoology, University of Stellenbosch (the maximum daily photosynthetically active irradiance was between 600 and 700 µmol m^{-2} s^{-1} and the average day and night temperatures and humidities were 25/15 $^{\circ}$C and 35/75% respectively). Plants were watered daily with 100 ml of distilled water (H_2O) to run off. After seedling emergence, they were transferred to clean sand and initially watered with distilled water for a week to acclimatize. Hereafter seedlings were supplemented with N_2 (500 µM) as ammonium nitrate (NH_4NO_3), quarter strength Long Ashton nutrient solution modified to high P (500 µM) and low P (5 µM) (pH 5.8) once a week and watered with distilled H_2O in between nutrient solution supply. The split design experiment was replicate 8 times per treatment.

Harvesting and nutrient analysis

Harvesting occurred at 60 days after seedling emergence. Upon harvesting, the plants were separated into nodules, roots, stems and leaves. The natural fynbos soil used for germinating seeds (natural inoculation) and nodules were analysed for bacterial population using PCR- Denaturing gradient gel electrophoresis (DGGE). DGGE is the most appropriate molecular method for monitoring microbial community ecology. It relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks & Alleman, 2002; Koizumi *et al*., 2002). The harvested 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

milled with a tissue-lyser. The milled samples were analysed for their respective C and N concentrations in the Archeometry Department, University of Cape Town, South Africa.

PCR-DGGE sample collection

Nodules were harvested and soil samples were collected. Nodules were separated from the roots by removing the roots on either side of each nodule. They were kept at -80°C, and were then sent to MicroScie, University of Pretoria, PO BOX 1387, Wapadrand 0050, Pretoria, South Africa.

Table 4.1 Nodules from *Virgilia divaricata* plants, grown in sand culture under high P (500µM P) and low P (5µM) concentrations and soil samples from which DNA was extracted for DGGE analyses.

Sample number	Description	Treatment
1	Nodules	5 µM P
2	Nodules	5 µM P
3	Nodules	5 µM P
4	Nodules	500 µM P
5	Nodules	500 µM P
6	Nodules	500 µM P
7	Fynbos soil samples	
8	Fynbos soil samples	
9	Fynbos soil samples	

DNA extraction and purification

At MicroScie both soil and nodules were maintained at 4°C until DNA could be extracted. Total DNA was extracted directly from 0.5 g of each soil sample and from surface sterilized nodules using the NucleoSpin®Soil Kit (Macherey-Nagel). DNA concentration was determined by agarose gel electrophoresis.

16S PCR

A portion of the 16S bacterial gene of the rDNA was amplified for DGEE through PCR analysis using the primers K and M below:

K: 5'ATT-ACC-GCG-GCT-GCT-GG3' (Siciliano *et al*., 2003)

M: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' (Fjellbirkeland *et al*., 2001)

A reaction with no template DNA was included as a negative control of each PCR. Each PCR tube was made up to a volume of 30 µl, which contained: 21.6 µl sterile milliQ water, 6 µl 5x MyTaq reaction buffer, 0.6 µl primer K (10 pM), 0.3 µl MyTaq polymerase, 0.6 µl primer M (10 pM) and 1.5 µl template DNA (ca. 27 ng/µl). Prokaryotic DNA amplification was performed in a PCR thermal cycler (BioRad Mini Opticon) using the following programme: 10 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, followed by 10 min at 72°C, and then held at 4°C. PCR product was analysed on a 1.5% TAE agarose gel (Fig. 1).

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 PCR product was loaded per lane onto 40-55% denaturing gradient gels. Gels were run at 70V for 17 hrs 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). Dominant bands were compared and analysed for population diversity determination (Fig. 2).

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 upPCR (re-amplification) and sequencing. The 16S bacterial sequences obtained after amplification were all subjected to a BLAST analysis (Altschul *et al*., 1990) on the GenBank database and matching hits were selected.

Calculations of %NDFA

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town. 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):

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

Where the reference plant was wheat (*Triticum aestivum*) 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 *Virgilia divaricata*, grown in a N-free solution. The B value of was determined as -0.71‰.

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):

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

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.DW^{-1}) (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) (mgN 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.

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):

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

Statistical analysis

The effects of phosphate on the plant physiological functions and their interactions were tested with an analysis of variance (ANOVA) (Super-Anova). Where the ANOVA revealed significant differences between treatments, the means (5-6) were separated using the post-hoc Student Newman Kuehl's (SNK) multiple-range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

4.3 Results

Bacterial species diversity

DNA was successfully isolated from *V. divaricata* nodules and the rhizosphere, and amplified using the 16S rDNA bacterial gene under 500 µM and 5 µM P conditions. The GenBank blast results revealed that bacterial species (Table 4.2) were able to effectively nodulate and fix N in *V. divaricata* plants irrespective of the P conditions. This suggests that these bacterial species have adapted to the low P availability in Fynbos soils.

This is the first study in which the nitrogen fixing bacterial composition of *V. divaricata* nodules is identified. Earlier research exploring the bacterial composition of the other *Virgilia* species, *V. oroboides*, suggested that *V. oroboides* may be nodulated by *Burkholderia tuberum* (Sprent 2009), while isolates from nodules of *V. oroboides* (Kirstenbosch, South Africa) were very similar to *B. fungorum* (LMG16225, AF215705), *B*. *megapolitana* (LMG23650, AM489502) and *B. phytofirmans* (LMG22487, AY497470) (Beukes, unpublished data).

N_2 fixation and N nutrition

When the seedlings were transplanted from germination, it was clear that nodules had developed on their roots. Nodule biomass was influenced by P supply, as nodule dry weight increased with P supply (500 µM). The nodule dry weight of plants treated with 500 µM P had more than doubled what it weighed at seedling stage compared to time at harvest (Fig. 4.3d). Despite the effects of P supply on nodule biomass, strains were isolated from *V. divaricata* nodules that developed under conditions of P deficiency and these were still efficient in fixing N_2. This suggests that the bacterial strains were as effective during P deficient conditions as they were under conditions of no P stress. *V. divaricata* plants maintained biological N_2 fixation, as indicated by the %NDFA (Table 4.3). The increasing P supply decreased the amount of N fixed per unit nodule, reducing nodule efficiency to fix atmospheric N (%NDFA/nodule, Table 4.3). The increase in nodule efficiency might explain how *V. divaricata* was able to maintain N fixation during P deficient conditions. The ability of *V. divaricata* plants to maintain the percentage N that is derived through fixation, and

the amount of N fixed per plant, suggest that this species may contribute N to its natural Fynbos ecosystem.

Besides N contribution from the atmosphere, all plants were also supplied with combined nitrogen during growth, and there was no significant difference in the amount of plant internal N derived from the soil (Table 4.3). In contrast, there was a decrease in the plant specific N absorption and utilization rate (SNAR & SNUR, Table 4.3) during P deficiency. This might explain the significant difference in the plant N concentration (Table 4.3), as a decrease in total plant N concentration was observed in P deficient plants.

Plant biomass

Plant biomass increased with increasing P supply, as plants grown under adequate P conditions accumulated more biomass during growth compared to plants grown under P deficient conditions (Fig. 4.3a). The greater biomass resulted firstly from shoot biomass, followed by nodule dry weight and root dry weight (Fig. 4.3b, c & d). In contrast, the root dry weight of P deficient plants was higher than the nodule dry weight (Fig. 4.3c & d). The response of plants to decreased P supply increased carbon construction costs (Fig. 4.4a), causing an increase in plant growth respiration (Fig. 4.4b). This might explain the decrease in plant biomass during P deficient conditions.

4.4 Discussion

Despite the low nutrient status of Fynbos soils, the indigenous legume *V. divaricata* showed remarkable nodulation, with highly effective symbionts, to maintain nitrogen fixation in their natural habitats. There was, however, a decrease in plant growth.

The results of the 16 S rDNA gene sequencing analyses showed that *V. divaricata* species are nodulated by a wide range of root-nodule bacterial strains, *Burkholderia phytofirmans*, *Burkholderia sp*. and *Bradyrhizobium sp.* (Table 4.2). Remarkably low levels of P are known to reduce the growth rate and efficiency of most rhizobial strains tested. Such conditions can even stop growth and function of these rhizobia

(Beck & Munns, 1984). The ability to store P and utilize it for subsequent growth is strain dependent (Beck & Munns, 1984). Studies on bacterial composition of indigenous legumes, including *V. oroboides*, have shown that few bacterial strains can tolerate such extremities of soil acidity and nutrient stress. Recent work on Fynbos soils characterized by low P nutrient levels showed that indigenous legumes are able to form effective symbiosis under these conditions (Muofhe & Dakora, 1999; Kanu & Dakora, 2012). *Aspalathus linearis* has been shown to form effective symbiosis with *Bradyrhizobium*, while *Psoralea* species can form root nodules with different soil bacterial, including *Rhizobium, Mesorhizobium* and *Burkholderia* strains (Muofhe & Dakora, 1999; Kanu & Dakora, 2012). This concurs with the results of the present study, as the *Burkholderia phytofirmans, Burkholderia sp.* and *Bradyrhizobium sp.* strains isolated here proved that successful nodulation occurred during P deficiency. We could also show that these strains fixed 40-50% of the total plant N from atmospheric N. This maintained the percentage of N fixation by *V. divaricata* during P deficient conditions compared to plants under conditions of adequate P. Enhanced nodule efficiency for nutrient utilization is considered a pivotal coping strategy during P deficiency (Høgh-Jensen *et al*., 2002; Le Roux *et al*., 2009; Lynch *et al*., 2005; Mortimer *et al*., 2008, 2009; Vance *et al*., 2003). Our results support this, as P deficient plant nodules had greater efficiency to BNF, shown by the increase in the %NDFA per nodule. The greater efficiency might further explain the ability of *V. divaricata* to maintain %N fixed during P deficiency.

