PHOSPHOROUS REQUIREMENT OF INDIGENOUS N_2-FIXING LEGUMES AND RHIZOBIAL DIVERSITY IN THE LOW P SOILS OF THE CAPE FLORISTIC REGION, SOUTH ARICA

by

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Declaration

I hereby declare that this thesis is my own work. It is submitted for the degree of Master of Science in the Department of Botany, University of Cape Town. It has not been submitted for any degree or examination at any other university.

Signature:

ABSTRACT

Soils of the Cape Floristic Region (CFR) vary in phosphorous (P) availability and legume species tend to be distributed in specific edaphic habitats. Eighteen indigenous CFR legume species were grouped as high-P, low-P, P-generalist and unclassified plants, by associating their distribution with soil [P] in the CFR. It was hypothesized that the low-P group would be superior N_2-fixers than the high-P group at low P supply. In a glasshouse study, the plants were inoculated with CFR soil rhizobia and grown for 22 weeks in sand supplied with low P of 0.1 μM P in the N-free nutrient solution. There was no difference in growth and symbiotic N_2-fixation (growth & SN_2F) between the low-P and high-P groups. However, *Aspalathus linearis*, a low-P species, demonstrated the greatest capacity to nodulate and fix N_2 at low P supply, followed by *Virgilia oroboides*, *Podalyria calyptrata* and *Cyclopia genistoides*. In contrast, *Psoralea pinnata*, *P. aphylla* and *Otholobium striatum* nodulated poorly at 0.1 μM P.

Most N_2-fixing crop legumes are reported to show a high P requirement for growth & SN_2F. However, N_2-fixing legumes indigenous to the low P soils of the CFR are hypothesized to have a low P requirement for growth & SN_2F. To test this hypothesis, the interaction between plants grown on symbiotic N_2-fixation (SN_2F) and combined-N (N-fed) in response to increasing P supply was examined. Indigenous CFR legumes namely *A. linearis, P. calyptrata* and *C. genistoides*, observed to nodulate well at 0.1 μM P supply, were fertilized with 0.1, 1.0, 10 and 100 μM P while both *P. pinnata* and *O. striatum* which nodulated poorly at 0.1 μM P supply, received 50, 100, 200 and 400 μM P. All five species were grown reliant either entirely on SN_2F for their N nutrition or supplied with NH_4NO_3. When P was increased from 1 to 10 μM P, a positive interaction between N and P in *P. calyptrata* indicated a low P requirement for growth & SN_2F while a negative interaction between N and P in *C. genistoides* showed a high P requirement for growth & SN_2F. In *P. pinnata* a zero interaction indicated a similar P requirement for N_2-fixing and

N-fed plants between 50 to 100 μM P. Increasing P supply increased plant growth and N_2-fixation in all legumes, with the N-fed *A. linearis* attaining maximum dry matter (DM) at 10 μM P. In addition, assessment of the relative effects of P supply on nodulation and N_2-fixation in comparison to host plant growth showed a higher P requirement for N_2-fixation than host plant growth in *C. genistoides*, *P. pinnata* and *O. striatum*, and indicates that P affected nodule functioning directly. This was due to the increasing nodule to whole plant DM ratio with increased P supply, highest nodule DM attained at a higher P supply than the highest total plant DM, or higher nodule [P] than shoot [P] recorded in these species. However, in plants of *P. calyptrata,* there was no change in the nodule to whole plant DM ratio with increasing P supply and nodule [P] was similar to shoot [P] at 10 μM P when maximum DM accumulation in the SN_2F plants was obtained. This suggests that in *P. calyptrata*, nodule function and host plant growth were similarly affected by P.

Soils of the CFR vary in available P and rhizobia strains may differ in their adaptation to low P soil. It was hypothesized that the rhizobia isolates in the soils of the CFR would cluster phylogenetically according to soil P levels. Cowpea traphosts were grown in soil from 34 CFR sites and the nodule bacteria were isolated and cultivated according to standard protocol. A total of 21 rhizobia isolates were obtained. The 16S rRNA gene was sequenced and the resulting sequences compared to type strains in the public domain nucleotide databases and then phylogenetic analysis was conducted. Sixteen isolates were most closely related to members of the α-Proteobacteria genera of *Rhizobium* and *Mesorhizobium* while five isolates were in the β-Proteobacteria genus *Burkholderia.* However, the clustering of rhizobia was independant of geography, soil fertility or available soil P with a clade consisting of isolates from different sites in the CFR while distantly related isolates were from soils with similar [P].

Overall, the P nutrition of wild legumes from the CFR indicated that there are exceptions to the dogma of a high P requirement for growth & SN_2F typical of crop legumes. Thus, plants of *P. calyptrata* and *A. linearis* showed a low P

requirement for growth & SN_2F and *P. pinnata* nodules showed maximum N_2-fixing efficiency at low nodule [P]. In terms of the microsymbiont, rhizobia isolates from low P CFR soils were identified.

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GENERAL INTRODUCTION

General Introduction

Phosphorous (P) is often the most limiting element for plant growth and development (Vance *et al.*, 2003) due to its essential role in genetic, metabolic, structural and regulatory macromolecules (Raghothama & Karthikeyan, 2005; White & Hammond, 2008). Plant available P is low in the Cape Floristic Region (CFR) soils (Mitchell *et al.*, 1984; Witkowski & Mitchell, 1987; Lambers *et al.*, 2007). However, indigenous CFR plants are adapted for growth in these soils (Lamont, 1982) with high P-use efficiency (PUE) (Hawkins *et al.*, 2006) and specialized P uptake strategies such as cluster roots (Shane *et al.*, 2008) and vesicular abuscular mycorrhizas (Hoffman & Mitchell, 1986). While the growth of N_2-fixing legumes is commonly limited by the availability of P (Zahran, 1999; Vance *et al.*, 2000), nodulated legumes grow successfully in the low P CFR soils (Lamont, 1982; Stock *et al.*, 1995; Muofhe & Dakora, 1999; Cocks & Stock, 2001), being the second largest plant family in the CFR with 761 species of which 627 species are endemic (Goldblatt & Manning, 2000).

The evidence indicating that legumes have a high P requirement for growth and symbiotic N_2-fixation (growth & SN_2F) is biased by the responses of crop species such as soybean, bean and clover to P (Israel, 1993; Almeida *et al.*, 2000; Hellsten & Huss-Danell, 2000; Olivera, 2004; Araujo *et al.*, 2008). However, these crop plants are normally selected on fertilized soils for high yield, which does not encourage a P-efficient nutrient economy in the plant (Chapin, 1980; Sprent, 1999). In contrast to the typical crop legume response of increased nodule biomass with P fertilization (Jakobsen, 1985; Israel, 1987; Pereira & Bliss, 1987), *Acacia urophylla, Paraserianthes lophantha,* and *Vimanaria juncea* demonstrated greater nodule biomass production in the low P than high P treatment (Adams *et al.*, 2002). These legume species are native to the nutrient poor Jarrah and Karri forests of south-western (SW) Australia. Therefore the overall understanding of the P requirement for growth & SN_2F remains incomplete, especially in wild legumes (Vitousek *et al.*, 2002) that are indigenous to low P soils.

Similarly, N_2-fixing legumes indigenous to the low P soils of the CFR that may have been naturally selected for efficient nutrient acquisition and use, may be adapted for growth & SN_2F at low P, showing little or no increase in plant dry matter (DM) production with increased P availability (Bradshaw *et al.*, 1964; Lynch & Brown, 2006). On the other hand, the growth & SN_2F process may be a high P requiring process with CFR legumes simply tolerating low P soil but significantly increasing N_2–fixation and host plant growth with higher P supply. Thus legumes may prefer spatial and temporal high P pockets such as near cultivated areas, in riparian vegetation with increased dissolved nutrient availability (Neff *et al.*, 2003), or in early post-fire succession when P levels are high (Brown & Mitchell, 1986; Eisele, 1989). Furthermore, plant physiologists disagree on whether it is nodule growth and N_2-fixation or plant growth and metabolism that has a higher P requirement so that the effect of P on N_2-fixation may be direct (Israel, 1993; Schulze & Drevon, 2005), or indirectly mediated by changes in plant growth and nitrogen (N) demand (Robson *et al.*, 1981; Jakobsen, 1985; Hartwig, 1998).

Low P soil in the Cape Floristic Region

The CFR is a highly distinctive phytogeographical unit of 90 000 km^2 (Cowling & Holmes, 1992) with low soil N (Stock & Lewis, 1986) and low soil P availability (Mitchell *et al.*, 1984, Witkowski & Mitchell, 1987). The region consists of five main vegetation types, namely strandveld, coastal and inland renosterveld, and coastal and mountain fynbos (Mitchell *et al.*, 1984) whose boundaries are proposed to be determined by the underlying parent material of the soil (Cowling & Holmes, 1992; Richard *et al.*, 1995; Mucina & Rutherford, 2006). Mountain fynbos occurs on sandstone and granite parent rock while the coastal fynbos is made up of sandstone fynbos in the west and limestone fynbos on the south coast (Witkowski & Mitchell, 1987). Renosterveld is situated upon more nutrient rich shale while the parent material for strandveld vegetation is Aoelian sand (Witkowski & Mitchell, 1987; Mucina & Rutherford, 2006). Within these broad categories, floristic and geological variation can also exist on a smaller scale such as the limestone, sandstone and deep colluvial sand slopes occurring adjacent to each other,

as reported in a 30 ha study site at Soetanysberg Hills in the Agulhas Plains (Richards *et al.*, 1995). The vegetation occurring on the different parent material was described as Proteoid, Mesic Ericoid and Dry Restioid Fynbos for the limestone, sandstone and colluvial sands respectively (Richards *et al.*, 1995). 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}. Both Beadle (1954, 1962) and Ozanne & Specht (1981) have also implicated soil P as a major factor determining species distribution of the heathland vegetation in SW Australia where soil P was between 1 to 19 μg P g soil^{-1} (Foulds, 1993). It is notable that Lambers *et al.* (2007) and Shane *et al.* (2008) reported that for plants in both the CFR and SW Australia, soil P availability was a key factor determining nutrient acquisition strategies and thus the distribution of plant species in these habitats.

P nutrition in plants

P is an essential nutrient for plant growth (Marschner, 1995) however more than 80% of P in the soil is unavailable to a plant due to either adsorption into highly insoluble compounds such as Al-P, Fe-P and Ca-P, or because P exists in the organic form (Schactman *et al.*, 1998). As a result the concentration of soil orthophosphate (P_i), the form of P most available to a plant, is often as low as 1 to 10 μM in solution (Schactman *et al.*, 1998; Vance *et al.*, 2003). Because of the low concentration in solution, diffusion, the principal means by which P_i is supplied to the root, is slow and plant growth is often P limited (Lambers *et al.*, 1998). Consequently, substantial energy is also required to transport the phosphate anion across the negative membrane potential and steep concentration gradient that exists between the plant and soil, as cytosol [Pi] can be as high as 5 – 10 mM (Schachtman *et al.*, 1998). The dual uptake model involving a high affinity transporter (HAT) that operates at a K_m of 5 μM and a low affinity transporter (LAT) with a K_m of 50 to 300 μM is used to explain the ATP dependant proton pump uptake of Pi. The

HAT is induced by low [P] and functions in root uptake while the constitutive LAT is involved in intracellular movement of P_i (Schachtman *et al.*, 1998; White and Hammond, 2008).

Plants indigenous to the low P soils of the CFR and SW Australia are generally susceptible to P toxicity even at relatively low P supply because of the inability to down regulate their HAT uptake system (Shane *et al.*, 2004b; Gikaara *et al.*, 2004; Hawkins *et al.*, 2006). However in this susceptibility to P toxicity, large variation in species sensitivity occurs. Two native Australian *Caustis* cultivars (Gikaara *et al.*, 2004) grown at 7 levels of P supply (0, 11, 22, 44, 88, 176 and 352 g P m^{-3}) showed no deficiency symptoms at the lowest P level but showed a distinct inability to reduce P uptake and P toxicity at a relatively low P (13 – 22 g P m^{-3}) supply with a shoot [P] of 2.1% and 2.0%. These values are similar to toxic leaf [P] of 2.2 – 2.4% reported by Ozanne & Specht (1981) for indigenous SW Australian *Banksia* species. However, Playstead *et al.* (2005) reported even lower shoot [P] toxicity levels of 0.36% at 10 μM P supply in another native *Caustis blakei* sedge, with shoot [P] eventually reaching 1.2% at 250 μM P supply. Thus *C. blakei* (Playstead *et al.*, 2005) reflects the P sensitive nature of low soil P plants since crops plants usually begin experiencing toxicity when shoot [P] increases over 1% (Marschner, 1995). However P toxicity is rare in wild plants in their natural environment because they would rarely encounter high concentrations of P and in cases where they did, the extra P would be stored in the vacuole (Schactman *et al.*, 1998; Shane *et al.*, 2004a). Thus P toxicity is apparent in low P adapted plants when grown experimentally at relatively higher P supply. Furthermore, while shoot [P] for optimal growth in crop plants is in the range of 0.3 – 0.5% (Marschner, 1995), plants indigenous to low P soils are able to achieve maximum growth with relatively lower shoot [P] due to high PUE. Playstead *et al.* (2005) explained the low P requirement of 0.03% shoot [P] for maximum growth at 1 μM P supply by *Caustis blakei* to be due to efficient internal redistribution of P. Hawkins *et al.* (2006) and Gikaara *et al.* (2004) also reported low shoot [P] of 0.06 – 0.08% and 0.15 – 0.20% for maximum

growth in two *Protea* and *Caustis* species achieved at low P supply of 10 μM P for the former and between 0 – 11 g P m^{-3} supply for the latter species.

High P requirement of legumes

Most studies show that plant growth and symbiotic N_2-fixation in legumes responds positively to increased P supply (Robson, 1981; Jakobsen, 1985; Israel, 1987; Sanginga *et al.*, 1989). This is thought to be because symbiotic N_2-fixation is an energy demanding process with a high ATP requirement of 16 ATP for the reduction of one mol of N_2 to $2NH_3$ (Burris, 2000). The energy demanding nature of the nitrogenase reaction was demonstrated by Ludden & Roberts (1989) who showed that N_2-fixing bacteria are capable of saving energy by deactivating the nitrogenase enzyme through ADP-ribosylation of the Fe protein in the Fe-MoFe (nitrogenase) complex when supplied fixed N and when the supply of fixed N was depleted the enzyme was reactivated through removal of the ADP-ribosyl group.

In addition, the C cost of N_2-fixation to the plant has been estimated at 6 - 12 mg C per mg N fixed (Gutshick, 1981; Vance & Heichel, 1991) which is far greater than the C cost for the acquisition of combined-N (Lambers *et al.*, 1998). Because P has a key role in C-fixation and energy metabolism, it is predicted that growth & SN_2F will have a higher P requirement than combined-N nutrition. Legumes experiencing P deficiency may show reduced N_2-fixation through lowered photosynthetic capacity due to reduced RuBP regeneration and ATP synthesis (Pieters *et al.*, 2000) decreasing C-fixation and carbohydrate (energy) (Rao *et al.*, 1990; Jakobsen, 1985; Crews, 1993), and ATP (Sa & Israel, 1991) supply to nodules. P deficient plants may also decrease shoot growth through lowered leaf expansion and lowered specific leaf area (SLA) (Freeden *et al.*, 1989; Radin & Eindenbock, 1984; Hogh-Jensen *et al.*, 2002) thus modulating N_2-fixation through low sink demand for N (Hartwig, 1998; Almeida *et al.*, 2000). Evidence for the high P requirement of growth & SN_2F also came from observations that in fertile soil, legume growth responded more than grasses to elevated CO_2 levels due to accessing

more N from N_2-fixation, but when P was deficient, the grass growth responded while legumes no longer increased biomass or N_2–fixation in response to elevated CO_2 (Almeida *et al.*, 2000).

In addition, the high P requirement of legumes for growth & SN_2F may be due to several processes such as membrane biosynthesis, plastid function, enzyme activation-inactivation and carbon partitioning within the nodule machinery being an extra P burden (Vance *et al.*, 2000) or metabolic cost to the plant. Furthermore, the nitrogenase enzyme requires a precise oxygen homeostasis to function since high rates of oxidative phosphorylation are required for the reduction of N_2 (Burris, 2000), but nitrogenase is rapidly denatured by free oxygen (Dakora & Atkins, 1989). The oxygen supply in the nodule is regulated by leghemoglobin, a heme-protein similar to the myoglobin of mammalian tissue. This protein constitutes up to 35% of the nodule protein content (Lambers *et al.*, 1998) thereby further increasing the metabolic costs to the plant. The influence of P availability on N_2-fixation was emphasized by the fact that during P stress in white lupin, 80% of new nodule growth preferentially located near cluster roots (Vance *et al.*, 2000) where P uptake is higher than non-cluster root parts of the root (Adams *et al.*, 2002).