Biological nitrogen fixation is assumed to require significantly more energy per N fixed than combined N uptake and reduction (Gentili & Huss-Danell, 2002; Valentine *et al*., 2011). Though 40-50% of the nitrogen economy was derived from atmospheric N, plants also assimilated soil N. Both plants grown during adequate and P deficient conditions assimilated soil N significantly, as equal amounts were supplied during growth. In contrast, there was enhanced N use efficiency in the adequate P treated plants, where absorption and utilization increased with P supply. This may explain the significance difference in the total internal N concentration of the plants. The limited P supply reduced plant growth and thereby may have limited the N demand and usage of P deficient plants compared to plants exposed to adequate P. This would further explain why adequate P plants had a higher N concentration to maintain the increased plant biomass.

The increased biomass of adequate P treated plants is consistent with results from previous studies, where an increase in P nutrition improved plant growth, increasing total plant dry weight (Almeida *et al*., 2000; Hernández *et al*., 2007; Le Roux *et al*., 2008, 2009; Mortimer *et al*., 2009; Muofhe & Dakora, 1999; Olivera *et al*., 2004). In contrast, during P deficiency reduction in plant biomass was observed, more strikingly as a decline of nodule dry weight. Our results thus demonstrate that, though nodules and their effective symbiotic partners were able to maintain the percentage N fixed during the experiment, nodules had a higher P requirement for growth. This agrees with results of Almeida *et al*. (2000), Tang *et al*. (2001), HØgh-Jensen *et al.* (2002) and Hernández *et al*. (2007), all of whom observed a drastic reduction in nodule dry weight during P stress conditions. Phosphorus status of the nutrient media supplied to the plants during the experiment may have affected C partitioning of the host plant.

Allocation of carbohydrates to various plant parts and functions is a principal parameter for plant growth and success (Nielsen *et al*., 2001). In legume plants resources generated through photosynthesis are either utilized for construction of plant tissues or to maintain bacterial physiology for effective N fixation (Nielsen *et al*., 2001). The success of plants under stressed conditions may be determined by the ability to control carbohydrate allocation (Nielsen *et al*., 2001). The cumulative sink effect by the host plant nodules imposes a drain of the host C reserves in order to maintain bacterial physiology and N fixation. The C allocation might have been more pronounced in P deficient plants, due to the observed increase in carbon construction costs attributed by the higher growth respiration rate. This concurs with findings of Jia *et al*. (2004) in *Vicia faba* L. and Lynch and Beebe (1995), Mortimer *et al*. (2008) and Nielsen *et al*. (1998) in *Phaseolus vulgaris* L.

4.5 Conclusion

Resembling other model legume species, example *Vicia faba* L. (Jia *et al*., 2004) and *Phaseolus vulgaris* L. (Mortimer *et al*., 2008), *Virgilia divaricata* showed a decrease in plant biomass during P deficient conditions, but most strikingly the legume plant was able to maintain an effective symbiosis with multiple strains of *Burkholderia phytofirmans*, *Burkholderia sp.* and *Bradyrhizobium sp.*, obtaining some of its N

nutrition by symbiotic fixation maintaining plant N nutrition. Therefore this study established that *Burkholderia phytofirmans*, *Burkholderia sp.* and *Bradyrhizobium sp.* may be highly adapted to the nutrient poor, acidic, sandy soils of the Cape Fynbos. These results provide a clear indication that the legume species *V. divaricata* maybe highly adapted to the low nutrient soils by its associated with the symbiotic nitrogen fixation bacteria mentioned above that were found to be adapted to P deficient soils.

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Table 4.2 Species diversity of the nitrogen fixing bacterial species revealed by DGGE band sequenced according to BLAST results from the NCBI GenBank database, from the 500 µM P and 5 µM P nodules isolated from *Virgilia divaricata* plants.

Virgilia divaricata	GenBank Bacterial ID	Accession no.	% match
	Burkholderia phytofirmans	NR_042931.1	98
	Burkholderia phytofirmans	AY836218.1	99
	Burkholderia phytofirmans	NR_042931.1	97
	Burkholderia phytofirmans	AY962606.1	99
	Burkholderia phytofirmans	AY836218.1	99
	Burkholderia phytofirmans	AY962606.1	99
	Burkholderia phytofirmans	NR_042931.1	99
	Burkholderia phytofirmans	HQ2422761.1	99
	Burkholderia phytofirmans	HQ2422761.1	96
	Burkholderia phytofirmans	AY836218.1	99
	Burkholderia phytofirmans	DQ387434.1	98
	Burkholderia phytofirmans	AY962606.1	82
	Burkholderia phytofirmans	HQ2422761.1	99
	Burkholderia phytofirmans	NR_042931.1	98
	Burkholderia sp.	DQ118949.1	99
	Burkholderia sp.	FJ422400.1	100
	Bradyrhizobiaceae bacterium	AB480363.1	82
	Bradyrhizobium sp.	AY547290.1	94
	Bradyrhizobium sp.	AB121773.1	89
	Bradyrhizobium sp.	GQ342569.1	90

Table 4.3 Nitrogen data of 81 days old *Virgilia divaricata* plants, grown in sand culture under high P (500µM P) and low P (5µM) concentrations. Values are presented as means (n=6). The different letters within the columns indicate significant differences among the treatments. (P≤0.05).

Treatment	Whole Plant																	
	%NDFA			Soil derived N ($mmolN.g^{-1}$)			N concentration ($mmolN.g^{-1}$)			SNAR ($mmolN.g^{-1}.d^{-1}$)			SNUR ($\mu molN.g^{-1}.d^{-1}$)			%NDFA/ Nodule		
500 µM P	35.83	±1.36	a	1.17	±0.086	a	2.03	±0.05	b	0.37	±0.041	b	9	±0.55	b	332.5	±20	a
5 µM P	39.85	±1.64	a	1.33	±0.095	a	1.69	±0.02	a	0.23	±0.002	a	8	±0.25	a	1930.3	±96	b

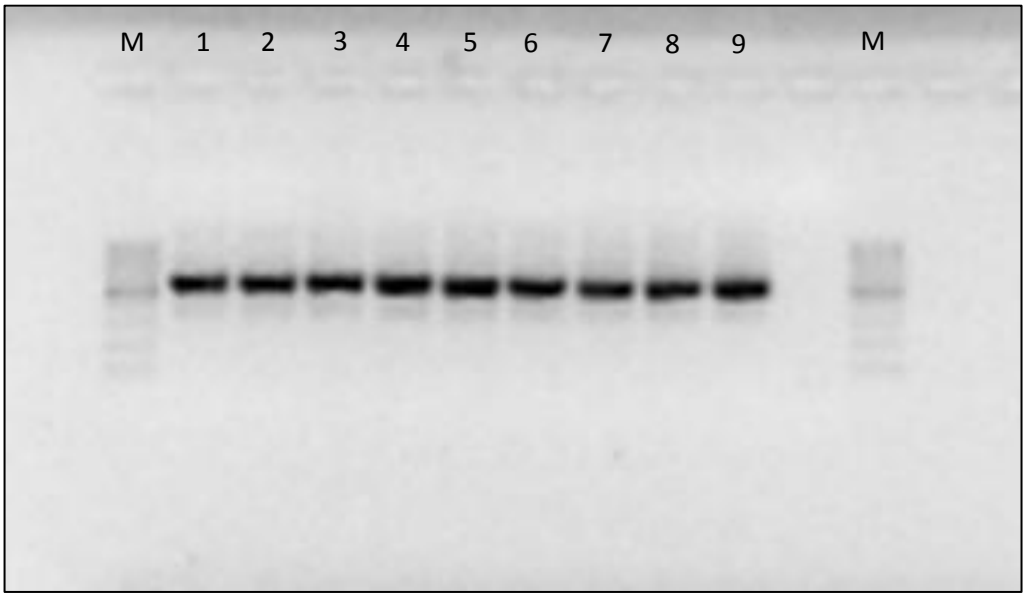

Figure 4.1 1.5% TAE agarose gel, showing 5 µl of PCR product from each of the 16S bacterial gene amplifications, of DNA isolated from *Virgilia divaricata* plant nodules and rhizosphere (7,8,9) treated with 500 µM P(4,5,6) and 5 µM P(1,2,3) and M abbreviation for DNA marker.