Most P fertilization studies on legumes link the high P requirement to N_2-fixation by reporting the higher [P] in nodules of P deficient legume plants compared to shoot or root [P] (Jakobsen, 1985; Israel, 1987; Pereira & Bliss, 1987; Drevon & Hartwig, 1997; Redell *et al.*, 1997; Hogh-Jensen *et al.*, 2002; Shulze *et al.*, 2006). For example, at low P supply, nodule [P] was 6.3 - 6.7 mg P g DM^{-1} compared to shoot [P] of 2.0 - 2.5 mg P g DM^{-1} (Jakobsen 1985), while Schulze *et al.* (2006) reported that the [P] threshold for optimal function in nodules of between 3.3 – 6.5 mg P g DM^{-1} was higher than shoot of 1.1 – 3.0 mg P g DM^{-1}. Similarly Hogh-Jensen *et al.* (2002) observed that following abrupt withdrawal of P, white clover plants growing in 100 mM P decreased tissue [P] more than tissue [N] and the decline in [P] was greater in roots and shoots than nodules. Nodule [P] was therefore two to three times greater than roots and shoots. The higher [P] in nodules indicated that nodules have a higher requirement for P than the shoot for effective functioning (Vance *et*

al., 2003). Legume physiology may thus be adapted to maintain N supply for plant growth by allocating more P to nodules where it is needed most, probably due to the high ATP requirement of nitrogenase.

P requirement of crop legumes

Due to the reported high P requirement of growth & SN_2F, upon P fertilization N_2-fixing crop legumes typically respond with increased plant and nodule DM accumulation and increased nodule number (Jakobsen, 1985; Israel, 1987; Pereira & Bliss, 1987; Drevon & Hartwig, 1997). Jakobsen (1985) observed that in pea, nodule DM at high P supply was 80 mg pot^{-1} which was 2.3 times greater than at low P, while in another study a three fold increase in nodule size was observed in 2 mM P treated soybean relative to the unfertilized counterparts (Israel, 1987). The ratio of green (ineffective) nodules to red (effective) nodules was higher at low than at high P in common bean (Pereira & Bliss, 1987), while acetylene reduction activity (ARA) which is a measure of nitrogenase activity per plant and specific nitrogenase activity (SNA), a measure of nitrogenase activity per nodule, was reduced in soybean and alfalfa plants supplied with lower levels of P (Crews, 1993; Drevon & Hartwig, 1997; Sa & Israel, 1991). In all cases, increased nodulation and nitrogenase activity with increased P supply led to a corresponding increase in total N in the plant (Jakobsen, 1985; Sa & Israel, 1991; Schulze *et al.*, 2006).

Some studies report more or the same number but smaller nodules with low P supply (Schulze *et al.*, 2006; Schulze & Drevon, 2005), indicating that nodule size is more sensitive to P stress than nodule number or mass per plant. The possible physiological significance of this was explained by Schulze & Drevon (2005) who demonstrated that P deficiency significantly increased nodule O_2 uptake to 17.3 $\mu m\ s^{-1}$ in 5 μM P grown alfalfa compared to 13.2 $\mu m\ s^{-1}$ for 20 μM P plants. Thus nitrogenase respiration per unit nodule in the low P plants consumed 23% more O_2 and the O_2 cost per unit N_2 fixed was double than that of the high P plants. Schulze & Drevon (2005) proposed that P stress contributed to higher respiratory costs for N_2-fixation causing higher O_2 uptake

by the nodules and related increased nodule O_2 permeability to smaller nodule size, which was the most apparent morphological response in the low P supplied alfalfa.

Adaptation and tolerance in wild plants

Whilst the above examples related the growth response of crop legumes to increased P supply, wild legumes would differ fundamentally from crop legumes in relative growth rate (RGR), a trait that integrates both nutrient uptake and utilization efficiency in the accumulation of DM (Chapin, 1980). Wild plants from infertile soils tend to have a lower RGR than crop plants and wild plants from fertile soils (Grime, 1977; Chapin, 1980) and are less responsive in growth to nutrient additions (Chapin *et al.*, 1986). Therefore it is expected that legume species from low P soils would be adapted for growth at low P, and would respond less in DM accumulation to increased P supply than legumes from high P soils. By comparing the growth response of plant species from fertile and infertile sites to increasing P, the reports of Bradshaw *et al.* (1960), Rorison (1968) and Clarkson (1967) provided evidence that species from low P soils are adapted to low P.

In their study, Bradshaw *et al.* (1960) compared the growth of grass species from habitats varying in soil P to find out if species from infertile soils were able to produce more DM than species from fertile soils when grown at low P. The results showed that plants of *Festuca ovina* and *Nardus stricta* from infertile soils accumulated the lowest DM at the lowest P level while *Lolium perenne* from fertile soils had the highest DM. Initially this would seem to indicate that the infertile soil species were not adapted to low P. However, *N. stricta* and *F. ovina* did not increase growth with P addition meaning that growth at the lowest P was their maximum, indicating low P adaptation. The problem with comparing biomass obtained at a single level of P as a measure of adaptation is that the potential low RGR of the low P species may result in a lower biomass at the lowest nutrient level but this biomass is still high relative to the plants maximum yield at a higher nutrient supply (Bradshaw *et al.*, 1964). Similarly a fast growing species may yield more than the low P

species at the lowest nutrient level but this biomass is far less relative to its maximum at a higher level. For instance, Rorison (1968) showed that at low P supply of 0.1 mM P, *Rumex acetosa*, a fast growing species from fertile soils, produced 19 mg DM plant^{-1} which was three times more than *Deschampsia flexuosa*, a species from low P soil. On closer examination it was evident that *D. flexuosa* achieved 63% of its maximum growth at 0.1 mM P, while *R. acetosa* only achieved 43% of its maximum growth at the same P level. Therefore relatively speaking, the low P *D. flexuosa* was better adapted for growth at low P than *R. acetosa* because it experienced lower yield depression at low P. These observations imply that plant adaptation to a nutrient should be assessed by exposing the plants to increasing levels of nutrient supply and assessing their growth response.

While species from low P soils may be better adapted than species from high P soils at maintaining growth under P stress (Bradshaw *et al.*, 1960; Rorison, 1968), the results by Clarkson (1967) on *Agrostis* species of *A. setacea* from low P soils, *A. stolonifera* from high P soils, and *A. canina* which has a wide edaphic tolerance proved inconclusive. In low P soil, RGR of *A. stolonifera* and *A. canina* declined after 10 weeks, while *A. setacea* did not show any deficiency and RGR did not decline. So although shoot DM in *A. stolonifera* was more than double that of *A. setacea* at 14 weeks, relative biomass accumulation was greater in the low P species. It is possible, given the trend of the curve in Clarkson (1967), that with time *A. setacea* would have outyielded the high P species in this nutrient poor soil. With increased P supply all three species increased biomass and RGR did not decline with the result that the low P species *A. setacea* produced six times more biomass at high P than it did at low P. Thus although the low P species had a lower growth rate than the high P species, it responded significantly more to P fertilization relative to the high P species, indicating that the low P *A. setacea* was merely tolerant of, and not adapted to low P soil. In another experiment Clarkson (1967) observed that both *A. stolonifera* and *A. setacea* grew best at a high P supply of 132 μg P plant^{-1} week^{-1} proving that the species from the low P soil was just tolerant of the low P supply.

P requirement of wild legumes from low P habitats

Muofhe & Dakora (1999) and Cocks & Stock (2001) suggested that indigenous CFR *Aspalathus* spp. may be adapted for growth & SN_2F in low soil P conditions. This was because N_2-fixing *A. linearis* reported to be growing in soils with extremely low available P of 0.03 μg P g $soil^{-1}$ contributed as much as 105 to 128 kg N ha^{-1} to the nitrogen budget of the ecosystem (Moufhe & Dakora, 1999). Cocks & Stock (2001) surveyed 15 *Aspalathus* spp. occurring in the CFR and indentified two *Aspalathus* species namely *A. abienta* and *A. ciliaris* that nodulated prolifically while growing in soil with low total P of 30 to 50 μg P g $soil^{-1}$ thus suggesting adaptation to low P. However, for these *Aspalathus* spp. the response or change in the N_2-fixation with increased P supply was not reported. On the other hand, Sanginga *et al.* (1995) showed that *Gliricidia sepium* a species used widely in agroforestry, did not increase growth, percentage N derived from the atmosphere or amount N_2-fixed when supplied with more P, thus showing low P adaptation. In contrast, indigenous herbaceous legumes growing on infertile soils in Zimbabwe with available P of less than 5 mg P kg $soil^{-1}$ responded to superphosphate addition (Mapfumo *et al.*, 2005). The proportion of legume biomass increased from 10% to 40% and total N in the legumes also increased.

To fully understand the relationship between P supply and growth & SN_2F in wild legumes it is pertinent to look at reports from SW Australia, an area well represented by low P soils and endemic N_2-fixing legumes (Lamont, 1982). Studies there reveal varied growth & SN_2F responses with some legumes showing possible adaptation for growth & SN_2F in low P soil (Lawrie, 1981; Langkamp & Dallling, 1982; Ribet & Drevon, 1996) while others showed a high P requirement (Hingston *et al.*, 1982; Hansen & Pate, 1987). For example, indigenous SW Australian species of *Acacia pulchella, Kennedia coccinea* and *Kennedia prostrata* increased plant DM, nodule DM, nodule number per plant and ARA with P fertilization in glasshouse and field experiments (Hingston *et al.*, 1982). Similarly, in a field study (Hansen &

Pate, 1987) plants of *Acacia alata* and *A. pulchella* showed greater reliance on N_2-fixation than soil-N in the first year after fire. This may suggest a high P requirement for growth & SN_2F given the high P content of soils after fire (Debano & Conrad, 1978; Brown & Mitchell, 1986; Eisele *et al.*, 1989).

In contrast, Langkamp & Dallling (1982) found that *Acacia holoserica*, noted for being a vigorous and successful colonizer of restored mining sites that were N and P depleted, was adapted to low levels of soil P. This was observed when inoculated *A. holoserica* were grown at six P levels of 4.4, 9.5, 12.4, 27.4, 33.7 and 60 μg P g soil^{-1} and after 4 months of plant growth, DM was measured as 26, 137, 96, 33, 26 and 5 g plant^{-1} respectively for the six P applications (Langkamp & Dallling, 1982). The maximum growth at 9.5 μg P g soil^{-1} followed by the sharp downward trend suggests adaptation for growth & SN_2F at low levels of soil P. In another study, Ribet & Drevon (1996) observed that although nodule DM decreased, greater nodule efficiency and nitrogenase activity was measured at low P than at high P supply and concluded that *Acacia mangium* was adapted to low P because of high PUE. Consequently this species was recommended as a model for investigating the mechanisms and genes involved in N_2-fixation during P deficiency (Ribet & Drevon, 1996). In addition, 10 indigenous Australian legumes occurring in low open forest, sandy heathland and costal sand dunes with low soil P of 0.009 - 0.57, 0.001 - 0.008 and 0.013 - 0.020% respectively, exhibited low P tolerant growth & SN_2F with high nodule number, mass and ARA (Lawrie, 1981).

Some studies in non-Mediterranean but similarly infertile ecosystems also show varied growth & SN_2F responses of legumes to increased P supply. In a three year field study (Bobbink, 1991), legumes in Dutch Chalk grassland were observed to increase DM by seven to 16 fold in two P-fertilized sites compared to the control sites, while in a serpentine plant community with soil P of 0.29 mg P g soil^{-1} the legume population did not respond to added P at only one site, while at the other site the legumes increased shoot growth (Koide *et al.*, 1988). Therefore, while growth & SN_2F in wild legumes from low P soils may be adapted to low P (Lawrie, 1981; Langkamp & Dallling, 1982;

Sanginga *et al.*, 1995; Muofhe & Dakora, 1999), with others not showing increased biomass with higher P applications (Koide *et al.*, 1988), there are exceptions with some having a higher P requirement commonly associated with crop legumes (Hansen & Pate, 1987; Bobbink, 1991; Mapfumo *et al.*, 2005).

Determining the P requirement for growth and symbiotic N_2-fixation

Robson (1983) proposed a simple approach to test whether legumes dependant on symbiotic N_2-fixation for growth actually have a high P requirement by examining the interaction between legume plants grown on SN_2F and combined-N (N-fed) in response to increasing P supply (N X P interaction). A negative interaction between combined-N and P supply occurs when there is greater DM and N accumulation response in the SN_2F plants relative to the N-fed plants with increased P supply and indicates that plants fixing N_2 have a higher P requirement than plants relying on combined-N acquisition. No or zero interaction due to similar growth responses in both N treatments means that plants have the same P requirement for SN_2F or combined-N uptake. A positive interaction between combined-N and P supply, due to a higher change in growth in the N-fed plants relative to the SN_2F plants when supplied more P, means that SN_2F plants have a lower P requirement than the N-fed plants.

Previous interaction studies have revealed varied P requirements for different legumes species by showing zero (Redell *et al.*, 1997; Ribet & Drevon, 1996), negative (Israel, 1987; Sanginga *et al.*, 1989) and positive interactions (Robson *et al.*, 1981; Jakobsen, 1985; Pereira & Bliss, 1987) between N and P supply. Redell *et al.* (1997) compared *Casuarina cunninghamiana* seedlings reliant on fixed-N to uninoculated seedlings receiving combined-N. In the inoculated plants, shoot DM and total N increased with additional P similar to the N-fed plants. Ribet & Drevon (1996) also found that in both N_2-fixing and urea-fed treatments on *A. mangium*, shoot DM responded equally to increased P supply. Both studies therefore concluded that the external P

requirement for SN_2F and N-fed plants was the same because of the zero interaction of N and P on shoot DM.

In contrast Sanginga *et al.* (1989) observed a negative interaction in the shoot DM response of *Casuarina equisitifolia* with P fertilization. Shoot DM increased 89% for the inoculated plants but only 14% for the N-fed plants. Similarly, Israel (1987) observed a negative interaction between N and P supply in the response of *Glycine max*, with the SN_2F soybean accumulating more DM and shoot N with increasing P supply than the N-fed treatment. The negative interactions imply that N_2-fixing soybean and *C. equisitifolia* have a greater requirement for P than plants assimilating combined-N and further indicates that growth & SN_2F has a high P requirement. However, in experiments with clover (*Trifolium subterraneum*), young pea (*Pisum sativa*) and the low P adapted bean (*Phaseolus vulgaris*) cultivar *Peubla-152*; Robson *et al.* (1981), Jakobsen (1985), and Pereira & Bliss (1987) found a positive interaction between combined-N and P on plant growth. This was due to a greater growth response by the N-fed plants than the N_2-fixing plants to increased P application and indicates that these N_2-fixing clover, pea and bean have a low P requirement for growth & SN_2F.

P requirement for N_2-fixation and nodule growth relative to host plant growth

To separate the direct effect of P upon N_2-fixation from an indirect effect via host plant growth, the relative effects of P supply upon N_2-fixation parameters and host plant growth should be compared (Israel, 1987). The inhibiting effect of low P upon N_2-fixation may be direct through nodule metabolism having a high P requirement (Israel, 1987; Israel, 1993; Sa & Israel, 1993) or indirectly mediated by decreased C-fixation resulting in either lower C supply to nodules or reduced shoot growth and lower plant N demand (Jakobsen, 1985; Almeida *et al.*, 2000). Evidence for an indirect effect of P has come from Redell *et al.* (1997), Jakobsen (1985) and Almeida *et al.* (2000) who interestingly used three different mechanistic approaches in coming to the same conclusion. Firstly, Redell *et al.* (1997) derived evidence for an indirect effect of P on N_2-

fixation from the observation that the external P requirement for maximum shoot dry DM (host plant growth) was at a higher P level than that for maximum nodulation. In Jakobsen (1985), nodulated pea plants were observed during recovery from P deficiency. The sequence of time course responses revealed that nodule [P] increased two days after shoot [P] and nodule ARA response also lagged but mimicked increasing shoot [P]. Jakobsen (1985) therefore concluded that P regulated N_2-fixation through its effects on photosynthesis and C supply to the nodule. Thirdly, Almeida *et al.* (2000) found that low P supply reduced plant growth but specific N_2-fixation rates in the nodules increased. The assimilation of N thus exceeded the amount of N required by the plant resulting in an accumulation of N in the plant tissue. This triggered a N feedback response leading to decreased nodule growth and decreased the proportion of whole plant N derived from N_2-fixation. Therefore the effect of P on N_2-fixation was indirectly mediated by shoot N demand (Almeida *et al.*, 2000).

The Almeida *et al.* (2000) study also reported that shoot [N] decreased with increasing P supply. This is in contrast to Israel (1987) and Sanginga *et al.* (1989) who reported an increase in shoot [N] with increasing P supply which implies that P is directly involved in N_2-fixation. In addition, Israel (1987) correlated whole plant [P] with whole plant [N] in SN_2F and N-fed soybean and showed that at similar whole plant [P] the N-fed soybean accumulated more biomass and N than the SN_2F soybean. This implied a higher internal P requirement by the N_2-fixation system in the SN_2F soybean, providing more evidence for a direct effect of P on nodule metabolism (Israel, 1987). A higher requirement for P by nodule growth relative to plant growth has also been suggested by an increase in the ratio of nodule to whole plant DM with increased P supply (Israel, 1987; Hellsten & Huss-Danell, 2002; Araujo *et al.*, 2008).