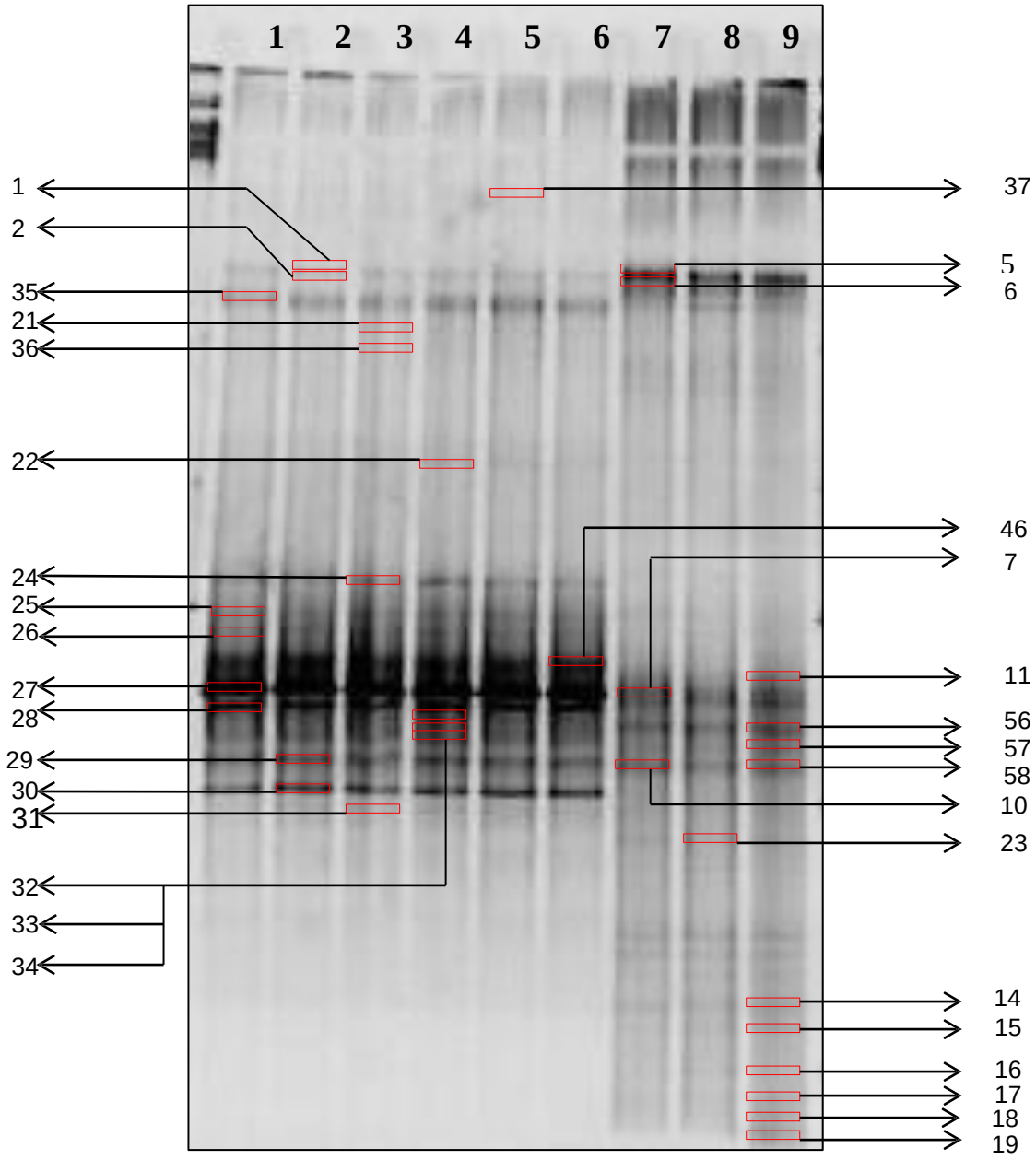

Figure 4.2 DGGE gel depicting the band pattern, indicating species diversity of bacteria from the 500 µM P and 5 µM P nodules isolated from *Virgilia divaricata* plants and rhizosphere soil sample, run at 40-45% denaturants; showing bands that were excised for sequencing and tentative bacterial identification.

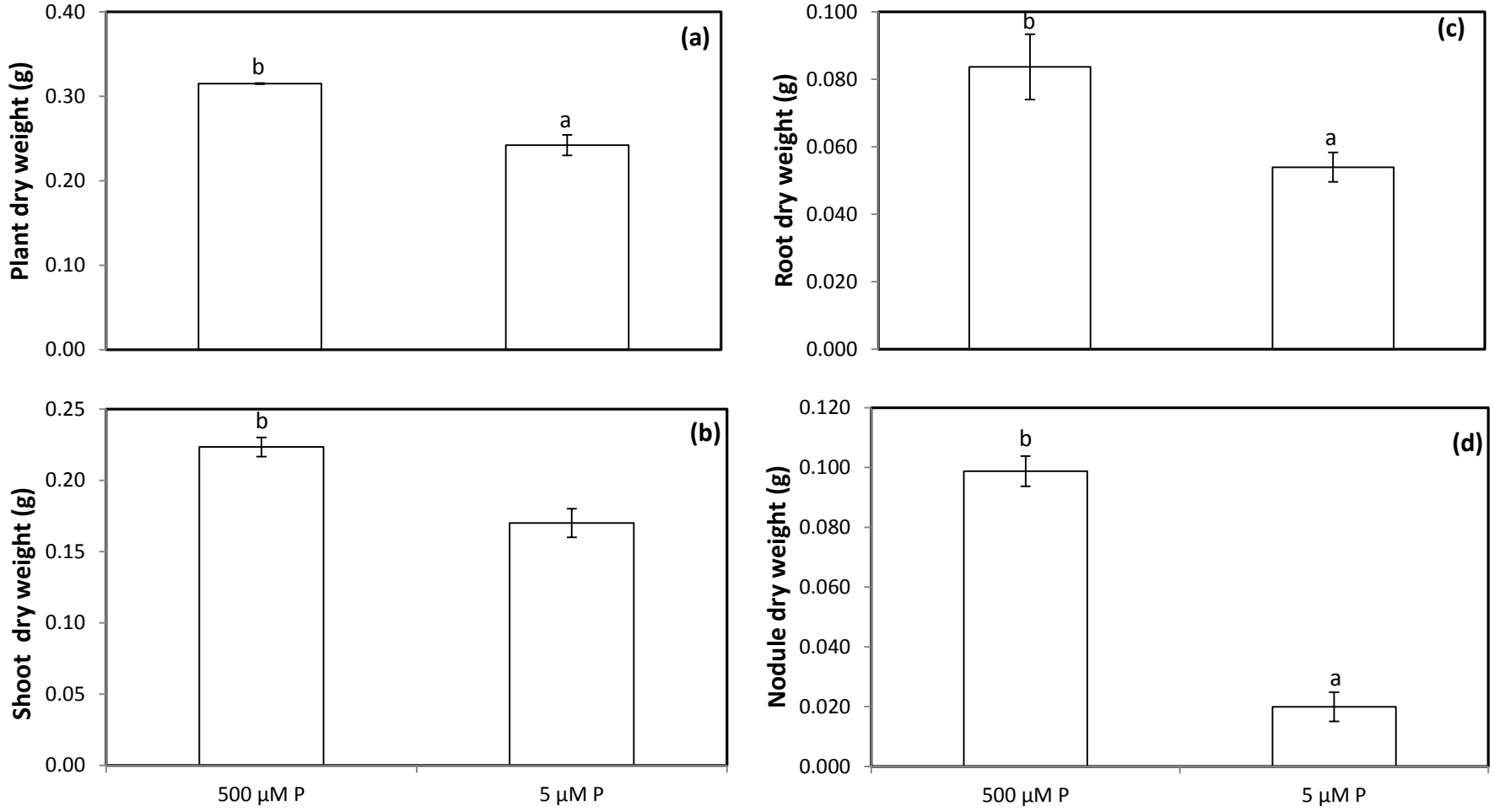

Figure 4.3 Biomass parameters (a) Plant dry weight (g), (b) Shoot dry weight (g), (c) root dry weight (g) and (d) nodule dry weight[a] (g) of 81 days old *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6). The different letters indicate significant differences among the treatments. (P≤0.05).

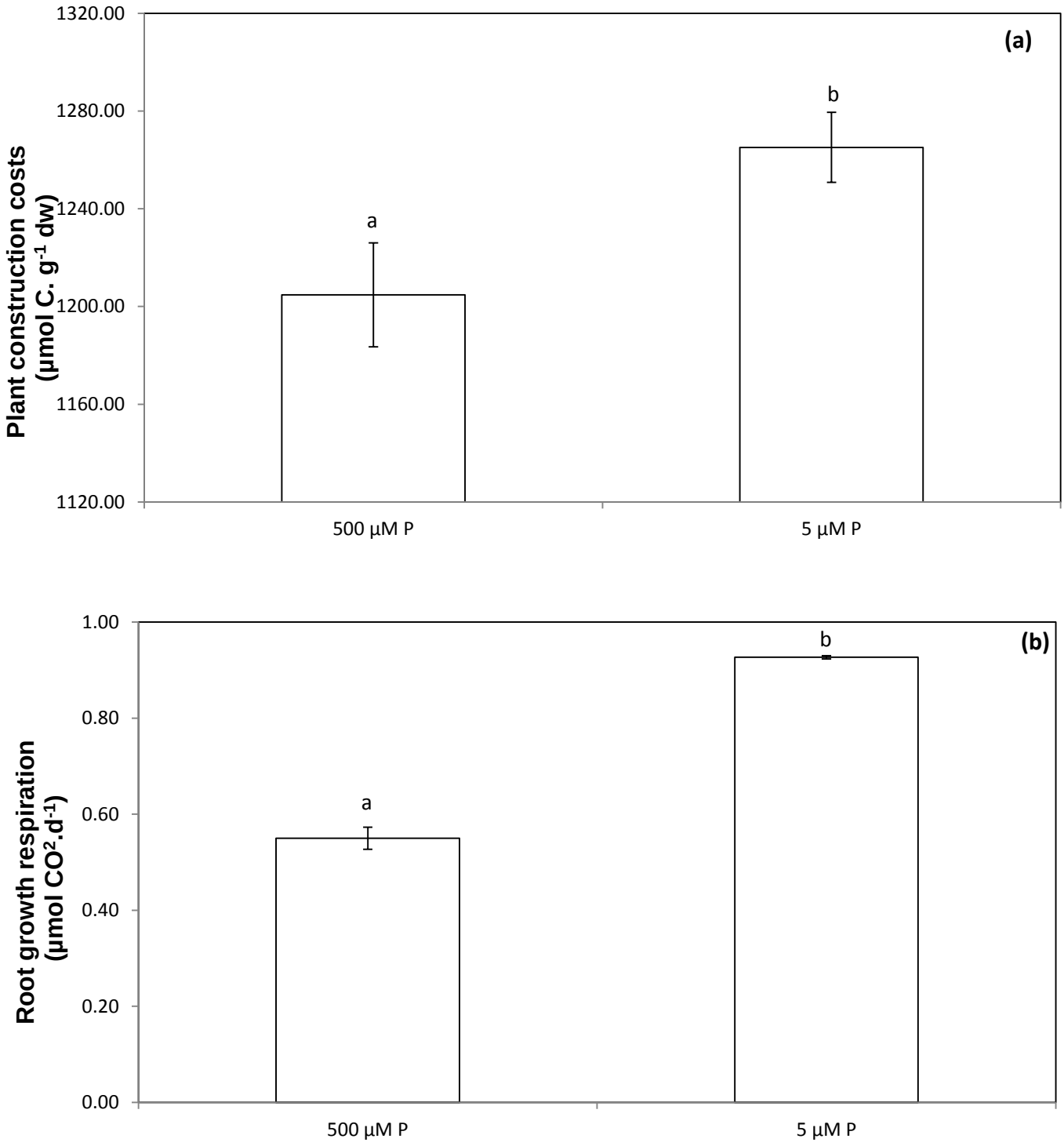

Figure 4.4 (a) Plant construction costs and (b) Root growth respiration of 81 days old *Virgilia divaricata* plants, grown in sand culture under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6). The different letters indicate significant differences among the treatments. (P≤0.05).