Selection for efficient growth and N_2-fixation at low P

With wild legumes from low P areas demonstrating that legumes may be adapted for growth & SN_2F at low P, studies aimed at selecting superior N_2-

fixers at low P have been conducted (Sprent, 1999), however mostly on crop species such as *P. vulgaris*. For example, from an initial screening of 51 genotypes, and a subsequent evaluation of the best eight performing lines at 5 μM P supply, Christiansen & Graham (2002) were able to identify two Andean *P. vulgaris* genotypes with superior growth, nodule mass, ARA and total plant N as efficient N_2-fixers. Similar to Vadez *et al.* (1999), they found a strong correlation between shoot DM and plant N per unit shoot P suggesting that plant N per unit shoot P was a key parameter for selecting low P adapted genotypes. Vadez *et al.* (1999) screened 220 common bean lines with one replicate per P treatment and classified 24 genotypes with superior growth & SN_2F as those that had more than 120 mg N $plant^{-1}$ at 72 μM P supply while 23 poor growth & SN_2F lines fixed less than 68 mg N $plant^{-1}$. Overall, N_2-fixation capacity at low P was associated with early nodulation, and low nodule [P] due to high PUE of the nodules. Both Christiansen & Graham (2002) and Vadez *et al.* (1999) cautioned that often when plants fix more N_2, larger shoots are produced resulting in a dilution of the N content in the plant. Thus increased N_2-fixation is not always associated with increased tissue [N]. Therefore tissue [N] is not considered to be a good selection criterion for superior growth & SN_2F. For example, the superior growth & SN_2F lines in Vadez *et al.* (1999) had 127.7 mg N $plant^{-1}$ compared to the 47.8 mg N $plant^{-1}$ in the poor growth & SN_2F lines; however plant [N] was similar at 8.74% and 8.21% respectively.

Rhizobia diversity and taxonomy

Traditionally, the term rhizobia referred to those genera of bacteria that are in the family Rhizobiaceae in the α-subclass of Proteobacteria and that are able to nodulate and fix N_2 in symbiosis with plants in the family Leguminosae and *Parasponia* in the family Ulmaceae (Willems, 2006; Sprent, 2007). Taxonomic evidence, based primarily on the phylogenetic analysis of the highly conserved 16S rRNA gene, which has been the single most important molecular marker for reconstructing phylogenies (Young & Haukka, 1996; Sessitsch, 2002), grouped rhizobia into four distinct branches within the α-

Proteobacteria domain. The four branches are *Mesorhizobium-Sinorhizobium-Rhizobium/Agrobacterium/Allorhizobium*, *Bradyrhizobium*, *Azorhizobium* and the fourth branch being the recently described *Methylobacterium* (Moulin *et al.*, 2001; Young *et al.*, 2001). While each of these branches form distinct and separate clusters within the α-Proteobacteria lineage they also include related non-rhizobia bacterial species (Young & Haukka, 1996).

Recently, the β-subclass of Proteobacteria has been reported to include rhizobia. For instance, several studies have reported the discovery of rhizobia belonging to the *Burkholderia* genus in the β-subclass of Proteobacteria (Moulin *et al.*, 2001; Kock, 2004; Spriggs, 2004; Hung *et al.*, 2005; Sprent, 2007). Kock (2004) and Spriggs (2004) demonstrated that indigenous *Cyclopia* species growing in undisturbed sites in the CFR nodulated predominantly with *Burkholderia* species with only six of the 55 isolates being α-Proteobacteria. Moulin *et al.* (2001), also through phylogenetic analysis of 16S rRNA gene sequences, found that strain STM678, isolated from the indigenous CFR legume *Aspalathus carnosa* initially thought to be nodulated by *Bradyrhizobium* rhizobia, was actually the β-Proteobacteria *Burkholderia tuberum*. Interestingly, 24 of the 49 *Burkholderia* isolates from *Cyclopia* spp. in the study by Kock (2004) were found to be closely related to the STM678 *A. carnosa* isolate from Moulin *et al.* (2001), demonstrating the prevalence of these *Burkholderia* rhizobia in the CFR.

With the contemporary advances in DNA sequencing technology, rhizobia taxonomy is progressing at a fast pace, and the classification of rhizobia is constantly changing. Proposals for new revisions are often reported, such as the proposal by Young *et al.* (2001) to incorporate the highly inter-related *Allorhizobium* and *Agrobacterium* genera into the *Rhizobium* genus. These debates continue as data from more gene sequences provide useful information resulting in an increasing number of genera and species being recognized (see Table 1).

Table 1. Chronology of rising number of species in the genera of rhizobia (from Willems, 2006).

Genus	Number of species					
	Before 1980	**1981-1985**	**1986-1990**	**1991-1995**	**1996-2000**	**2001-2006**
Agrobacterium	4	4	5	5	5	5
Rhizobium	4	5	5	10	10	16
Bradyrhizobium		1	1	3	3	7
Sinorhizobium			2	5	8	11
Azorhizobium			1	1	1	2
Mesorhizobium					7	11
Allorhizobium					1	1
TOTAL	8	9	13	23	34	53

The current taxonomy of rhizobia consists of 12 genera, nine of which are α-Proteobacteria, now also termed α-rhizobia, and three are β-Proteobacteria or β-rhizobia. Bacteria genera that are phylogenetically outside the traditional Rhizobiaceae family but that are now considered rhizobia include *Methylbacterium*, *Devosia*, *Ochrobactrum* and *Phyllobacterium* in the families Methylobacteriaceae, Hyphomicrobiaceae, Brucellaceae and Phyllobacteriaceae respectively. In the β-rhizobia the genera are *Burkholderia*, *Ralstonia* and *Cuprividis* in the family Burkholderiaceae (see Table 2).

Table 2. The 12 genera of nodule forming N_2-fixing bacteria (Young & Haukka, 1996; Moulin *et al.*, 2001; Young *et al.*, 2001; Willems, 2006)

α-Proteobacteria/ α-rhizobia	**β-Proteobacteria/ β-rhizobia**
Rhizobium/Agrobacterium/Allorhizobium	*Burkholderia*
Bradyrhizobium	*Ralstonia*
Sinorhizobium	*Cuprividis*
Mesorhizobium	
Azorhizobium	
Phyllobacterium	
Ochrobactrum	
Devosia	
Methylbacterium	

Recently, in addition to Kock (2004) and Spriggs (2004); Joubert (2002), Le Roux (2003), and Phalane (2008) have also investigated the diversity of root nodule bacteria associated with *Acacia*, *Lotonis* and *Lebeckia* legume species

growing in South African (SA) soils. Their research identified a diversity of rhizobia symbionts from the nodules of the legume genus studied. However none of the studies correlated the diversity of rhizobia with edaphic factors such as soil P.

Aims and Rationale

The CFR is renowned for its high biodiversity and edaphic variation (Goldblatt & Manning, 2000). Therefore, with indigenous legumes growing successfully in low P CFR soils, there exists the potential for discovering species with a low P requirement for growth & SN_2F. Additionally, observations on the P requirement of CFR legumes indigenous to low P soil could address some of the inconsistent results obtained in previous N X P interaction studies, thus improving our understanding of the role of P in N_2-fixation. In this study it was hypothesized that N_2-fixing legumes indigenous to the low P soils of the CFR have a low P requirement for growth & SN_2F.

Vitousek *et al.* (2002) defined a factor P as a constraint to N_2-fixation when a legume fixing N_2 requires more P than when acquiring combined-N as its source of N nutrition. This study adopted the Vitousek *et al.* (2002) conceptual approach by growing indigenous N_2-fixing CFR legume genotypes at low P supply (Chapter 2), then examining the response of selected species to increasing levels of P supply while reliant on fixed-N or combined-N for their N nutrition (Chapter 3).

Thus, the objectives were to:

- identify indigenous CFR legume species with superior nodulation and N_2–fixation at low P supply,
- to determine whether the N_2-fixing CFR plants require more P than N-fed plants with increased P supply,
- to examine the effect of increasing P supply on nodulation and N_2-fixation in the N_2-fixing CFR plants, and

- to determine whether the positive N_2-fixation response to P is because of a direct effect of P on nodule functioning or an indirect effect via changes in host plant growth.

In addition, the mineral nutrition of legumes is more complex than non-legumes because of the possible effects of the mineral nutrient on both the host plant and the rhizobia (Robson, 1983). Therefore, the strain of rhizobia involved may be as important as the species of the host legume plant in determining the low P requirement of the symbiosis. This is possible because certain strains of rhizobia may be adapted for growth and function at low P (Cassman *et al.*, 1981; Graham & Vance, 2000). With this in mind, a study on CFR rhizobia (Chapter 4) was conducted with the hypothesis that rhizobia isolates from the soils of the CFR would cluster phylogenetically according to soil P levels.

The objectives of this study were to:

- investigate the diversity of rhizobia in the CFR soil,
- to associate rhizobia isolates from the low P sites with rhizobia type strains, and
- to assess fertility levels of the soil from the different CFR sites using cowpea as a bioassay plant.

CHAPTER 2

VARIATION IN SYMBIOTIC N_2-FIXATION OF INDIGENOUS CAPE FLORISTIC REGION LEGUMES GROWN AT LOW P SUPPLY

2.1 INTRODUCTION

Soils of the CFR are low in total and available P that varies with season, soil depth, parent material, age of soil, extent of leaching and chemical form of P (Mitchell *et al.*, 1984; Witkowski & Mitchell, 1987; Lambers *et al.*, 2007). For instance, aoelian derived soils have relatively high available P levels of 34 to 70 μg P g soil^{-1} (Witkowski & Mitchell, 1987; Hawkins *et al.*, 2006); available P is 1 to 4 μg P g soil^{-1} in highly leached steep mountain slopes and coastal lowland soils on sandstone parent rock (Mitchell *et al.*, 1984; Witkowski & Mitchell, 1987) while limestone derived alluvial sands have slightly higher levels of 6 to 8 μg P g soil^{-1} (Witkowski & Mitchell, 1987). Furthermore, Lambers *et al.* (2007) showed variation in soil age and total soil P, with P as high as 800 μg P g soil^{-1} in "young soils" and as low as 30 μg P g soil^{-1} in "ancient, highly weathered soil", such as in the mountain fynbos of the CFR.

Several authors (Kruger *et al.*, 1983; Cowling & Holmes, 1992; Richards *et al.*, 1995; Richards *et al.*, 1997) have proposed that variation in edaphic factors such as P availability may be responsible for the distribution patterns of species, and high levels of endemism present in the CFR. For example, *Protea compacta* is endemic to shallow colluvial sands with low available P of 0.5 μg P g soil^{-1} and does not occur with *Protea obtusifolia* and *Leucadendron meridianum* in adjacent limestone soils with 3 μg P g soil^{-1} probably because of its inability to down regulate P uptake at higher levels of P availability (Richards *et al.*, 1997; Shane *et al.*, 2008). In addition, species such as *P. susannae* and *L. coniferum* dominated on more fertile limestone derived deep colluvial sand while mostly ericoid fynbos occurred on less fertile sandstone slopes (Richards *et al.*, 1997). Ozanne & Specht (1981) also implicated soil P variation as a major factor determining species distribution in Western Australian soils that are similar to the low P CFR soils (Foulds, 1993; Cowling & Witkowski, 1994; Herppich *et al.*, 2002). This association between species distribution and soil nutrient factors may arise through physiological differences in nutrient use and uptake strategies amongst plant species (Lamont, 1982; Richards *et al.*, 1997; Orians & Milewski, 2007).

Indigenous CFR legumes are able to circumvent CFR N-limitation (Stock & Lewis, 1986; Stock *et al.*, 1995) by fixing N_2 in a symbiosis with rhizobia (Lamont, 1982; Stock *et al.*, 1995; Muofhe & Dakora, 1999; Cocks & Stock, 2001). However, the distribution of these indigenous legumes in the CFR is patchy and is partly attributed to edaphic factors such as P availability since Cocks & Stock (2001) were able to demonstrate species variation in nodulation with soil P. In a survey of 15 *Aspalathus* spp. across nine CFR sites with total P of 30 – 50 to 100 – 300 μg P g soil^{-1}, nodulation differences were evident between species, with *A. abienta* and *A. ciliaris* nodulating prolifically in the lowest P soil while *A. larcifolia*, *A. ericifolia* and *A. neglecta* nodulating poorly in the same soil. It is expected that the former two species would grow well in low P CFR areas or fix N_2 at low P supply. More specifically, *A. linearis* which is able to fix N_2 in soil with P as low as 0.03 μg P g soil^{-1} (Muofhe & Dakora, 1999) is edaphically restricted to the highly nutrient deficient soils of the Cedarberg Mountains between Gifberg in the north and Elandskloof in the south (van der Bank *et al.*, 1999).

Due to variation in available P in the CFR and the patchy distribution of N_2-fixing species, CFR legumes occurring in low P soils (low-P) may be more tolerant of low P than legumes from high P soils (high-P) and consequently would be superior N_2-fixers at low P supply. For instance, wild *P. vulgaris* genotypes from low P soils were found to be superior N_2-fixers at low P supply, with higher growth, nodule biomass and shoot N content than high-P genotypes (Pereira & Bliss, 1987; Lynch & Beebe, 1995; Vadez *et al.*, 1999; Christiansen & Graham, 2002). Sanginga *et al.* (2000) grouped four lines of cowpea as low-P adapted and four others as the high-P lines. At 0 kg P ha^{-1}, the low-P group had higher biomass, shoot to root ratio, total shoot N and total N_2-fixed indicating that the low-P group was superior to the high-P group in N_2-fixation at low P supply. Vadez *et al.* (1999) also compared the N_2-fixation performance of four groups of beans: bushy (late or early flowering) and climbing (early or late flowering) at 72 μM P and found that the climbing-late flowering genotypes were tolerant of low P.

In this study CFR legumes species were grouped as either low-P, high-P, P-generalist and unclassified. It was hypothesized that the low-P group would be superior N_2-fixers than the high-P group at low P supply. The study objective was to identify indigenous CFR legume species with superior nodulation and N_2–fixation at low P supply. Therefore 18 indigenous CFR legume species, inoculated with CFR soil, were grown with no N supplied at 0.1 μM P supply and assessed for growth & SN_2F.

2.2 MATERIALS AND METHODS

Species selection

Literature was analyzed for information on the distribution of indigenous legume species and for data on soil [P] in the CFR. The datasets were combined to determine species distribution in association with soil [P]. Sites with total soil [P] greater than 100 μg P g $soil^{-1}$ were classified as high-P, while those with [P] less than 100 μg P g $soil^{-1}$ were designated low-P soil, based on the range 5.91 - 365.35 μg P g $soil^{-1}$ obtained from the literature (see references in Table 1). Hence a total of 18 species were selected for the study and were grouped as four low-P species, four high-P, five P-generalists, and five unclassified legume species (Table 1).

Plant germination and growth

Seeds were obtained from Silverhill Seeds, Claremont, Cape Town. Seeds of *A. linearis* were scarified in concentrated H_2SO_4 for ½ an hour, thoroughly rinsed in six changes of sterile distilled water and then soaked in water overnight to aid germination, while the seeds of the other species were only soaked overnight in boiling water to aid germination. All seeds were sown in seedlings trays containing acid washed (0.1% HCl) silica sand. Four weeks after emergence seedlings of similar size were transplanted to 18 cm pots containing 3 kg of acid washed silica sand and 10-12 g of CFR soil composed of soil collected from legume habitats in the CFR, .was placed around the root

and stem base of the plant as rhizobia inoculum. The seedlings were watered with strength N-free Hoagland solution with P in the form of K_2HPO_4 (0.5 M) and KH_2PO_4 (0.5 M) adjusted to supply 0.1 μM P in the nutrient solution, to simulate infertile low P CFR soils. This nutrient solution was administered in 400 ml per pot twice a week and pots were flushed with 1 L tap water once a week to prevent accumulation of salts. Plants grew for 22 weeks from September to February in pots randomly placed on trolleys in the glasshouse under natural light and natural temperature. There were seven replicates for each species.

Plant harvest and measurement of biomass

Plants were harvested by gently washing off the sand around roots and the plant separated into nodules, roots, and shoots. The plant material was dabbed dry with paper towels and fresh weight (FW) measured. Nodules were counted for each plant. Plant material was dried at 60˚C for 72 hours in a forced draught oven and then reweighed for DM. The dried shoot material was ground in a steel ball mill (MM200, Retsch®, Haan, Germany) to a fine powder for N analysis.

Shoot N analysis

Approximately 2 mg of each plant sample was weighed into a tin foil cup to an accuracy of 1 μg on a Sartorius micro balance. The cups were then folded to enclose the sample. The samples were combusted in a Flash EA 1112 series elemental analyzer (Thermo Finnigan, Italy). The resulting gases were automatically fed into a Delta Plus XP isotope ratio mass spectrometer (Thermo Electron, Germany), via a Conflo III gas control unit (Thermo Finnigan, Germany). The standards used to calibrate the results were Merck Gel, a proteinaceous gel produced by Merck and dried nasturtium leaves collected from Woodbine Lane, University of Cape Town campus. Shoot [N] was expressed as a percentage of shoot DM, from which total amount of N in the shoot was calculated by obtaining the product of [N] and DM.