CHAPTER 5

The role of P nutrition in N acquisition from different inorganic N sources in the legume tree species, *Virgilia divaricata* in native Cape Fynbos soils.

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The role of P nutrition in N acquisition from different inorganic N sources in the legume tree species, *Virgilia divaricata* in native Cape Fynbos soils.

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Abstract

In nutrient poor environments, plants may be able to switch between soil N and atmospheric N_2 sources during growth to adjust the carbon costs of N acquisition. This study investigated the effects of different inorganic N sources on the costs N nutrition and assimilation of the legume, *Virgilia divaricata*, during prolonged P deficiency. Legume plants were cultivated in sterilze quartz sand supplied with N-free quarter strength Long Ashton nutrient solution, modified to contain either high P (500 µM) or low P (5 µM). In addition, plants were treated with 500 µM NH_4NO_3 (+N), inoculated with effective *Burkholderia sp.* (+Bact) or treated with combined N sources (500 µM NH_4NO_3) and inoculated with effective *Burkholderia sp.* (+N+Bact). The application NH_4NO_3 to the legumes resulted in a greater increase in plant dry matter. Carbon construction costs were more pronounced in plants supplied with two N sources and during P deficiency. Maximum photosynthetic rates per leaf area increased during prolonged P deficiency, irrespective of the N sources. Although the plant roots were nodulated, the plant dependence on N_2 fixation decreased with addition of +N. Roots and nodules of the P deficient plants showed an increase in asparagine content, most strikingly so in plants treated with a single source of N. These results show that *V. divaricata* is highly adapted to low nutrient soils by utilizing both atmospheric and soil N sources.

Keywords: *Virgilia divaricata*, NH_4NO_3, P deficiency, N_2 fixation, P and N interaction, C costs, asparagine.

5.1 Introduction

Large portions of the Mediterranean-type ecosystem of the Cape Floristic Region (CFR) in South Africa has sandstone-derived soils (Goldblatt and Manning, 2000), which are typically very acidic and nutrient poor (Kruger *et al*., 1983). These soils typically house Fynbos vegetation, which thrive on these soil conditions. These soils typically have low concentrations of nitrogen and phosphate, and are more similar to the soil of the Western Australian heathlands than other Mediterranean-climate regions (Mitchell *et al*., 1984). The Fynbos soils usually have different concentrations of elements, resulting in nutrient (typically P) deficiencies (Bordeleau and Prevost, 1994; Grigg, 2008; Von Uexkull and Mutert, 1998).

Virgilia L. is one of the legume genera endemic to Fynbos vegetation in the CFR. Soil acidity is a significant problem facing legume production in many areas of the world, including southern Africa (Bordeleau and Prevost, 1994; Correa and Barneix, 1997; Graham, 1992; Marschner, 1995). Most legume plants require neutral to slightly acidic soils for growth, but experience problems with nodulation if the pH drops to a very acidic state. Soil acidity adversely affects the survival, growth and nitrogen fixation of micro-organisms, while nutritional disorders affect legume-*Rhizobium* symbiosis (Graham, 1992; Lie, 1981; Munns, 1986). P remains mostly unavailable for plant uptake, specifically in acid-weathered soils in tropical and subtropical regions (Bieleski, 1973; Schactman *et al*., 1998; Vance, 2011; Von Uexkull and Mutert, 1998). It has been reported that host legume nodules require comparatively high amounts of P and energy during N_2 fixation. P deficiency, which is common in Fynbos, can thus impair both nodulation and symbiotic nitrogen fixation. This will negatively affect photosynthesis, respiration, growth, organic acid supply, amino acid biosynthesis and production by such legume host (Almeida *et al*., 2000; Drevon and Hartwig, 1997; Harrison *et al*., 2009; Lynch and Ho, 2005; Olivera *et al*., 2004; Vadez *et al*., 1997). Low P soils will limit legume growth to a greater extent than low nitrogen soils, since during symbiosis with *Rhizobia* legumes can utilise both atmospheric nitrogen and soil nitrogen acquired through *Rhizobia* in their nodules (Mortimer *et al*., 2008). Nitrogen fixation is the most energetically expensive reaction known to occur in any plant cell (Lodwig and Poole, 2003; Raven and Johnson, 2008; Streeter, 1981; Valentine *et al*., 2011). The process is presumed to require significantly more energy per N fixed than mineral or soil N assimilation

(Gentili and Huss-Danell, 2002; Gibson and Pagan, 1977; Pate and Dart, 1961; Valentine *et al*., 2011).

Numerous studies have explored the effects of mineral and soil N on the nodulation of legumes. It has been observed that high levels of soil N reduce nodule number and inhibit nodule growth and fixation (Gentili and Huss-Danell, 2002; Gibson and Pagan, 1977; Hellsten and Huss-Danell, 2000; Pate and Dart, 1961). The inhibition of nitrogen fixation parameters may be caused by the diminishing supply of photosynthate to the nodules due to the high levels of supplied soil N. The inhibition factor is dependent on the effectiveness of the bacterial strain during mineral N supply (Gibson and Pagan, 1977; Nelson, 1987). In earlier studies long term addition of NH_4NO_3 to inoculated peas led to a decrease in C_2H_2 activities of peas relative to peas in the absence of NH_4NO_3. This led to inhibition of N_2 fixation and the increase in dry weight due to the addition of mineral nitrogen (Pate and Dart, 1961; Nelson and Edie, 1991). Robson *et al.* (1981) investigated the nature of the interaction between mineral N and P on the growth of nodulated subterranean clover, under different levels of P and NH_4NO_3. They observed that at all the levels of P supply, particularly at the lowest P levels, plants relied more on mineral N as shoots of plants reliant on symbiotic N fixation had accumulated less N concentration than those in the shoots of plants supplied with NH_4NO_3. P supply has stimulating effects on the sources of N utilization to legumes that affect plant growth (Hellsten and Huss-Danell, 2000). The effects on N_2-fixation parameters are stronger for plants given high concentrations mineral N (Hellsten and Huss-Danell, 2000). The high sensitivity of legumes to varying environmental conditions and mineral concentrations during the N_2 fixation process may be attributed to the carbon and energy costs during plant growth (Le Roux *et al*., 2009; Schulze *et al*., 1999; Schulze, 2004). Nodulation and plant growth respond to mineral N and P concentrations. Mineral N assimilation requires lower output of the respired carbon than N fixation, improving plant growth, while P deficiency impairs nodulation and limits growth (Gentili and Huss-Danell, 2002; Le Roux *et al*., 2009; Pate *et al*., 1979; Schulze *et al*., 1999; Schulze, 2004). Legume plants, including legumes endemic to Mediterranean-type ecosystems like *Virgilia divaricata*, may change their N preference in their nutrient deficient and acidic ecosystems during growth. *V. divaricata* belongs to the taxonomically isolated genus *Virgilia* in the Fabaceae subfamily Papilionoideae (Van Wyk, 1986). Its native range

includes the southwestern and southern coastal regions of the CFR, South Africa (Greinwald *et al*., 1989).

The objective of this study was to examine the role of P nutrition in indigenous legume N acquisition from various inorganic sources and the associated carbon costs and amino acid synthesis in *Virgilia divaricata*.

5.2 Materials and Methods

Plant material and growth conditions

Seeds of *V*. *divaricata* were obtained from Kirstenbosch Botanical Gardens, Private Bag X7, Claremont 7735, 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. Hereafter seeds were treated overnight with diluted smoke water, which was also obtained from Kirstenbosch. The seeds were germinated in sterile sand. Plants were grown under the ambient conditions in the glasshouse (the maximum daily photosynthetically active irradiance was between 600 and 700 µmol m^{-2} s^{-1} and the average day and night temperatures and humidities were 25/15 $^{\circ}$C and 35/75% respectively) of the Department of Botany and Zoology, University of Stellenbosch. After seedling emergence, they were transferred to pots with clean sand and were either treated with 500 µM NH_4NO_3 (+N), inoculated with effective *Burkholderia sp.* (+Bact) or treated with combined N sources (500 µM NH_4NO_3) and inoculated with effective *Burkholderia sp.* (+N+Bact). Each of these three treatments was replicated eight times. In each treatment, the 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). Seedlings were supplied with nutrient solution once a week and watered with 100ml of distilled H_2O in between nutrient solution supply.