Statistical analysis

To reduce inequality of variance in the raw data, all measurements were $\log_e$ transformed before statistical analysis. Data was analyzed using a Nested ANOVA in the STATISTICA software package with species as random effects nested in soil P groups. Means that were significantly different at $P < 0.05$ were separated by Duncan's multiple range test.

Table 1. Legume species distribution associated with total soil [P] of sites in the CFR. ([P] > 100 μg P g soil^{-1} = High-P; [P] < 100 μg P g soil^{-1} = Low-P; [P] < 100 and > 100μg P g soil^{-1} = P-generalist)

[a]**Total soil [P] > 100 μg P g^{-1}**	[a]**Total soil [P] < 100 μg P g^{-1}**
• Swartboskloof cliffs • De Hoop Nature Reserve • DuToitskloof	• Swartboskloof lowlands • Pella • Cape Point Nature Reserve • Silvermine Nature Reserve • Soetanysberg, Cape Agulhas • Bainskloof

[b]**High-P species**	[b]**P-Generalist species**	[b]**Low-P species**
Cyclopia genistoides	*Podalyria calyptrata*	*Podalyria sericea*
Bolusafra bituminosa	*Liparia splendins*	*Virgilia oroboides*
Lessertia frutescens	*Psoralea pinnata*	*Aspalathus linearis*
Lessertia capensis	*Psoralea aphylla*	*Indigofera filifolia*
	Otholobium fruticans	

[b]**Unclassified P species:** *Cyclopia intermedia, Cyclopia subternata, Virgilia divaricata, Otholobium striatum, Indigofera lyalli*

[a]Literature reviewed for total soil [P] data:
Read & Mitchell, 1983; Mitchell *et al.*, 1984; Lambrechts *et al.*, 1986; Witkowski & Mitchell, 1987; Van Reenen *et al.*, 1992; Marumo, 1996; Richards *et al.*, 1997.

[b]Literature reviewed for legume distribution data:
Van Wilgen & Kruger, 1981; Lamont, 1982; Taylor, 1983a; Taylor, 1983b; Hoffman & Mitchell, 1986; McDonald, 1988; McDonald & Morley, 1988; Van Wilgen & Forsyth, 1992a; Van Wilgen & Forsyth, 1992b; Allsopp & Stock, 1993; Musil, 1993; Cocks, 1994; Marumo, 1996; Masutha *et al.*, 1997; Muofhe & Dakora, 1999; Cocks & Stock, 2001; Spriggs *et al.*, 2003; Trinder-Smith, 2003; Brown & Duncan, 2006.

2.3 RESULTS

Comparison of the groups of plants grown with 0.1 μM P supply showed that there was no difference in total fresh biomass (Fig. 1A), root to shoot FW ratio (Fig. 2A), nodule FW (Fig. 3A), nodule number plant^{-1} (Fig. 4A), nodule

size (Fig. 5A), nodule to root FW ratio (Fig. 6A), and shoot [N] and shoot N content (Table 2).

However at a species level, *V. oroboides* and *V. divaricata*, produced the highest total fresh biomass (Fig. 1B) followed by *P. calyptrata* and *A. linearis*. The low-P legumes of *V. oroboides*, *A. linearis*, *P. sericea* and *I. filifolia* accumulated significantly different total fresh biomass of 2530, 660, 330 and 150 mg plant^{-1} respectively. Three of the four species in the high-P group namely *L. capensis*, *L. frutescens*, and *B. bituminosa* had the lowest total growth with 32, 72 and 82 mg plant^{-1} while the other high-P plant *C. genistoides* produced higher biomass of 169 mg plant^{-1}. The root to shoot FW ratio for individual species also varied (Fig. 2B). *V. oroboides* had the highest root to shoot FW ratio whereas *A. linearis* attained the lowest ratio.

In terms of N_2-fixation performance, *O. striatum*, *P. pinnata* and *P. aphylla* nodulated poorly and had the lowest nodule FW (Fig. 3B), nodule size (Fig. 5B) and nodule to root FW ratio (Fig. 6B). In contrast, *V. oroboides*, *P. calyptrata*, *V. divaricata* and *A. linearis* nodulated well, attaining the highest nodule FW in the range of 43 to 80 mg plant^{-1} (Fig. 3B), with the former three species also having the highest nodule number plant^{-1} (Fig. 4B). *A. linearis* also produced the largest nodules of 15 mg nodule^{-1} (Fig. 5B), which where greater than *V. oroboides* and *I. filifolia* with 7 and 4 mg nodule^{-1} respectively. In addition, *A. linearis* had the highest nodule to root FW ratio together with *B. bituminosa*, with both species more than five times greater than *V. oroboides* and *L. capensis* (Fig. 6B). *A. linearis* also accumulated significantly more N than any of the eight species shown in Table 2. However, shoot [N] was highest in *L. frutescens*, *O. striatum* and *O. fruticans* probably due to the developmental effect of very small plants and not because of high N_2-fixation (Vadez *et al.*, 1999; Christiansen & Graham, 2002).

2.4 DISCUSSION

In this study 18 indigenous CFR legumes species were grouped according to soil [P] of their natural habitat and assessed for differences in growth and N_2-

fixation at low P supply. There was no difference in growth and N_2-fixation between the low-P and high-P groups due mainly to the fact that within the group, variation amongst species was high. For instance, within the low-P group (Fig. 1B) *V. oroboides* had 16 times more total fresh biomass than *I. filifolia* and four times more biomass than *A. linearis* which in turn was double

Table 2. N parameters for CFR legumes grouped according to soil [P] groups and for individual species grown at 0.1μM P for 22 weeks. Means (n=7) with similar letters are not significantly different at **P < 0.01. (ns = not significant). Tissue analysis was not possible for 9 of the 18 species (Table 1) which were lost (burnt) in an oven failure during the drying process, and for *L. capensis* due to insufficient biomass.

	Shoot [N] (%)	Shoot N content (mg plant^{-1})
Soil [P] groups		
Low-P	1.29	1.019
High-P	1.56	0.587
P-Generalist	1.58	0.703
Unclassified	1.27	0.575
F-statistic $_{(3,48)}$	0.60 ns	0.26 ns
Species		
Aspalathus linearis	1.25bc	1.544a
Cyclopia genistoides	1.28b	0.719bc
Cyclopia intermedia	1.05c	0.496cd
Cyclopia subternata	1.15bc	0.942b
Lessertia frutescens	1.83a	0.455de
Otholobium fruticans	1.58a	0.703bc
Otholobium striatum	1.63a	0.288e
Indigofera filifolia	1.33b	0.493cde
F-statistic $_{(4,48)}$	11.80**	14.70**

than that of *P. sericea*. Similar variation was also evident, for example, in root to shoot FW ratio (Fig. 2B), nodule FW (Fig. 3B) and nodule size (Fig. 5B). The variation in total fresh biomass amongst low-P legume species may also reflect variation in N_2-fixation, with the species producing higher biomass being able to fix more N_2 than those with lower biomass. Numerous studies have similarly correlated superior biomass accumulation with high N_2-fixation (Pereira & Bliss, 1987; Vadez *et al.*, 1999; Christiansen & Graham, 2002).

Therefore the species within each group were too different to each other in plant and nodule growth, for any difference to be observed between the

groups. This species variation within each group could be due to the wide range of P (< 100 μg P g soil^{-1} for the low-P group and > 100 μg P g soil^{-1} for the high-P group) that was used in this study. For instance, the similarity between groups obtained in this study was different to Sanginga *et al.* (2000) who reported that low-P grouped legumes were superior N_2-fixers than high-P legumes at low P supply. However, in the Sanginga *et al.* (2000) study, the cowpea plants were first observed for a growth response to increased P supply in a pot experiment. Thereafter, four non-responding lines were grouped as low-P, and four responders were grouped as high-P, to examine growth at low P supply (0 kg P ha^{-1} added in low P field soil). Thus a finer scale classification of CFR legume species distribution and soil [P] may be needed to properly test the hypothesis that the low-P group would be superior N_2-fixers than the high-P group at low P supply.

However, the consistently superior nodulation and N_2-fixation by one low-P species *A. linearis* was apparent when species were ranked according to cumulative performance in SN_2F parameters. For instance, *A. linearis* had the largest nodules (Fig. 5B), the best root nodulation efficiency (Fig. 6B) and fixed the most N_2 with highest shoot N content of 1.54 mg N plant^{-1} compared to all the species tested (Table 2). This may explain why *A. linearis* was reported to nodulate and fix N_2 in field soil with low soil P (Muofhe & Dakora, 1999), and has its natural distribution restricted to the highly nutrient deficient soils of the Cedarberg Mountains (van der Bank *et al.*, 1999). The performance of *A. linearis* means that it may indeed be a model for further study (Muofhe & Dakora, 1999), similar to *A. mangium* (Ribet & Drevon, 1996) regarding adaptation for SN_2F at low P. In addition to *A. linearis*, *V. oroboides*, *P. calyptrata* and *C. genistoides* also ranked as the best N_2-fixing CFR legume species at low P. In contrast, *P. pinnata*, and *O. striatum* nodulated poorly with the lowest nodule FW, nodule size, nodule to root ratio, and fixed N_2.

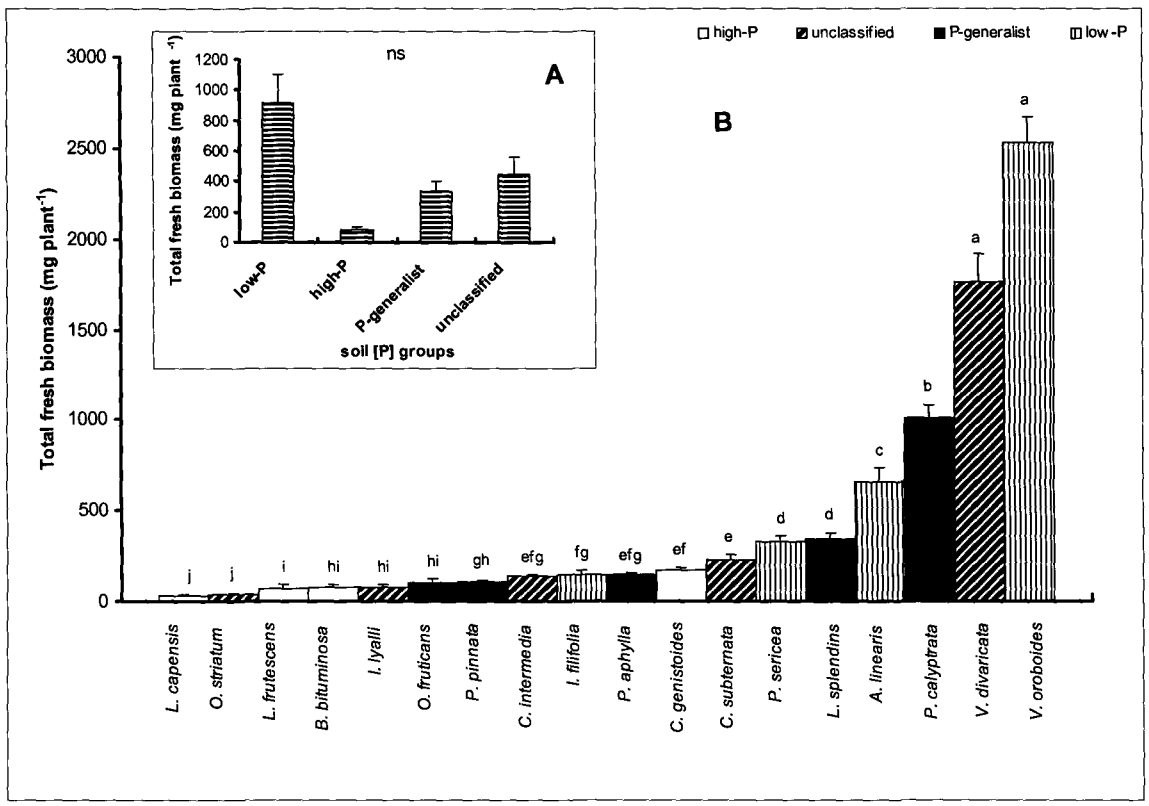


Figure 1A & 1B. 1A. Total fresh biomass of 18 CFR legume species grown at 0.1μM P for 22 weeks. 1B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 1A: $F_{3,108}$ 2.11; ns = not significant).

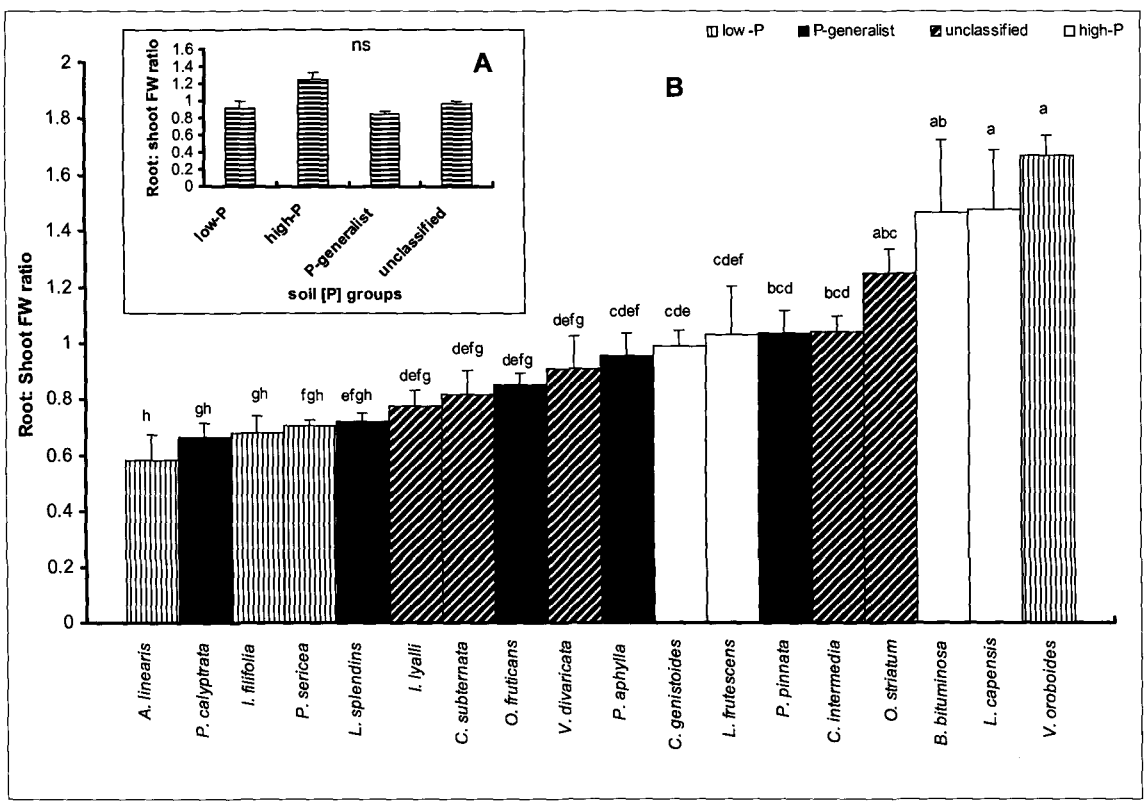


Figure 2A & 2B. 2A. Root to shoot fresh weight (FW) ratio of 18 CFR legume species grown at 0.1μM P for 22 weeks. 2B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 2A: $F_{3,108}$ 1.37; ns = not significant).

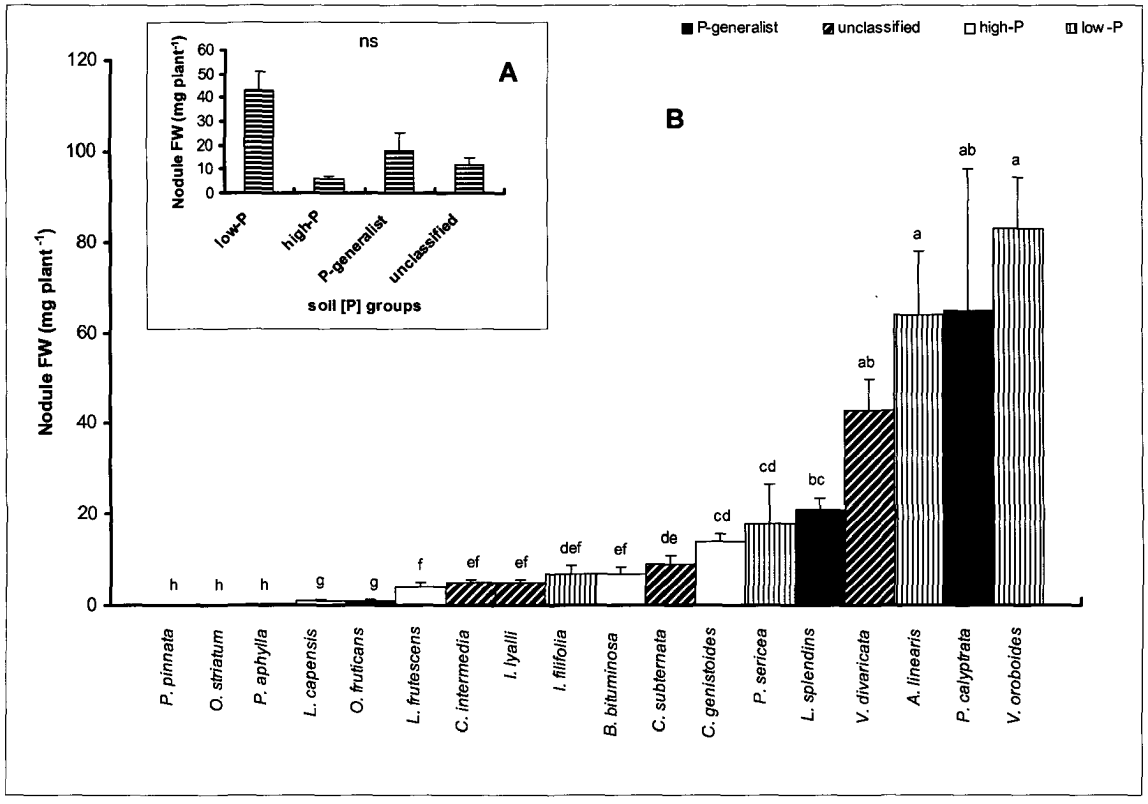


Figure 3A & 3B. 3A. Nodule fresh weight (FW) of 18 CFR legume species grown at 0.1µM P for 22 weeks. 3B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 3A: $F_{3,108}$ 1.35; ns= not significant).

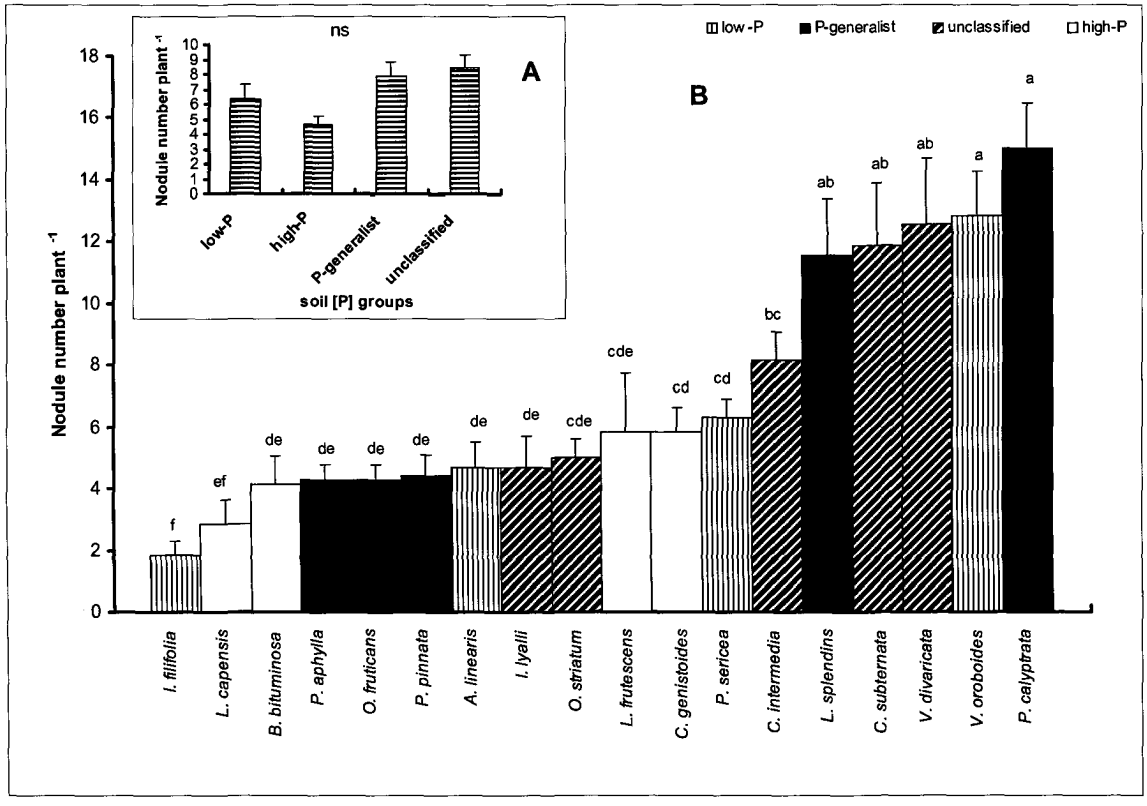


Figure 4A & 4B. 4A. Nodule number of 18 CFR legume species grown at 0.1µM P for 22 weeks. 4B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 4A: $F_{3,108}$ 0.88; ns = not significant).

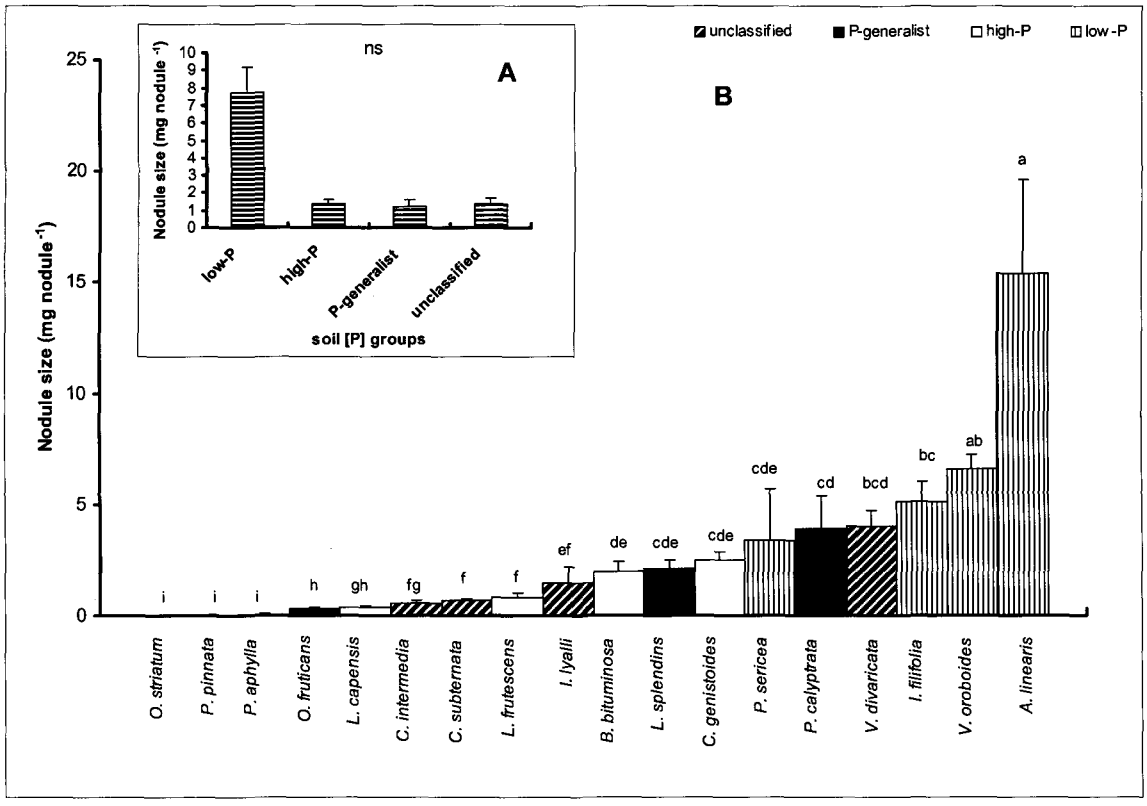


Figure 5A & 5B. 5A. Nodule size of 18 CFR legume species grown at 0.1µM P for 22 weeks. 5B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 5A: $F_{3,108}$ 2.65; ns = not significant)

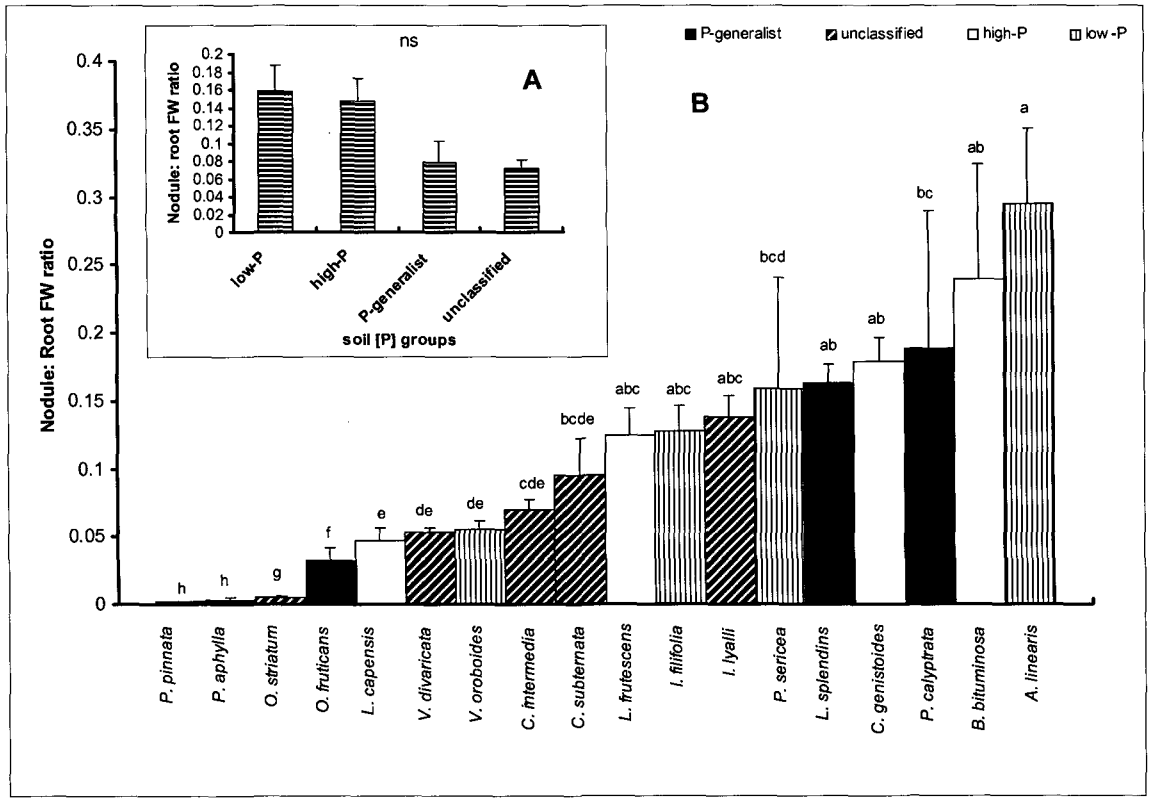


Figure 6A & 6B. 6A. Nodule to root fresh weight (FW) ratio of 18 CFR legume species grown at 0.1µM P for 22 weeks. 6B. Species were grouped as low-P, high-P, P-generalist, and unclassified. Bars are means ± SE. Different letters on bars indicate significantly different means at $P < 0.01$. (Fig. 6A: $F_{3,108}$ 1.91; ns = not significant).

2.5 CONCLUSION

Growth of N_2-fixing CFR legumes at 0.1 μM P showed that there was no difference in plant growth and symbiotic N_2-fixation between the low-P group and the high-P group. However, at a species level, *A. linearis* a low-P grouped legume, demonstrated the greatest capacity to nodulate and fix N_2 at low P, followed by *V. oroboides*, *P. calyptrata* and *C. genistoides*. In contrast, *P. pinnata*, *P. aphylla* and *O. striatum* nodulated poorly at 0.1 μM P supply.

CHAPTER THREE

DO N_2-FIXING LEGUMES INDIGENOUS TO THE LOW P SOILS OF THE CAPE FLORISTIC REGION, SA HAVE A LOW P REQUIREMENT?

3.1 INTRODUCTION

P and N most commonly limit plant growth and ecosystem productivity (Lambers *et al.*, 1998), however growth & SN_2F in legumes is most likely limited by the availability of nutrients other than N, particularly P. There is considerable evidence showing a positive response in growth & SN_2F with increased P supply such as increased plant growth, nodule growth, shoot N content and nitrogenase activity of nodules (Jakobsen, 1985; Olivera *et al.*, 2004; Israel, 1987; Crews, 1993). However most of these responses were obtained from legumes such as pea, bean, soybean and alfalfa which are crop species usually selected for high yield on fertilized soils that does not encourage nutrient use efficiency (Chapin, 1980). This is in contrast to wild legumes that are indigenous to infertile soils (Vitousek *et al.*, 2002). These legumes may be adapted for growth & SN_2F at low P supply, showing little or no increase in growth with P addition (Koide *et al.*, 1988; Sanginga *et al.*, 1995). Overall, the relationship between P supply and growth & SN_2F remains incompletely understood especially in wild legumes (Sprent, 1999).

The soils of the CFR are low in available P (Mitchell *et al.*, 1984; Witkowski & Mitchell, 1987) and indigenous CFR legumes such as *A. linearis, P. calyptrata* and *C. genistoides* have been observed to fix N_2 at a low P supply of 0.1 μM P (Chapter 1), while *P. pinnata* and *O. striatum* nodulated poorly at the same level of P. The CFR legumes such as *A. linearis, P. calyptrata* and *C. genistoides* may have evolved and adapted to low soil P conditions and are hypothesized to show a low P requirement for growth & SN_2F. Alternatively the growth & SN_2F process may be an inherently P demanding process with legumes requiring more P for growth & SN_2F regardless of low P adaptation.

To test whether N_2-fixing legumes have a low P requirement, Robson (1983) proposed that the nature of the interaction between legumes growing on SN_2F and N-fed in response to increasing P supply should be examined. By assessing whether the supply of P affects plant DM and N accumulation in SN_2F plants differently from the N-fed plants, it is possible to determine

whether growth & SN_2F in legumes actually requires more P (Robson, 1983; Israel, 1987). The assumption for a correct interpretation of the interaction is that plants in the two N treatments should have similar tissue [N] so that where the response is different it is due to P and not an artefact of N. A negative interaction between combined-N and P (when the rate of increase in DM and N accumulation in N-fed plants is less than SN_2F plants with increasing P supply) indicates that SN_2F plants require more P than N-fed plants, and that growth & SN_2F has a high P requirement. A positive interaction between combined-N and P (when N-fed plants increase DM and N accumulation more than SN_2F plants with increasing P supply) indicates that SN_2F plants require less P than N-fed plants, and that growth & SN_2F has a low P requirement. A zero interaction (a similar response to P fertilization in SN_2F and N-fed plants) indicates that both forms of N nutrition have the same external P requirement. The results of past interaction studies have been varied with some data showing that SN_2F legumes require more P than N-fed legumes (Israel, 1987; Sanginga *et al.*, 1989); others report a zero interaction (Ribet & Drevon, 1996; Redell *et al.*, 1997) while some interactions were positive (Robson *et al.*, 1981; Jakobsen, 1985; Pereira & Bliss, 1987). The findings of these studies are confounded by the fact that they were based on crop species such as soybean, pea, bean, clover; woody *Casuarina* species and with only one on a low P adapted species, *A. mangium*. Therefore the question of whether growth & SN_2F in legumes has a low or high P requirement remains unresolved, particularly in wild plants.

Furthermore, because P is essential for cellular metabolism in host plant growth (White & Hammond, 2008) as well as for the N_2-fixation process (Burris, 2000) it has been difficult to separate its direct effect upon nitrogenase activity and nodule function from an indirect effect mediated via changes in the host plant. Almeida *et al.* (2000) and Hartwig (1998) have proposed that nodule growth is inhibited by a N feedback mechanism associated with low demand when plant growth is constrained, and thus plant N requirement is reduced by low P supply. Jakobsen (1985) also concluded that reduced nodulation at low P levels was due to impaired photosynthesis and reduced C supply to nodules; while Redell *et al.* (1997) interpreted

maximum shoot growth at 50 μM P relative to maximum nodulation at the 25 μM P application to imply a higher P requirement for host plant growth and an indirect effect rather than a direct effect of P on nodule functioning. In contrast, by correlating increased nitrogenase activity with recovery from P stress, and decreased nitrogenase activity with the onset of P stress Israel (1993) suggested a direct effect of P upon nodule function. Similar direct effects of P upon nodule function have been proposed by Hellsten & Huss-Danell (2002) and Israel (1987) due to a higher increase in nodule growth relative to whole plant growth with increased P supply.

In this study it was hypothesized that N_2-fixing legumes indigenous to the low P soils of the CFR have a low P requirement for growth & SN_2F. The study objectives were to determine whether the N_2-fixing CFR plants require more P than the N-fed plants, to examine the effect of increasing P supply on nodulation and N_2-fixation in the N_2-fixing plants, and to determine whether the positive N_2-fixation response to P is because of a direct effect of P on nodule functioning or an indirect effect via changes in host plant growth. Thus, indigenous CFR legumes were grown in a glasshouse experiment, reliant either entirely on SN_2F for their N nutrition or N-fed and subjected to different levels of P supply.