Gas exchange measurements

The photosynthesis response to varying intercellular CO_2 concentrations was determined to measure maximum photosynthesis (Pmax), Rubisco activity (Vc) and electron transport (Jmax). Measurements were performed on the youngest fully expanded leaves (5 replicates in each treatment per species), using an open gas exchange system Li-6400 (LI-COR Inc., IRGA, Lincolin, NE, USA). Measurements were taken from 9:00 to 16:00h over a period of a week. A full response curve took 45 minutes to 1 hour to complete. The leaves were enclosed in a leaf chamber (6 cm^2), which received steady light of 1800 $\mu mol.m^{-2}.s^{-1}$ at leaf temperature of 24 ˚C. The carbon varied from 50, 100, 150, 200, 250, 400, 500, 650, 800, 900, 1000, 1500 to 2000 ppm.

Harvesting and nutrient analysis

Harvesting intervals occurred at 60 days after seedling emergence. Upon harvesting, the plants were separated into nodules, roots, stems and leaves. The harvested plant material was placed in a drying oven, at 50˚C for 3 days, and their dry weights (DW) were recorded. The dried material was ground with a tissue-lyser (Central Analytical Facilities, Stellenbosch University). The ground samples were analysed for their respective C, N and P concentrations by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (BemLab, De Beers Road, Somerset West, SA.).

Amino acid concentration

Freshly harvested roots and nodules from some of the *V. divaricata* legume plants in each treatment were harvested, freeze-dried and ground with a tissue-lyser. The ground samples were hydrolysed in preparation for the analysis of their total amino acids concentration. The samples were subjected to the Waters AccQ Tag Ultra Derivitization Kit and Liquid Chromatography Mass Spectrometry (LC-MS) was used to analyse for amino acids at the Central Analytical Facilities (CAF), Stellenbosch University.

Carbon and nutrition cost calculations

Construction costs, CW (mmol C g^{-1} DW), were calculated according to the methods of Mortimer *et al*. (2005):

CW = [C+ kN/14 x 180/24] (1/0.89) (6000/180)

Where CW 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 NH_3) and N is the organic nitrogen content of the tissue (g DW^{-1}) (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) ($mgNg^{-1}$ 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 to the N source per plant and R is either nodule of root dry weight.

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):

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

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

The utilization rate of the specific N taken up by the plant was also measured per unit nodule DW and per unit root DW according to N sources.

Where M is the N content specific to the N source per plant and W is the plant DW.

Calculations of %NDFA

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town. 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):

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

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‰.

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 (5-6) were separated using post-hoc Student Newman Kuehl's (SNK) multiple-range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

5.3 Results

Biomass

Continued application of high phosphate and NH_4NO_3 (+N) to the legume plants during growth had major effects on legume plant biomass accumulation, as plants grown under these conditions accumulated the most biomass (Table 5.1). However, there was no significant difference between plants supplied with NH_4NO_3 (+N) during P deficient conditions. Furthermore legume plants given combined N sources (+N+Bact), irrespective of P supply, showed a decrease in biomass compared to plants grown during adequate P conditions supplied with +N (Table 5.1). Plants inoculated with effective bacterial strain (+Bact) grown during both adequate P and P deficiency conditions showed a decrease in biomass compare to plants grown under P adequate conditions (+N). Plants grown during P deficient conditions +Bact maintained their biomass compared to plants grown during adequate P conditions +Bact (Table5.1). Legume plants with two N sources (+N+Bact) during adequate P conditions accumulated the most nodule biomass, while there were no significant differences between other treatments (Table 5.1). In this aspect the interaction between P and nutritional N supply on biomass production was positive.

Photosynthetic and respiratory C costs

During adequate P conditions, carbon construction costs were more pronounced in plants supplied with two N sources (+N+Bact) compared to plants grown under similar P conditions, but with a single N source (Fig. 5.1a). Plants grown during P deficiency irrespective of N sources showed the same significant increase in carbon construction cost as +N+Bact during adequate P. This is further shown by the increase in maximum photosynthesis per leaf area (Pmax) (Fig. 5.1b) during P deficiency, while there was no increase in Pmax in adequate P +N+Bact treated plants. This may explain the major decline in biomass observed in these plants, as the two N sources might have increased the carbon demand in addition to plant growth increase. The supply of carbon (Pmax) was not balanced with the sink during plant growth. The increase in P max during P deficiency might be explained by the

increase in both electron transport activities (Jmax) and Rubisco activities (Vc) (Fig. 5.2a & b).

Nutrition

The dependence of plants on N_2 fixation decreased with the addition of NH_4NO_3, shown by the decline in %NDFA in +N+Bact treated plants irrespective of the P supply, compared to +Bact inoculated plants (Fig. 5.3a). This means that plants supplied with combined N sources relied more on mineral N assimilation, but were still able to fix atmospheric N. In such plants, 40% of their total plant N was derived from the atmosphere and 60% from mineral N. This may be because less energy is used for nutrient N assimilation than N_2 fixation. This is further shown by the increase in the N_2 fixation efficiency in +Bact inoculated plants, shown by %NDFA/nodule dry weight (Fig. 5.3b), while there was a decrease in efficiency in plant supplied with combined N sources. Though plants inoculated with the effective N_2 fixing bacterial strain showed an increase in %NDFA, the increase in N_2 fixation increased with P supply, where 20% more N_2 was fixed during adequate P conditions compared to under P deficiency (Fig. 5.3a).

Irrespective of N sources, N concentration was similar in all treatments (Table 5.2). Despite the differences in plant biomass, plants maintained their total plant nutrient concentrations in all treatments, including P concentration (Table 5.2). This is also shown by the specific N absorption rate (SNAR), as there is no significant difference in the rate of absorption during any of these treatments, while there were significant differences in the specific N utilization rate (SNUR) (Table 5.2). The major increase in the SNUR during adequate P +N may have been to maintain its increased biomass. Our results further shows that adequate P plants inoculated with bacteria were more efficient in fixing N, shown by the higher rates of nodule specific N absorption (Table 5.2), while legume plants with two N sources were more efficient in utilizing atmospheric N at a higher rate than mineral N, shown by the nodule specific N utilization rate (Table 5.2).

Amino acid concentration in roots and nodules

During adequate P conditions there was no significant difference in asparagine concentration in the nodules and roots between treatments (Fig. 5.4a). Plants inoculated with bacterial strain (+Bact) under the same P conditions showed an increase in glutamine concentration compared to plant supplied with +N, while plants with combined N sources (+N+Bact) showed no significant differences (Fig. 5.4a). The glutamine content showed the same sequence of differences as adequate P plants during P deficient conditions. By contrast, the roots and nodules P deficient plants showed an increase in asparagine concentration, most strikingly so in plants treated with a single source of N (+N/+Bact). This is further shown by the asparagine: glutamine ratio, which clearly shows that these plants produced more asparagine than glutamine during P deficiency (Fig. 5.4b)

5.4 Discussion

The present study demonstrates a positive interaction between P and NH_4NO_3 during the legume plant growth, as legume plants supplied with NH_4NO_3 accumulated the most biomass irrespective of the P supply.

Our results validate that *V. divaricata* plants supplied with NH_4NO_3 accumulated more biomass than N_2 fixing *V. divaricata* plants. This is because it may have been less expensive to assimilate NH_4NO_3 than to fix N_2, and thereby making more organic C available for growth. 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 (Minchin and Witty, 2005). 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 and Witty, 2005). Several studies have demonstrated that legumes spend larger amounts of photosynthates to acquire N through symbiotic N_2 fixation than through assimilation of NO_3^- directly from soil solution, affecting productivity (Kaschuk *et al*., 2009; 2012). Furthermore the combination of two N sources, (atmospheric N_2 and NH_4NO_3 supply) irrespective of P treatment, may have increased the C sink strength of *V. divaricata* plants during growth, as carbon might

have been supplied to maintain both N fixation and soil N assimilation. The C drain imposed by the respective N sources can amount to a relatively large amount of the host C budget, which may have caused the decline in biomass compared to plants supplied with NH_4NO_3 (Kaschuk *et al*., 2009; 2012). The larger differences in plant biomass accumulation under different nutrient supply during this experiment may have been influenced by the carbon sink strength and photosynthetic rate of *V. divaricata* during growth.

During limited P supply, legume plants have been reported to increase both nodule and root C construction costs during growth as greater sink strength is imposed by these organs during P starvation (Johnson *et al*., 1996; Nielsen *et al*., 2001; Le Roux *et al*., 2009). These findings were confirmed by results of the present study, where there was an increase in carbon construction costs during P deficiency, irrespective N source. The increase in carbon construction costs was also observed in the adequate P grown plants with combined N sources. If the rate of photosynthesis remained the same, then higher costs of nutrient acquisition coupled with plant growth would lead to a lower biomas. This concurs with the results by Harris *et al*., 1985, where *Glycine max* (L. Merr.) was either inoculated with bacterial strains or un-inoculated supplied with P and N fertilizer, inoculated host plant had higher carbon construction costs and there was no increase in photosynthetic rate, which may have led to the decrease in biomass. This was also observed in our results, where the increased carbon construction costs during P deficient conditions may have led to increase in the photosynthesis rates to maintain their functions. This suggests that the C sink strength stimulated the rate of photosynthesis during P deficient conditions, where C costs were probably covered by compensatory photosynthesis (Paul and Foyer, 2001; Kaschuk *et al*., 2009; 2012). Furthermore the increased photosynthetic rate was underpinned by the equal and balanced increases in both electron transport and Rubisco activities, as found in previous work (Bukhov 2004; Farquhar *et al*. 1980; Harley *et al*. 1992; Paul and Foyer 2001).