3.2 MATERIALS AND METHODS

CFR legume species and P treatments

Three indigenous CFR legumes namely *A. linearis*, *C. genistoides,* and *P. calyptrata* previously observed to effectively nodulate and fix N_2 at low P (0.1 μM P) supply were grown in potted sand reliant either entirely on SN_2F for their N nutrition or supplied with 0.3 mM NH_4NO_3 and both N treatments received 0.1, 1.0, 10 and 100 μM P. Another two indigenous legumes *P. pinnata* and *O. striatum* observed to nodulate poorly at 0.1 μM P were also grown entirely on SN_2F for their N nutrition or supplied with 0.75 mM NH_4NO_3 with both N treatments receiving 50, 100, 200 and 400 μM P. The latter two

species received higher levels of N and P than the former three species because of their higher nutrient requirements observed in the experiment in Chapter 2.

Seed germination and plant culture

Seeds of plants were obtained from Silverhill Seeds, Claremont, Cape Town. Seeds of *A. linearis* were scarified in concentrated H_2SO_4 for ½ an hour, thoroughly rinsed in six changes of sterile distilled water and then soaked in water overnight to aid germination, while the seeds of *C. genistoides, P. calyptrata, P. pinnata* and *O. striatum* were only soaked overnight in boiling water. Seeds were then sown in seedling trays containing acid washed (0.1% HCl) silica sand and placed on trolleys in the glasshouse under natural light. After emergence, seedlings were inoculated with rhizobia isolated from the nodules of plants of the same species grown in Chapter 2 (see below) by applying rhizobia inoculum to the sand at the base of each seedling. The rhizbobia were effective at N_2-fixation because of the observed mass of nodules, plant DM and shoot [N] of SN_2F plants that grew vigourously without signs of N limitation. N-fed seedlings were similarly administered an equal amount of sterile yeast extract mannitol (YEM) solution (Vincent, 1970). Four weeks after emergence three seedlings were transplanted to 18 cm pots (later thinned to one seedling per pot) containing 3 kg of acid washed sand and inoculated again as above. For each treatment there were six replicates.

From the day of transplanting, the seedlings were fed with ¼ strength N-free Hoagland solution with P supplied as K_2HPO_4 (0.5 M) and KH_2PO_4 (0.5 M) adjusted to supply the appropriate P concentration per treatment. The amount of K_2SO_4 in the Hoagland solution was also adjusted accordingly to compensate for the 10 fold changes in K_2HPO_4 and KH_2PO_4. The nutrient solution was administered in 400 ml per pot, twice a week, and pots were flushed with 1 L tap water once a week to prevent accumulation of salts. For four weeks after transplanting, all plants received a start up N of 0.25 mM NH_4NO_3. Thereafter, N was not supplied to the inoculated plants so that

these plants were reliant solely on SN_2F. Plants grew during spring and summer on trolleys in the glasshouse under natural light and temperature.

Preparation of rhizobia inoculum

Broth cultures were prepared with rhizobia isolates from nodules of *A. linearis, Cyclopia subternata* for *C. genistoides* (Spriggs, 2004), *P. calyptrata, P. pinnata* and *O. striatum* plants. Using a sterile wire loop in a laminar flow, rhizobia cultures of each species growing on YEM agar (Vincent, 1970) were transferred into 200 ml sterile YEM in 250 ml conical flasks. Each flask was clearly labelled according to species and stoppered with its sterile cotton wool and tin foil. This broth culture was transferred to a rotary shaker at medium velocity in a 25˚C constant temperature room. The culture was incubated for five days until milky and turbid in appearance and then stored at 0˚C. Absorbance readings of the broth at 600 nm were obtained to indicate the concentration of rhizobia in the culture so that similar quantities of rhizobia were applied to each legume species.

Plant harvest and measurement of biomass

Seedlings were harvested 130 d after transplanting for assessment of plant and nodule growth and concentration of nutrients. Sand around the roots was removed by gently washing it off with water and each plant was separated into nodules, roots, and shoots. Nodules were counted for each plant. Plant material was dried in a forced draught oven at 60˚C to constant dry weight. After recording DM for each plant organ, the dried shoots were milled in a Wiley Mill using a 0.5 mm mesh (Arthur H. Thomas Co. Philadelphia, CA, USA) and nodule DM were milled in a steel ball mill (MM200, Retsch®, Haan, Germany) to a fine powder for N and P analysis.

Shoot N and P analysis

For N analysis about 2 mg of each shoot, root, and nodule sample was weighed into a tin foil cup on a Sartorius micro balance. The cups were then

folded to enclose the sample. The samples were combusted in a Flash EA 1112 series elemental analyzer (Thermo Finnigan, Italy). The resulting gases were automatically fed into a Delta Plus XP isotope ratio mass spectrometer (Thermo Electron, Germany), via a Conflo III gas control unit (Thermo Finnigan, Germany). The standards used to calibrate the results were Merck Gel, a proteinaceous gel produced by Merck and dried nasturtium leaves collected from Woodbine Lane, University of Cape Town campus. Shoot [N] was expressed as a percentage of shoot DM, from which total amount of N in the shoot was calculated by obtaining the product of [N] and DM. For P analysis, plant tissue samples were sent to BemLab (Pty) Ltd., Stellenbosch. Shoot and nodule P was determined by dry-ashing pulverized plant material at 480°C for 8 h, dissolving in HCl (Kalra, 1998) and analyzing using inductively coupled plasma atomic emission spectrometry (Varian Vista MPX ICP-AES; Varian, Mulgrave Australia).

Statistical analysis

To reduce inequality of variance in the raw data, all measurements were $\log_e$ transformed before statistical analysis. Data for each legume species was analyzed separately. The interaction between N-source and P levels in each species was assessed using Factorial ANOVA while N_2-fixation responses to P that occurred only in SN_2F plants was assessed using One-way ANOVA in the STATISTICA software package. Means that were significantly different at $P < 0.05$ were separated by Duncan's multiple range test.

3.3 RESULTS

Plant growth

In *P. pinnata* and *O. striatum,* both N sources increased total DM accumulation from 50 to 100 μM P and decreased DM with higher P supply, but DM accumulation in SN_2F plants was higher than N-fed plants at all P levels (Figs. 1A & 2A). From 50 to 100 μM P supply the increase in growth

response was the same for SN_2F and N-fed plants of *P. pinnata*, while N_2-fixing *O. striatum* plants increased growth almost two times more than the N-fed plants, at the same range of P supply.

In *C. genistoides* and *P. calyptrata*, there was no growth response to increasing P supply from 0.1 to 1 μM P in both N treatments (Figs. 3A & 5A respectively). However, in *C. genistoides*, the DM increase from 1 μM P to 10 μM P supply was six times greater in SN_2F plants than in N-fed plants (Fig. 3A). In contrast, the increase from 10 to 100 μM P was two times greater in the N-fed than in SN_2F plants. *P. calyptrata* N-fed plants responded to increased P supply from 1 to 10 μM P with 30% greater DM accumulation relative to the SN_2F plants and with 18 times more growth from 10 μM P to 100 μM P supply (Fig. 5A).

In *A. linearis*, there was no growth response to increasing P supply from 0.1 to 1 μM P in both N treatments (Fig. 4A), while only the N-fed plants increased growth, producing four times greater DM when P supply increased from 1 to 10 μM P, with no further change from 10 to 100 μM P supply.

Nitrogen and Phosphorous concentration and content in shoots

Nitrogen

In *P. pinnata*, N concentration was the same in both N treatments at 50 and 100 μM P supply, but lower in N-fed than SN_2F plants at 200 and 400 μM P (Fig. 1B). Although SN_2F plants accumulated more N than N-fed plants at all P levels, increasing P supply from 50 to 100 μM P increased N accumulation equally in both SN_2F and N-fed plants reaching a maximum at the 100 μM P (Fig. 1C). However, in SN_2F plants of *O. striatum*, the increase in N accumulation from 50 to 100 μM P was over three times greater than the N-fed plants (Fig. 2C). This was due to a higher [N] in the SN_2F *O. striatum* compared to the N-fed plants. For instance, at 50 μM P, the shoot of N-fed *O. striatum* plants contained 2.7% N compared to 2% N in the SN_2F shoot (Fig.

2B). However, the shoot N of the 100 μM P N-fed plants was 1.4% compared to 2% N in SN_2F plants. The lower shoot [N] of the SN_2F plants at 50 μM P was a dilution effect associated with higher biomass in SN_2F plants (Fig. 2A), but the lower shoot [N] in the N-fed plants at 100 μM P was due to actual lower N in the tissue and not a developmental effect of plant size.

With P supply increasing from 1 to 10 μM P the shoot [N] of SN_2F plants of *C. genistoides* and *P. calyptrata* was lower than the N-fed counterparts (Figs. 3B & 5B respectively) probably due to dilution effect because the N content was higher in the SN_2F than N-fed plants (Figs. 3C & 5C respectively). However at 100 μM P, the similar shoot [N] in *C. genistoides* and higher shoot [N] in *P. calyptrata* N-fed plants relative to the SN_2F plants (Figs. 3B & 5B) that corresponded with higher total biomass relative to the SN_2F plants receiving 100 μM P (Figs. 3A & 5A) suggests that the N-fed plants had access to more N. When P supply was changed from 1 to 10 μM P, SN_2F plants of *C. genistoides* increased N_2 accumulation by 2.5 times but the increase in the N-fed treatment was not significant ($P < 0.05$) (Fig. 3C). In contrast, a further 100 μM P supply elicited a 50% greater increase in the N-fed plants relative to the SN_2F plants. In *P. calyptrata*, however, N-fed plants were more responsive, accumulating nearly two times more N than the SN_2F plants when P supply was increased from 1 to 10 μM P, while only N-fed plants accumulated more N with a further increase to 100 μM P supply (Fig. 5C).

The similar and lower [N] of the N-fed *A. linearis* plants at 10 and 100 μM P (Fig. 4B) relative to the SN_2F plants is due to the diluting effect of higher biomass (Fig. 4A) in the N-fed plants because shoot N content of the N-fed plants was higher than the SN_2F plants at all levels of P supply (Fig. 4C). When P supply was increased from 1 to 10 μM P, N-fed *A. linearis* plants accumulated two times more N whereas there was no response in the SN_2F plants.

Phosphorous

Plants of *P. pinnata* and *O. striatum* exposed to both N treatments increased tissue [P] (Figs. 1D & 2D) and P uptake (Figs. 1E & 2E) with increasing P supply from 50 to 400 μM P. The concentration of P in SN_2F plants of both species was consistently lower than the N-fed plants at all levels of P supply probably due to the dilution effect. P uptake was similar in both N treatments in *P. pinnata* (Fig. 1E) and *O. striatum* (Fig. 2E) except that in the former species, SN_2F plants accumulated nearly twice more P than N-fed plants at 400 μM P supply.

Similarly, shoot [P] increased with increasing P supply from 0.1 to 100 μM P in SN_2F plants of *C. genistoides* (Fig. 3D), and in both N treatments of *P. calyptrata* (Fig. 5D). However, the concentration of P in SN_2F plants of *P. calyptrata* was consistently higher than the [P] in the respective N-fed plants at all levels of P supply. In addition, SN_2F *P. calyptrata* plants at 100 μM P contained eight times more P in their shoots than at 10 μM P (Fig. 5E).

Shoot [P] increased with increasing P supply from 0.1 to 100 μM P in both N treatments of *A. linearis* (Fig. 4D). The concentration of P in SN_2F *A. linearis* plants was also higher than the [P] in the respective N-fed plants at all levels of P supply. With the 100 μM P application, *A. linearis* N-fed plants had accumulated 11 times more P than plants at 10 μM P (Fig. 4E).

N_2-fixation parameters

Nodule DM

Nodule DM increased with increasing P supply in both *P. pinnata* and *O. striatum* up to 200 μM P but decreased in *O. striatum* with 400 μM P supply (Fig. 6A). In *C. genistoides* and *P. calyptrata* nodule DM increased by 40% and 200% respectively when P supply increased from 1 to 10 μM P, while *C. genistoides* produced seven times more nodule DM with 100 μM P compared

to 10 μM P supply (Fig. 6B). In contrast, *A. linearis* plants decreased nodule DM production with increased P supply (Fig. 6B).

Nodule DM to whole plant DM ratio

The nodule DM to whole plant DM ratio increased with increasing P supply in both *P. pinnata* and *O. striatum* to a maximum when P supply was 200 and 400 μM P respectively (Fig. 6C). Similarly, the nodule DM to plant DM ratio increased in *C. genistoides* from 1 to 100 μM P supply (Fig. 6D), but the ratio was not altered with increased P supply in *P. calyptrata* and *A. linearis*.

Shoot N to nodule DM ratio

In both *P. pinnata* and *O. striatum* the shoot N to nodule DM ratio, an estimate of nodule N_2-fixing efficiency (Redell *et al.*, 1997; Vadez *et al.*, 1999), decreased with increasing P supply up to 200 μM P but did not change thereafter (Table 1). The shoot N to nodule DM ratio did not change with increasing P in *P. calyptrata* and *C. genistoides* but did increase in *A. linearis* (Fig. 6E), due to poor nodulation (Fig. 6B).

Nodule [P]

Nodule [P] in both *P. pinnata* and *O. striatum* and in *P. calyptrata* increased with increasing P supply, although the effect of P-level treatments varied with species (Table 1). Specifically, the nodule [P] in *P. pinnata and O. striatum* at 100 μM P, where maximum biomass accumulation was recorded (Figs. 1A & 2A respectively), was about three times more than the shoot [P] (Table 1). In *P calyptrata*, nodule [P] was similar to the shoot [P] at 10 μM P (Table 1) where highest biomass yield was obtained (Fig. 5A).

Table 1. Effect of P supply on P concentration in the shoot and nodules, and on the shoot N to nodule dry matter (DM) ratio in inoculated *P. pinnata, O. striatum* and *P. calyptrata*. Data for each legumes species was analyzed separately. Two-way ANOVA assessed the effect of P levels and P concentration in organs (shoot and nodule) whereas one-way ANOVA assessed the effect of P levels on shoot N: nodule DM ratio. Means followed by the same letter are not significantly different at ***P < 0.001. (ns = not significant). Nodule [P] was not determined for *C. genistoides* and *A. linearis* due to insufficient nodule biomass.

Species	P-levels (μM P)	Shoot [P]	Nodule [P]	Shoot N: Nodule DM (mg Shoot N mg nodule DM^{-1})
P. pinnata	50	0.057d	0.195b	0.97a
	100	0.060d	0.191b	0.75b
	200	0.155c	0.287a	0.64c
	400	0.308a	0.310a	0.57c
F-statistic			$F_{(3,40)}$ 99.77***	$F_{(3,20)}$ 31.67***
O. striatum	50	0.062f	0.130e	0.37a
	100	0.063f	0.180cd	0.34a
	200	0.165d	0.227bc	0.29b
	400	0.538a	0.280b	0.28b
F-statistic			$F_{(3,40)}$ 35.66***	$F_{(3,20)}$ 12.45***
P. calyptrata	1	0.043c	0.031d	0.099
	10	0.073b	0.065bc	0.096
	100	0.542a	0.082b	0.097
F-statistic			$F_{(2,30)}$ 16.94***	ns

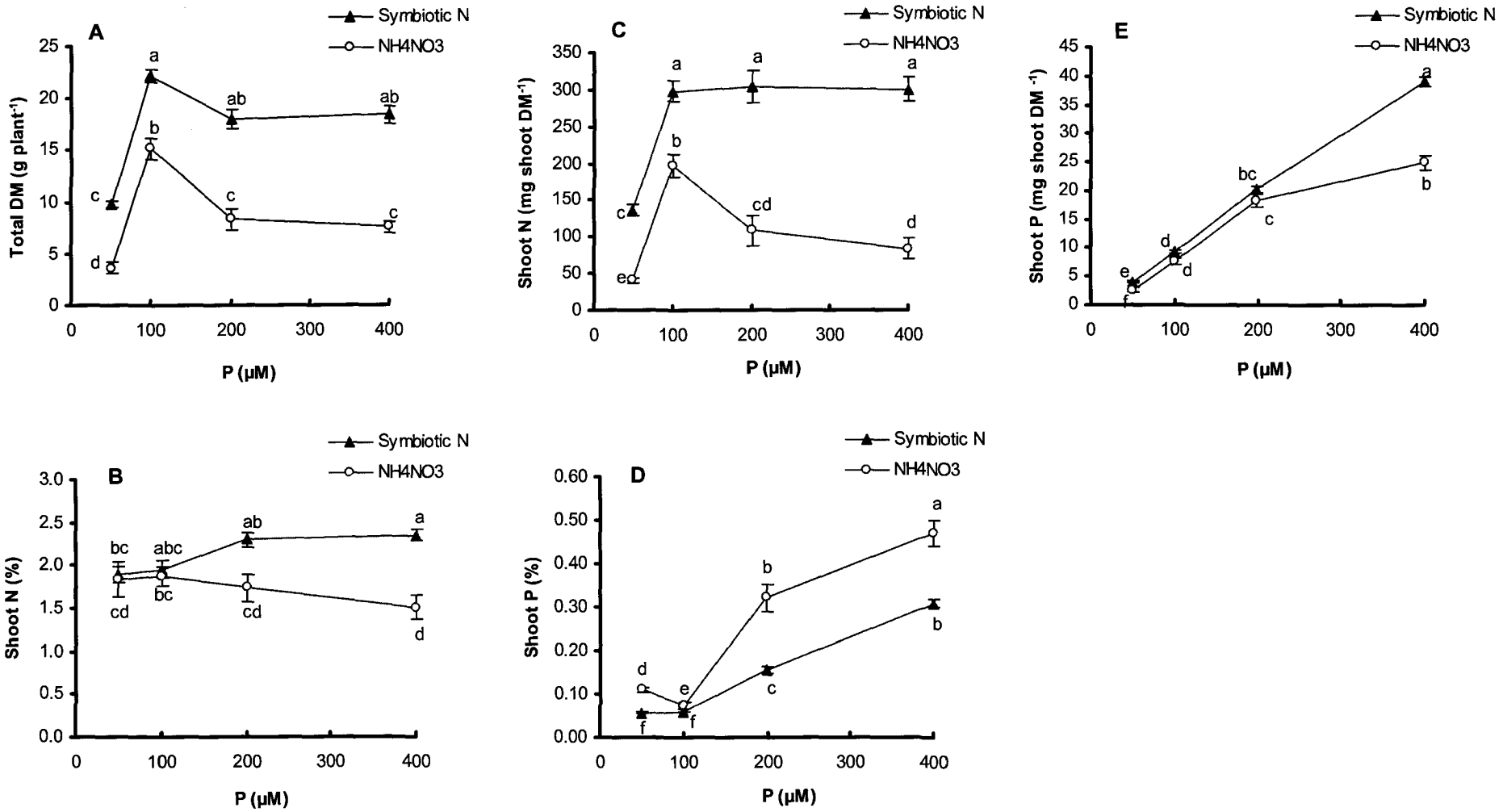


Figure 1 (A – E). Effect of P supply and N source on a) Total dry matter (DM), b) Shoot [N], c) Shoot N content, d) Shoot [P] and e) Shoot P content in *P. pinnata*. Means ± SE of 6 replicates. Different letters indicate significantly different means at $P < 0.05$.