During the supply of mineral N, a range of inhibiting effects on nodulation which vary among bacterial strains, have been reported for legume species (Harper and Gibson, 1984). Pea and soybean have been reported to nodulate in the presence of high

concentrations of nitrate (Carroll *et al*., 1985ab; Nelson, 1987). This concurs with the results of the present study, where bacterial inoculated *V. divaricata* plants were able to nodulate when supplied with 500 µM of NH_4NO_3. Many tropical tree legumes prefer ammonium to nitrate and are able to fix N_2 and assimilate ammonium at the same time (Sprent, 1999). Bremer *et al*. (1989) also observed under conditions of high soil N availability, the legume lentil can take up N from the soil, rather than fix N_2 from the atmosphere. This agrees with our results, where a reduction in %NDFA is observed in plants supplied with the combined sources of N, with only 40% of the plant N derived from the atmosphere. Bremer *et al*. (1989) assumed the change in N preference of the legume plant to be due to energy requirements of soil N assimilation being lower than N_2 fixation. Nelson and Edie (1991) also observed a decrease, or rather a diversion, of photosynthetically-derived energy and reductant from the nodules to the assimilation site in pea plants when supplied with NH_4NO_3. This might explain why there was a decrease in %NDFA observed when plants were supplied with combined N sources. This was also shown by the reduction in N_2 efficiency due to the combined N sources. In this study, the differences in N sources, N preferences and plant growth may have been the reason why there were no significant difference in specific N absorption rate and total plant N concentration. Significant differences in specific N utilization rate were to maintain biomass of the legume plants during growth, showing legume plants treated with two N sources were more efficient in utilizing atmospheric N_2. It has been mentioned that P plays a direct role in nodule functioning and N_2 fixation and a reduction in P supply result in a reduction in N_2 fixation parameters and yield (HØgh-Jensen *et al*., 2002; Le Roux *et al*., 2009; Muofhe and Dakora, 1999; Tang *et al*., 2001; Vadez *et al*., 1999). This concurs with the results of this study, where a 20% decline of N derived from the atmosphere was observed in plant grown under P deficient conditions inoculated with bacteria, with atmospheric N_2 as N source in comparison to plants grown during adequate P conditions. Phosphorus status of the nutrient media and different N sources supplied to the plants during the experiment may have affected amino acid production of the host plant nodules and roots.

Early literature by Stewart and Larher (1980) indicated that mineral nutrient deficiencies stimulated large increases in asparagine concentration. 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 this, the present study found an increase in the accumulation of asparagine in roots and nodules of *V. divaricata,* grown under P deficient conditions. This accumulation was more pronounced in plants grown with a single N source. Since asparagine is a substrate for only a few enzymatic reactions in 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*., 2007). The increase in the accumulation of asparagine during P deficiency may result because of the regulation of the N feedback mechanism induced during P deficiency as reported by Almeida *et al*. (2000). 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), so that any excess oxaloacetate may be available for the transamination reaction with glutamine to produce asparagine (Prell and Poole, 2006). Although it is speculative, this might also explain the accumulation of asparagine in *V. divaricata* in the current study during P deficiency.

5.5 Conclusion

This positive interaction between P and mineral N may mean that endemic South African legume plants may have adapted to growing in the P deficient Fynbos environment, and would prefer soil N and atmospheric N_2 fixation, as it requires less energy to assimilate, so it might utilize both soil N and atmospheric N. The present study agrees with earlier studies of model leguminous plants that showed that during P deficiency there is an increase in the accumulation of asparagine, this might be to preserve energy during P deficiency.

5.6 Acknowledgements

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Table 5.1 Biomass parameter data of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters within the rows indicate significant differences among the treatments. (P≤0.05).

Parameters	500 µM P									5 µM P								
	NH_4NO_3			NH_4NO_3+Bact			Bact			NH_4NO_3			NH_4NO_3+Bact			Bact		
Biomass (g)																		
Plant	0.39	±0.06	b	0.17	±0.000	a	0.23	±0.003	a	0.26	±0.025	ab	0.22	±0.018	a	0.18	±0.020	a
Shoot	0.27	±0.07	c	0.08	±0.008	a	0.13	±0.003	ab	0.17	±0.018	b	0.12	±0.012	ab	0.11	±0.014	ab
Root	0.12	±0.02	b	0.06	±0.008	a	0.07	±0.005	a	0.09	±0.008	ab	0.09	±0.019	ab	0.06	±0.005	a
Nodule	-	-	-	0.03	±0.005	b	0.02	±0.008	a	-	-	-	0.01	±0.002	a	0.01	±0.002	a

Table 5.2 Growth N nutrition data of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

Parameters	500 µMP						5 µM P					
	NH_4NO_3		NH_4NO_3+Bact		Bact		NH_4NO_3		NH_4NO_3+Bact		Bact	
Growth N nutrition												
N conc.(mmol $N.g^{-1}$ dw)	1.68 ±0.097	a	1.97 ±0.230	a	1.9 ±0.34	a	1.9 ±0.103	a	1.99 ±0.142	a	1.94 ±0.07	a
Plant Specific N Absoption Rate (SNAR) (mmol $N.g^{-1}$ d^{-1})	0.48 ±0.149	a	0.38 ±0.105	a	0.43 ±0.05	a	0.43 ±0.046	a	0.40 ±0.068	a	0.47 ±0.08	a
Nodule SNAR	-	-	0.03 ±0.01	a	1.01 ±0.14	b	-	-	0.35 ±0.11	a	0.24 ±0.00	a
Root SNAR	-	-	0.01 ±0.00	a	0 ±0.00	-	-	-	0.02 ±0.005	b	0 ±0.00	-
Plant Specific N Utilization Rate (SNUR) (g $DW.g^{-1}N.day^{-1}$)	0.02 ±0.002	b	0.01 ±0.001	a	0.01 ±0.00	a	0.014 ±0.001	ab	0.01 ±0.0003	a	0.01 ±0.08	a
Nodule SNUR	-	-	0.03 ±0.01	a	0.01 ±0.00	a	-	-	0.04 ±0.01	a	0.02 ±0.00	a
Root SNUR	-	-	0.01 ±0.002	a	0 ±0.00	-	-	-	0.01 ±0.002	a	0 ±0.00	-
P conc.(mmol $P.g^{-1}$ DW)	0.21 ±0.061	a	0.20 ±0.061	a	0.20 ±0.02	a	0.19 ±0.066	a	0.12 ±0.017	a	0.23 ±0.02	a

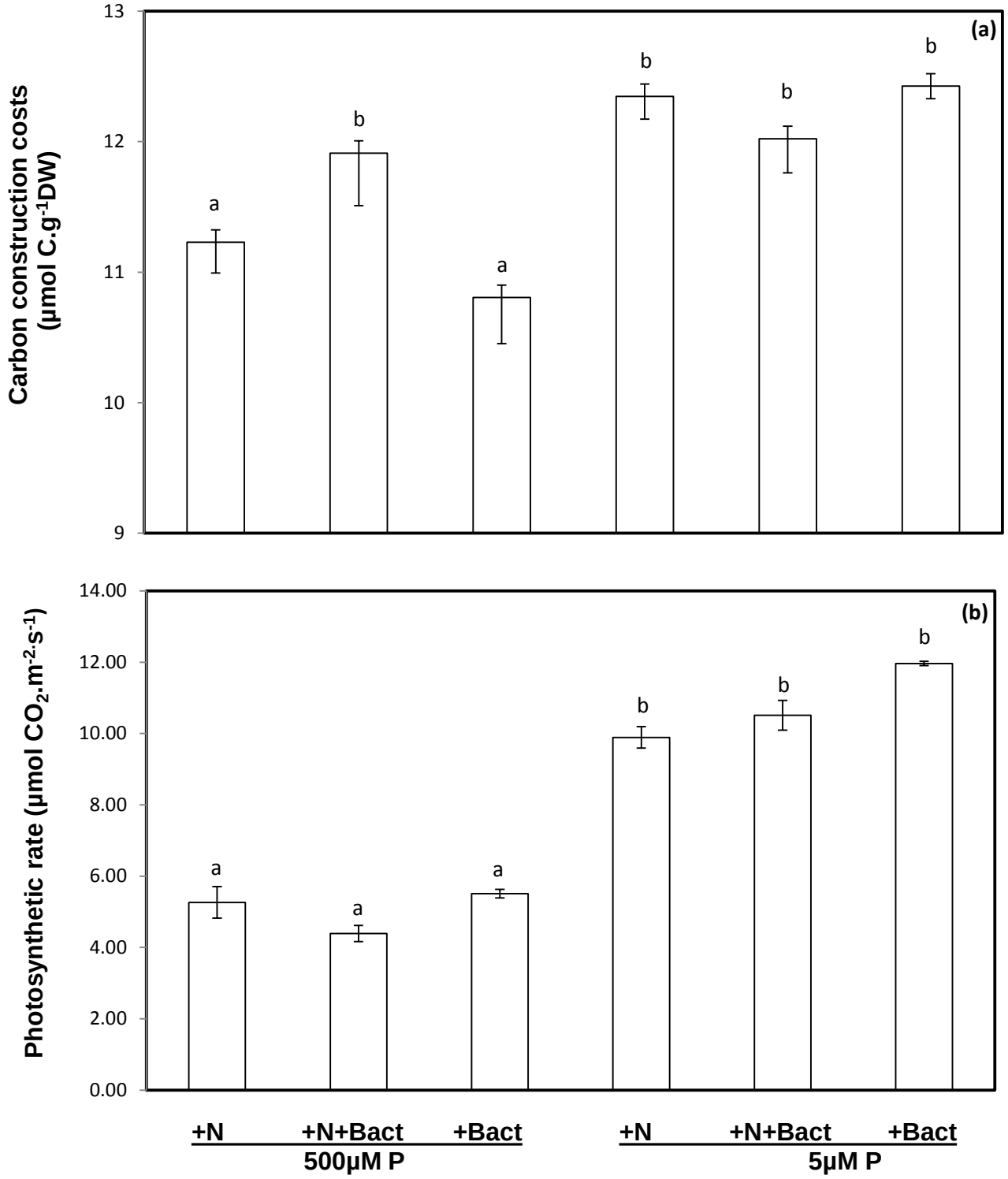

Figure 5.1 (a) Plant construction costs and (b) Photosynthetic rate of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