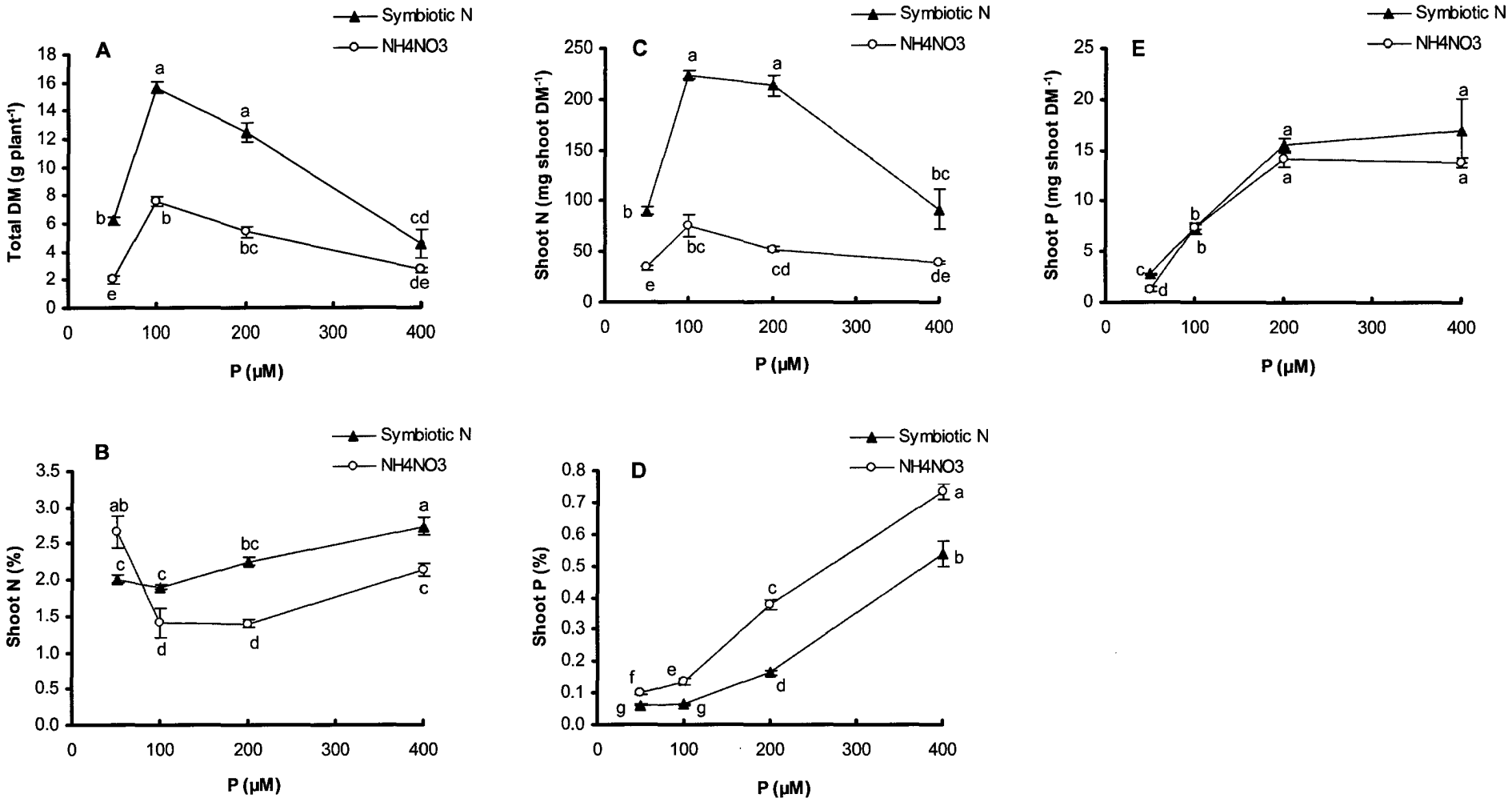


Figure 2 (A – E). Effect of P supply and N source on a) Total dry matter (DM), b) Shoot [N], c) Shoot N content, d) Shoot [P] and e) Shoot P content in *O. striatum*. Means ± SE of 6 replicates. Different letters indicate significantly different means at $P < 0.05$.

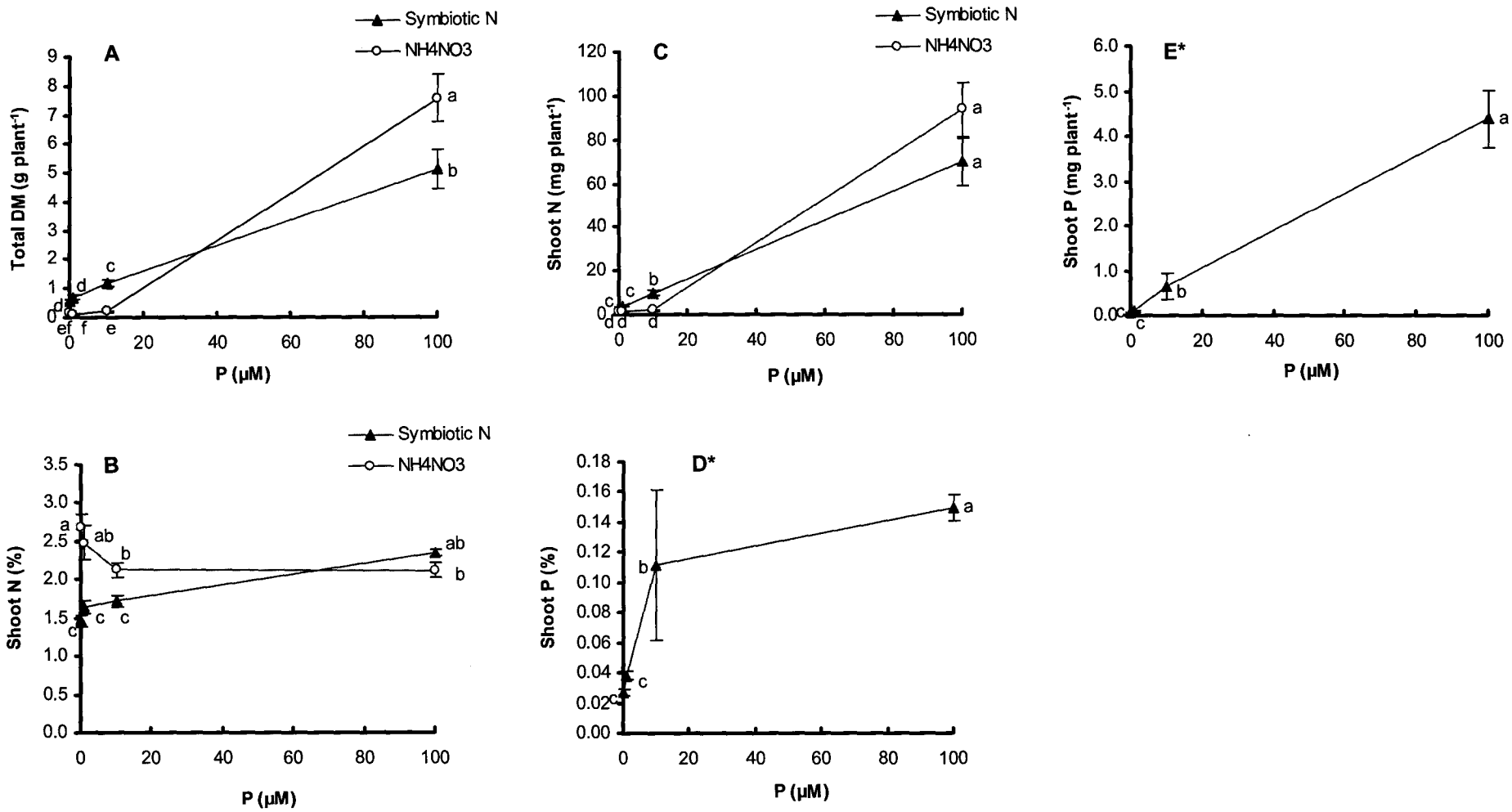


Figure 3 (A – E). Effect of P supply and N source on a) Total dry matter (DM), b) Shoot [N], c) Shoot N content, and effect of P supply on d) Shoot [P] and e) Shoot P content in *C. genistoides*. Means ± SE of 6 replicates. Different letters indicate significantly different means at $P < 0.05$. *Shoot P was not determined for N-fed 0.1 to 10 µM P supplied plants due to insufficient DM.

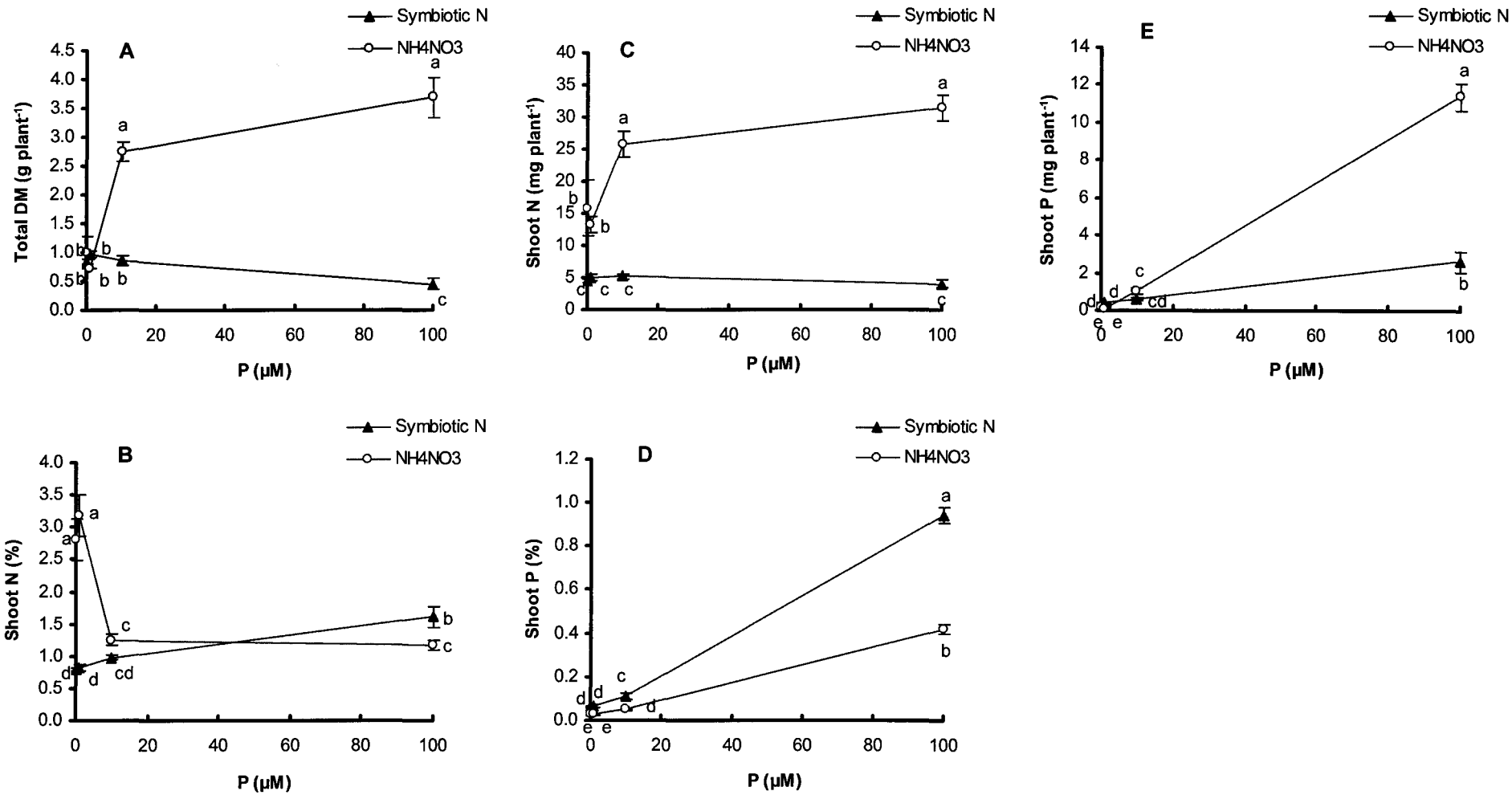


Figure 4 (A – E). Effect of P supply and N source on a) Total dry matter (DM), b) Shoot [N], c) Shoot N content, d) Shoot [P] and e) Shoot P content in *A. linearis*. Means ± SE of 6 replicates. Different letters indicate significantly different means at $P < 0.05$.

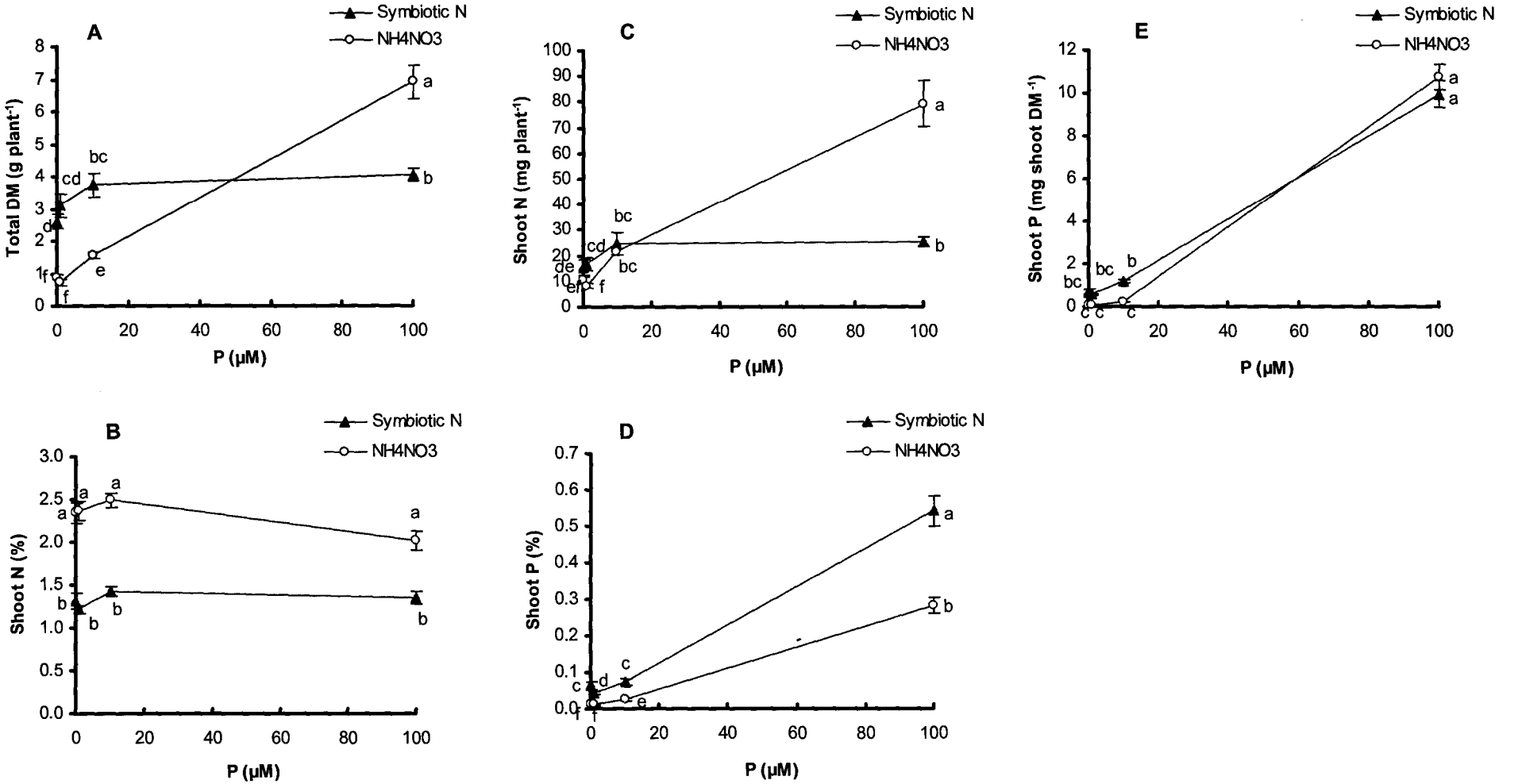


Figure 5 (A – E). Effect of P supply and N source on a) Total dry matter (DM), b) Shoot [N], c) Shoot N content, d) Shoot [P] and e) Shoot P content in *P. calyptrata*. Means ± SE of 6 replicates. Different letters indicate significantly different means at $P < 0.05$.