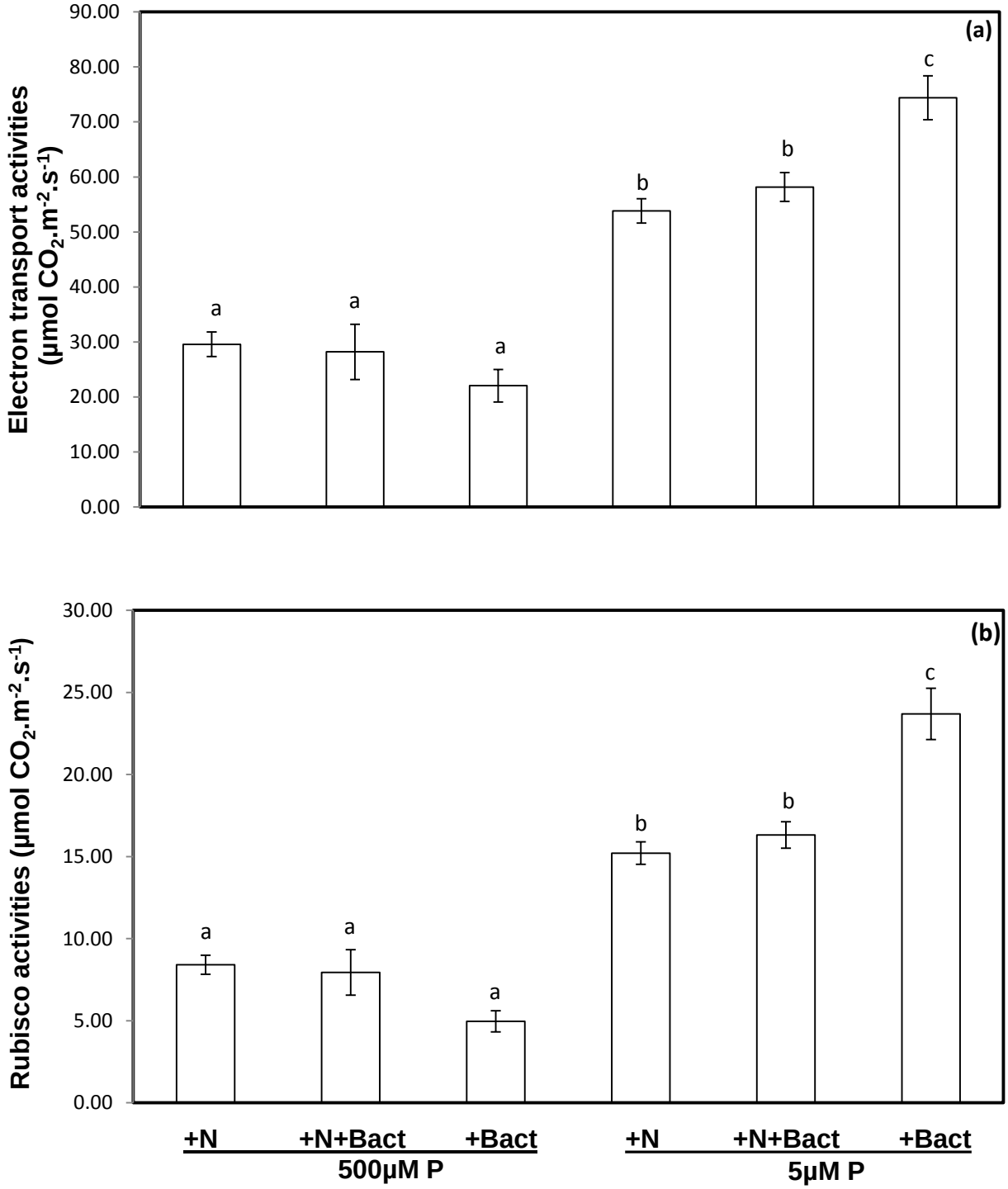

Figure 5.2 (a) Electron transport capacity (Jmax) and (b) Rubisco activities (Vc) of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

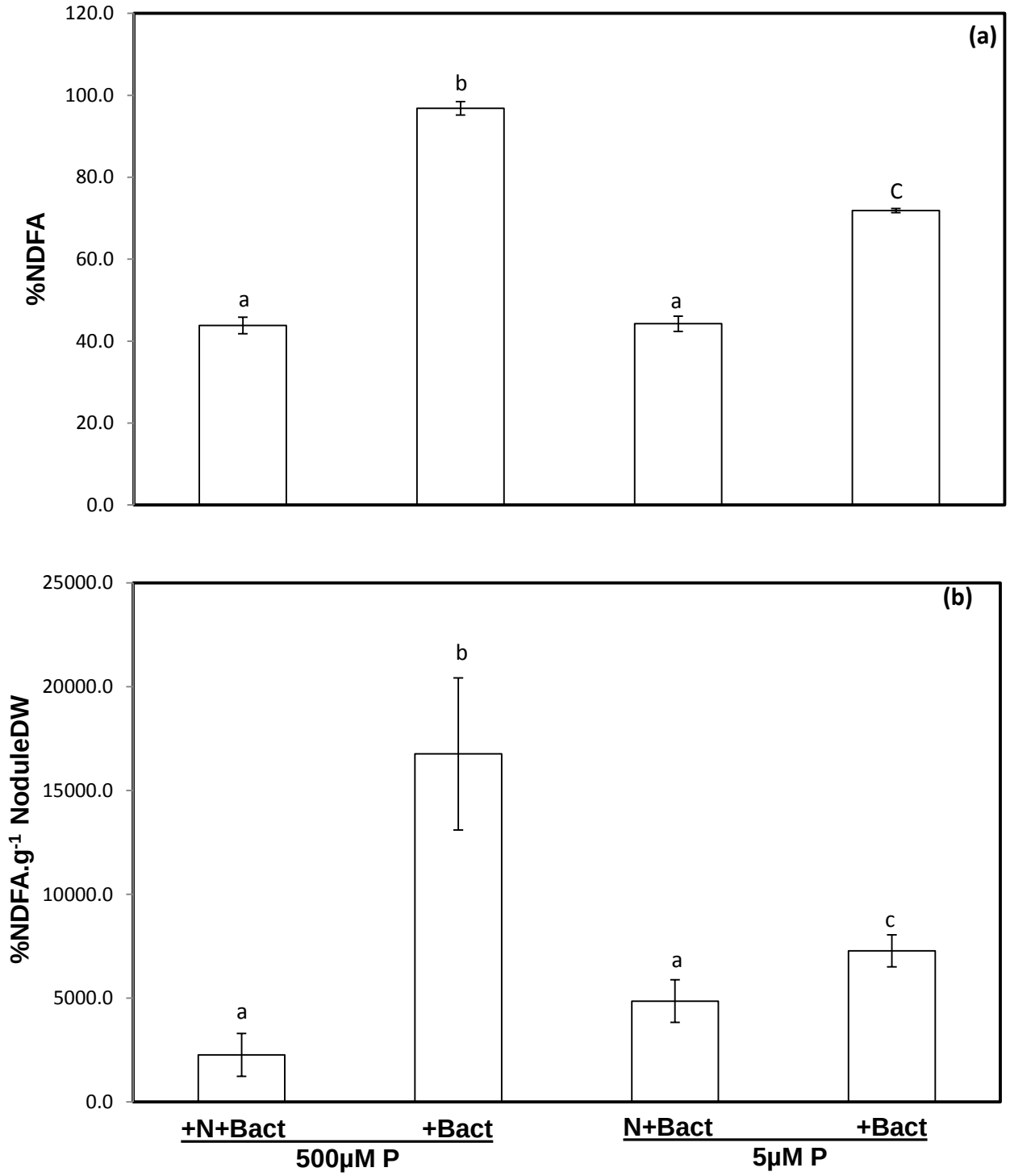

Figure 5.3 (a) Percentage N derived from the atmosphere (%NDFA) and (b) %NDFA per nodule of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

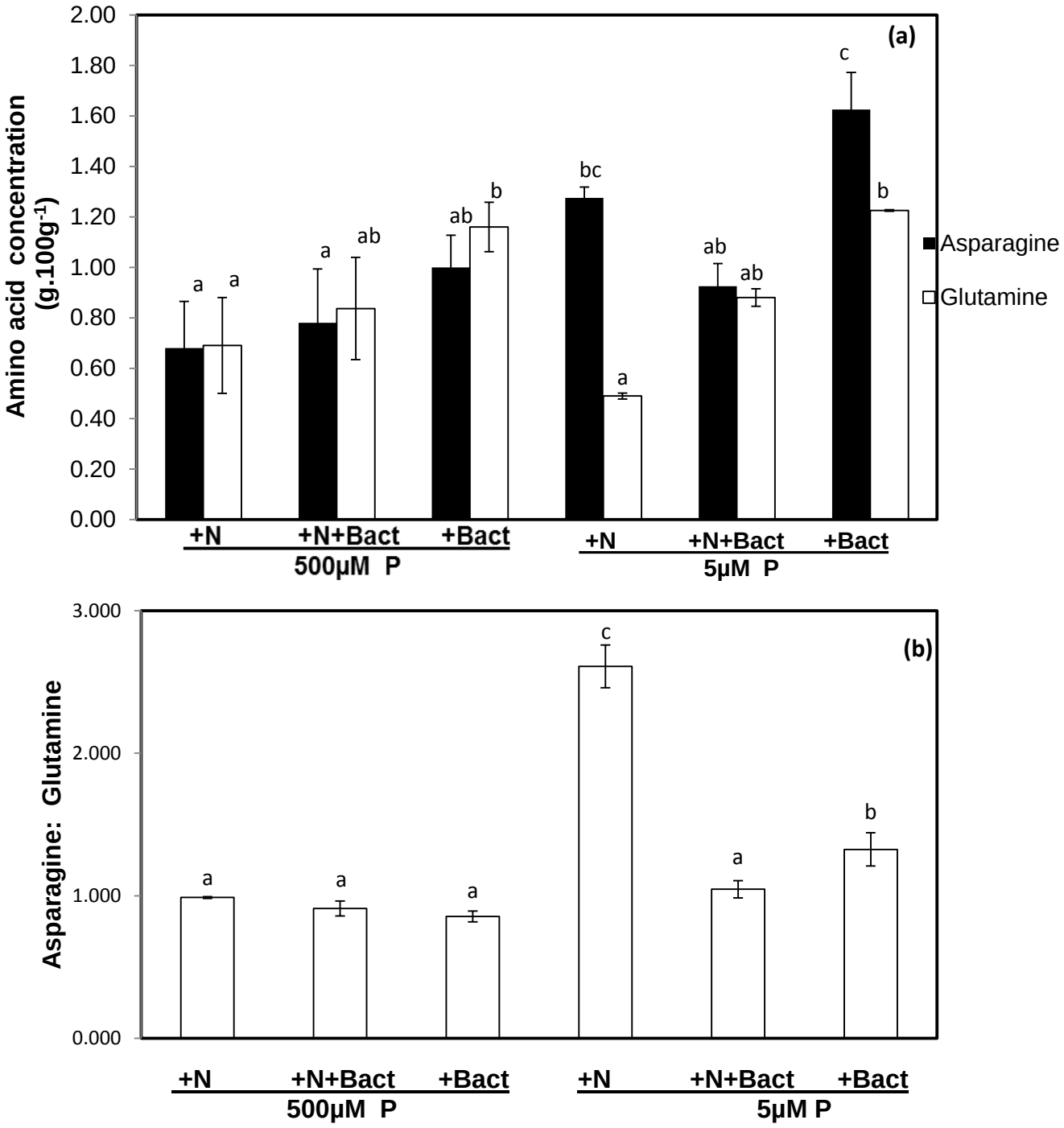

Figure 5.4 (a) Amino acid concentration (b) Asparagine: Glutamine ratio of 87 days old *Virgilia divaricata* saplings, grown in sand culture treated with +N, +N+Bact and +Bact under high P (500 µM) and low P (5 µM) concentrations. Values are presented as means (n=6) with standard error bars. The different letters indicate significant differences among the treatments. (P≤0.05).

CHAPTER 6

General Discussion and Conclusion

6.1 General Discussion and Conclusion

The Cape Fynbos ecosystem is generally known of its nutrient poor soils, characteristically low in N and P (Spriggs & Dakora, 2008). Both these nutrients have primary importance to legume plants as nodulation and photosynthesis requires increased levels of P and N (Mortimer *et al*., 2012). Members of the Fabaceae that are native to the Fynbos depend heavily on N_2 fixation for their N nutrition (Spriggs & Dakora, 2008). It is commonly known that low P supply can reduce nodulation and N_2 fixation in legumes (Muofhe & Dakora, 1999). However these native legume plants might have evolved adaptations to obtain adequate P under these conditions (Vance *et al*. 2003).

Clearly there is a need for information on the dynamics of N and P uptake and utilisation 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. A better understanding of metabolic and ecological costs associated with P acquisition strategies is needed (Lynch & Ho, 2005), which may be important for the management and future conservation of Fynbos legume species. Therefore, we need to understand the mechanisms of legume adaptations to fix N_2 in a low P environment. For this reason, this study was undertaken to increase our knowledge and understanding of the role of phosphorus in the carbon costs, nodulation and efficiencies of N acquisition from the soil and atmosphere in indigenous legumes. In understanding the phosphorus (P) requirements during growth and symbiotic nitrogen (N_2) fixation in legume species native to low P soils of Fynbos vegetation in the Cape Floristic Region (CFR) was the main focus of the present study. This was determined through a series of experiments, focussing on N acquisition from various inorganic sources during P deficiency.

The experiments in Chapter 3 aimed to determine how P deficiency affects growth and nitrogen nutrition via soil and atmospheric sources in the two Fynbos legume species, *V. divaricata* and *V. oroboides*. The variation in growth of the two Mediterranean-type legumes revealed that they have different adaptations to P starvation. This may influence their performance in their naturally low P environment, as the different ecosystems have different nutrient patterns (Cowling, 1992). *V.*

divaricata maintained its biomass and other physiological functions (N efficiency and photosynthetic rate) during low P supply by altering its N and P economy. It did so more efficiently than *V. oroboides.* This concurs with work of Power *et al*. (2010) on Fynbos native legumes supplied with 1 mg and 10 mg $P.kg^{-1}$, amongst others, *Cyclopia genistoides* maintained its plant dry weight during P deficiency. This shows that indeginous legume species cope differently throughout the Fynbos ecosystem, but it has never been reported that species within the same genus may cope differently under P deficiency, as the current experiment has revealed. Our findings agree with previous work that showed that both model and native legume plants might have evolved coordinated different gene expressions as broad adaptations to maintain their growth during P deficiency (Horst *et al*., 2001; Lajtha & Harrison, 1995; Power *et al*. 2010; Raghothama, 1999; Vance, 2011). Results from these experiments in Chapter 3, The results of this study suggest that *Virgilia divaricata* is more resilient, and would do well both in habitats with high or low P. This is interesting, as *V. oroboides* is more specifically associated with nutrient poor Fynbos soils than *V. divaricata*, the latter species often growing in richer P nutrient soils.

In Chapter 5, further research on the role of P nutrition of *V. divaricata* was thus expanded upon by assessing its physiological effects (including N_2 fixation parameters, N preference, plant dependence on N_2 fixation, plant growth, carbon costs and amino acid biosynthesis) during P deficiency and mineral N supply as NH_4NO_3. Results of this short-term study of *V. divaricata* demonstrated a positive interaction between P and nutritional N supply on biomass production. Maximum biomass accumulation was reached during the supply of soil N, irrespective of the P supply. Collectively the positive interaction may mean that endemic South African legume plants have adapted to growing in the P deficient Fynbos environment. They have achieved this by altering soil N and atmospheric N_2 fixation, as mineral N assimilation requires less energy to assimilate (Minchin & Witty, 2005). *V. divaricata* might hold similar features as rooibos legume plant, *Aspalathus linearis* as they are both native to the Cape Fynbos, the application of N, P, and Ca to field plants of *Aspalathus linearis* increased total biomass compared to unfertilized plants (Muofhe & Dakora, 1999). It was further observed that during the supply of mineral N (NH_4NO_3), mineral N is preferred as atmospheric N_2 fixation was extremely reduced.

As has been shown in model leguminous plants, results of this study showed that during P deficiency, the synthesis of amino acids change. In this case an increase in the accumulation of asparagine was observed, the increased asparagine might be an energy preserving adaptation (Stewart & Larher, 1980; Rufty *et al*., 1993; Almeida *et al*., 2000). The N preference under P deficiency indicated that bacterial strains and species within nodules may play an important role in facilitating BNF during mineral N supply. The role of bacteria in nodulation of certain wild legumes with various sources of inorganic N, further demonstrates the significance of studying wild legumes when aiming to identify superior N_2-fixers at low P (Muofhe & Dakora, 1999; Sprent, 1999; Elliot *et al*., 2007; Kanu & Dakora, 2012).

Therefore, in Chapter 4, we explored the effect of the relationship that low phosphate supply and N_2 fixation bacterial strains has on Fynbos legumes. *V. divaricata* was able to maintain an effective symbiosis with multiple strains of *Burkholderia phytofirmans*, *Burkholderia sp*. and *Bradyrhizobium sp*., obtaining some of its N nutrition by symbiotic fixation. This suggests that *Burkholderia phytofirmans*, *Burkholderia sp.* and *Bradyrhizobium sp.* may be highly adapted to the nutrient poor, acidic and sandy soils of the CFR. This further supports evidence that the legume species *V. divaricata* is highly adapted to the low nutrient soils of its native range by its associated N_2 fixing bacteria, and may contribute to the N cycle in the Fynbos ecosystem. Studies of other Cape Fynbos native legumes have also been undertaken where similar results were found, e.g. *Psoralea* species were found to be associated with different soil bacteria, *Burkholderia*, *Rhizobium* and *Mesorhizobium* and maintain N_2 fixation (Kanu & Dakora, 2012). Furthermore, unfertilized *A. linearis* contributed to the N economy of the ecosystem by its association with *Bradyrhizobium*. This means that there are probably a larger number of N_2 fixing bacterial strains that are dominant symbionts to the Fynbos vegetation and environmental conditions.

The collective experiments of this study revealed that different legume species of the same genus, may employ contrasting adaptations in order to maintain N nutrition under P deficiency. The application of these findings is important to understanding

that legumes employ different genetic mechanisms to adapt to P starvation. Therefore, a weakness of this study, is that only whole-plant physiological events were observed, without any knowledge of the molecular biological components that underpin these phenotypic responses.

6.2 Future Prospects

Most of the current model legumes are herbaceous crops, with an annual growth form. Therefore, our use of a legume tree from a Medditaranean-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, Medditaranean-type ecosystems. It is therefore pertinent for *V. divaricata* to be used as a model legume, which should be proposed for exposure to the modern genomics tools.

In this regard, an initial RNA-sequencing project should be undertaken, so that the P-deficiency syndrome can be explored at the molecular level, in order to assess the associated gene 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 proteiomic profile investigation, in order to compliment the transcriptome of the P-deficient responses. The knowledge of the transcriptome can then be used for a follow-up genome sequencing project. The use of these “omics” technologies in a legume from a Medditaranean-type ecosystem will therefore be able to establish *V. divaricata* as a model legume for nutrient-poor ecosystems. 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.

6.3 References

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