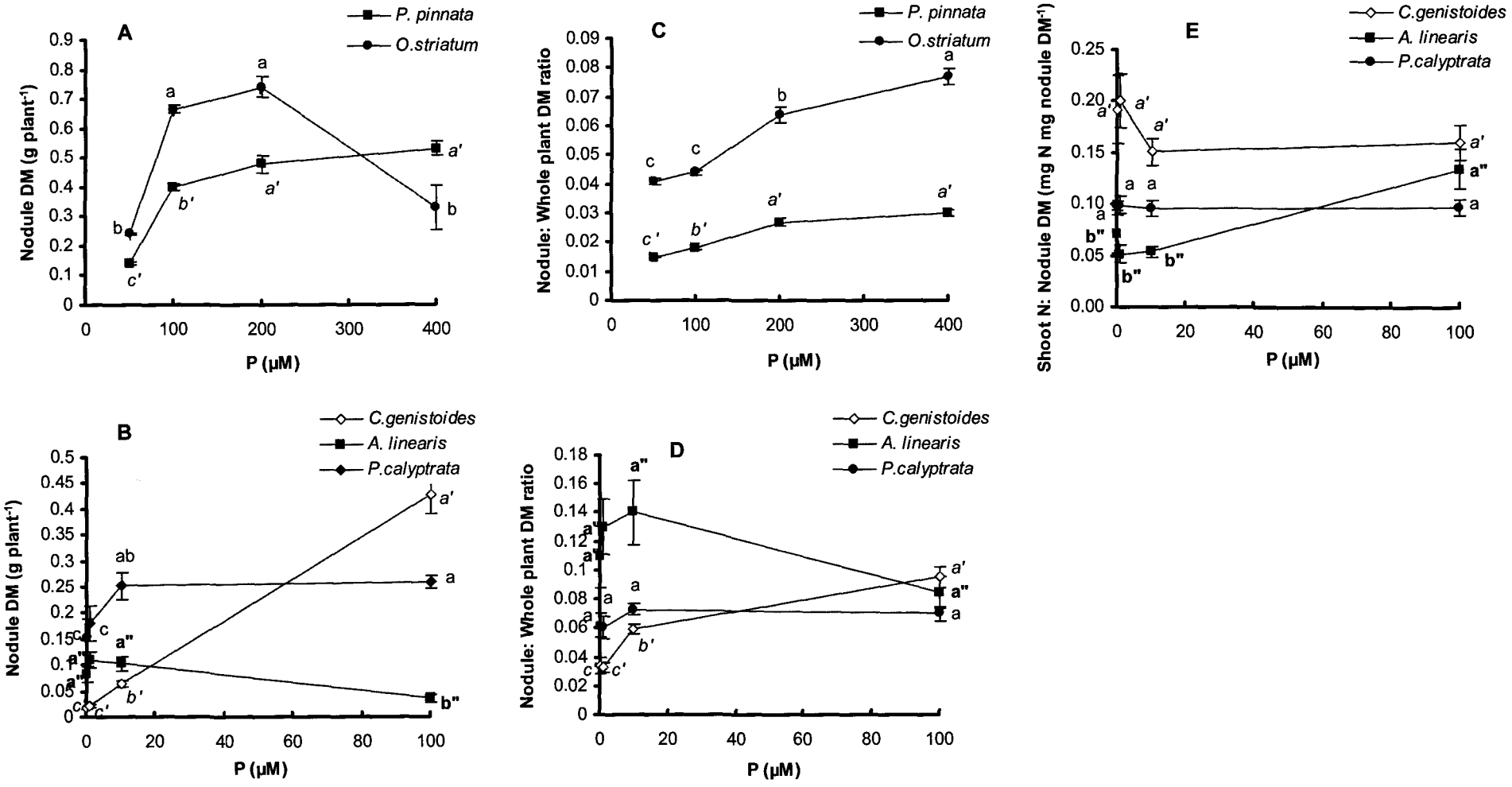


Figure 6 (A – E). Effect of P supply on a) & b) Nodule dry matter (DM), c) & d) Nodule to whole plant DM ratio and e) Shoot N to nodule DM ratio in five CFR legume species. Means ± SE of 6 replicates. Different letters indicate significantly different means for each species at $P < 0.05$.

3.4 DISCUSSION

N X P interaction

In this study increasing P supply improved plant growth and N accumulation in all species and in both N treatments to varying degrees except the SN_2F plants of *A. linearis*, possibly due to inadequate nodule formation and hence low N supply. However, the N-fed *A. linearis* yielded highest biomass and N accumulation at 10 μM P but did not increase further with supply of 100 μM P. The observed highest biomass and shoot N content at 10 μM P suggests that *A. linearis* is adapted to low P similar to CFR Proteaceae cultivars *Leucadendron* and *Leucospermum* (Hawkins *et al.*, 2006) where maximum growth was also at 10 μM P. However, the interaction of N and P as well as the nodulation response to increased P supply was not assessed in *A. linearis* because of the observed absence of nodulation and SN_2F.

The N X P interaction showed that *P. calyptrata* had a low P requirement for growth & SN_2F due to a positive interaction between combined-N and P from 1 to 10 μM P supply. This positive interaction was due to a 30% greater DM increase (Fig. 5A) and twice the N accumulation response (Fig. 5C) in the N-fed plants relative to the SN_2F plants when P supply was increased from 1 to 10 μM P. The positive interaction suggests a low P requirement for growth & SN_2F in *P. calyptrata*, a result similar to the findings of Robson *et al.* (1981), Jakobsen (1985), and Pereira & Bliss (1987) who assessed the low P adapted *P. vulgaris* cultivar *Puebla-152.*

In contrast, a negative interaction between N and P observed in *C. genistoides* showed that N_2-fixing plants had a high P requirement. This was because in SN_2F *C. genistoides* plants, there was a six times greater increase in total DM (Fig. 3A), and higher N accumulation (Fig. 3C), between 1 and 10 μM P supply relative to N-fed plants. This result is similar to the observations on symbiotic *G. max* and *C. equisitifolia* (Israel, 1987; Sanginga *et al.*, 1989). Furthermore, when P shows a negative interaction with combined-N on

legume growth, increasing its supply should also increase shoot [N] in the SN_2F plants (Robson, 1983; Israel, 1987). This did occur in the SN_2F *C. genistoides* plants, with shoot [N] concentration increasing when P supply was increased (Fig. 3B).

In *P. pinnata* however, SN_2F and N-fed plants responded equally between 50 and 100 μM P supply in DM (Fig. 1A) and N accumulation (Fig. 1C). This response indicates a zero interaction between combined-N and P and signifies that in *P. pinnata*, the external P requirement for fixed-N_2 and combined-N acquisition is the same. This result is similar to the low P tolerant *A. mangium* (Ribet & Drevon, 1996) and the *Frankia* inoculated *C. cunninghamiana* seedlings (Redell *et al.*, 1997).

It is notable that the different P requirements of the three N_2-fixing CFR species included the range of P requirements reported by previous studies on species as diverse as crop legumes such as pea, clover and soybean, *Frankia* inoculated *Casuarina* spp. and the low P adapted *Acacia* sp. This variation in P requirement within the CFR species may reflect the high level of soil [P] variation (Mitchell *et al.*, 1984) and diversity in nutrient use and acquisition strategies amongst the indigenous N_2-fixing legumes prevalent in the CFR. The low P requirement for growth & SN_2F in *P. calyptrata* and the high P requirement for growth & SN_2F in *C. genistoides* is consistent with their ecological distribution in the CFR. Thus, *C. genistoides* grows in areas with relatively high soil P levels due to its high P requirement, whereas *P. calyptrata* (a P-generalist) is found in both low and high P soils (Table 1, Chapter 1) because of its ability to tolerate low levels of P in the soil.

There was also clearly a greater DM increase in the N-fed *C. genistoides* and *P. calyptrata* plants relative to the SN_2F plants when P was increased from 10 to 100 μM P supply (Figs. 3A & 5A). This is actually a false positive interaction associated with higher N availability in the N-fed plants than the SN_2F plants (Robson, 1983; Israel, 1987), because unexpectedly, of the N-fed *C. genistoides* and *P. calyptrata* plants, only those receiving 100 μM P

nodulated (0.66 and 0.34 g nodule DM plant^{-1} respectively), and therefore received extra N from N_2-fixation. Similarly, the greater total plant DM and N accumulation response of SN_2F *O. striatum* from 50 to 100 μM P supply (Figs. 2A & 2C), relative to their N-fed counterparts, was actually a false negative interaction due to the lower shoot [N] of the N-fed plants that may have restricted their growth. These observations show that consideration of both tissue concentration of N and biomass (i.e. the dilution effect) is critical in the interpretation of interaction studies comparing the requirement of a nutrient (in this case P) in symbiotic and N-fed legumes.

Effects of P supply on nodule N_2-fixing efficiency

In plants of *P. pinnata*, the ratio of shoot N to nodule DM, a measure of the N_2-fixing efficiency of nodules (Redell *et al.*, 1997; Vadez *et al.*, 1999), decreased with increasing supply of P (Table 1), a result similar to reports on *A. mangium* and white lupin (Ribet & Drevon, 1996; Schulze *et al.*, 2006). In contrast, Redell *et al.* (1997) reported increasing shoot N to nodule DM ratio with increasing P supply in C. *cunninghamiana*. The negative correlation (R^2 = 0.71 for the *P. pinnata* plants) between nodule [P] and the ratio of shoot N to nodule DM means that the most efficient N_2-fixing nodules were those with lower [P] obtained at low P supply. For example, plants receiving 50 μM P with a nodule [P] of 0.20% supported N_2-fixation of 0.97 mg shoot N mg nodule DM^{-1} while for those at 100 μM P and with nodule [P] of 0.20%, N_2-fixation was significantly lower ($P < 0.001$) at 0.75 mg shoot N mg nodule DM^{-1}. This observation is similar to that of Vadez *et al.* (1999) who demonstrated high PUE in nodules of low P tolerant wild bean genotypes at low P supply. Therefore in *P. pinnata*, nodules at lower P supply were able to use internal P more efficiently for N_2-fixation than nodules at higher levels of P supply. However, nodule efficiency was unchanged in *P. calyptrata* at 0.10 mg shoot N mg nodule DM^{-1} (Fig. 6E) with increasing P supply, showing that the additional P did not alter the functioning of the nodules which is probably a unique characteristic of a P-generalist (Chapter 1).

The relative effects of P supply on N_2-fixation and host plant growth

All the N_2-fixing legume species, except *A. linearis*, increased nodule DM and total DM with increased levels of P supply. This is typical of N_2-fixing legume responses to P fertilization because P is required for both the process of N_2-fixation and host plant growth (Pereira & Bliss, 1987; Hellsten & Huss-Danell, 2000; Araujo *et al.*, 2008). However, there is uncertainty as to whether the effect of P upon increased N_2-fixation is directly on nodule growth and function (Sa & Israel, 1991; Hellsten & Huss-Danell, 2000) or indirectly mediated through increased supply of photosynthate (Jakobsen, 1985; Redell *et al.*, 1997) or through high plant N demand caused by increased shoot growth (Hartwig, 1998; Almeida *et al.*, 2000).

Assessment of the relative effects of P supply on nodulation and N_2-fixation in comparison to plant growth showed a higher P requirement for N_2-fixation than host plant growth in *P. pinnata* and *O. striatum*, and indicates that P may affect nodule functioning directly. This observation is based on the result that although both nodule growth and plant growth increased with P fertilization, the external P requirement for highest nodule DM (Fig. 6A) and nodule DM to whole plant DM ratio (Fig. 6C) was 200 μM P which was higher than the P requirement for highest plant DM which occurred at 100 μM P (Figs. 1A & 2A). This was in contrast to the P requirement of *C. cunninghamiana* reported in Redell *et al.* (1997) where the nodulation parameters such as nodule DM, nodule number, nodule size and nodule DM to whole plant DM ratio reached their maximum between 10 to 50 μM P supply which was below that required for maximum host plant growth between 50 to 100 μM. Therefore, Redell *et al.* (1997) concluded that the P requirement for N_2-fixation was lower than the P requirement for host plant growth. However, a similar assessment on the relative effects of P in growth & SN_2F was not possible for *C. genistoides* and *P. calyptrata* because nodulation (Fig. 6B) and plant growth (Figs. 3A & 5A) did not reach a clear maximum with 100 μM P supply. In addition, in *P. pinnata* and *O. striatum* the nodule [P] at 100 μM P where maximum DM yield was observed was about three times more than shoot [P] (Table 1). A higher

nodule [P] than shoot [P] has also been reported in clover (Hogh-Jensen *et al.*, 2002), white lupin (Schulze *et al.*, 2006) and bean nodules (Vadez *et al.*, 1999; Christiansen & Graham, 2002; Araujo *et al.*, 2008) and indicates that nodules have a higher demand for P than the shoot for effective functioning (Marschner, 1995; Vance *et al.*, 2000).

Furthermore, the ratio of nodule to whole plant DM increased with increasing P supply in *P. pinnata, O. striatum* (Fig. 6C) and *C. genistoides* (Fig. 6D) but remained the same in *P. calyptrata* (Fig. 6D). The increase in this ratio in the three species suggests that there was greater response of nodule growth relative to host plant growth with increased P supply which also implies a higher P requirement for nodule functioning (direct effect) than host plant growth (indirect effect), a result consistent with several reports (Israel, 1987; Ribet & Drevon, 1996; Hellsten & Huss-Danell, 2000). However, in plants of *P. calyptrata,* the ratio of nodule to whole plant DM was not altered with increasing P supply and nodule [P] was similar to shoot [P] at 10 μM P where maximum DM accumulation in SN_2F plants was obtained. This suggests that in *P. calyptrata*, nodule function and host plant growth were similarly affected by P.

Effect of P supply on shoot P concentration in CFR legumes

The effect of increasing P supply also showed that the CFR legumes observed in this study have physiological traits such as high PUE and poor down-regulation of P uptake that are typical of plants from low P soils. For example, in the SN_2F plants, the shoot [P] associated with maximum plant growth was 0.06% for *P. pinnata* and *O. striatum*, 0.15% for *C. genistoides* at 100 μM P supply and 0.07% for *P. calyptrata* at 10 μM P. In the N-fed plants receiving 100 μM P, *P. pinnata*, *O. striatum*, *C. genistoides* and *P. calyptrata* plants had shoot [P] of 0.08, 0.14, 0.15 and 0.28% P respectively at maximum plant growth, while the N-fed *A. linearis* growing optimally at 10 μM P had a shoot [P] of 0.05%. The shoot [P] values compare favourably with shoot [P] of 0.15 – 0.20% P for optimum growth in two native Australian low P adapted

Caustis cultivars (Gikaara *et al.*, 2004). Furthermore the shoot [P] for *A. linearis* (0.05%) is comparable to *Leucadendron* and *Leucospermum* (Hawkins *et al.*, 2006) which also showed low shoot [P] of 0.08% and 0.06% respectively at maximum growth. These tissue concentrations are typical of plants from low P nutrient poor soils because standard tissue P concentrations in healthy well fertilized crop plants approximate 0.4 – 1.5% (White & Hammond, 2008).

Furthermore, the growth response of *O. striatum* and *P. pinnata* SN_2F plants from 200 to 400 μM P was very different, revealing variation in susceptibility to P toxicity. While there were non-significant ($P < 0.05$) decreases in both species at 200 μM P, biomass accumulation did not decrease for *P. pinnata* at 400 μM P (Fig. 1A) in contrast to *O. striatum* which showed a significant ($P < 0.05$) decline (Fig. 2A). The shoot [P] of *O. striatum* at 400 μM P was 0.54% and clearly toxic to growth due to noticeable grey necrosis at the leaf tips (Hawkins *et al.*, 2006) and with plants unhealthier and stunted compared to those at 100 μM P supply. However, *P. pinnata* was able to maintain a lower shoot [P] of 0.31% at 400 μM P with growth not adversely affected. Thus plants of *P. pinnata* showed characteristics of tolerant species at relatively higher P levels.

Although biomass accumulation of the N-fed *A. linearis* from 10 to 100 μM P supply was similar (Fig. 4A), the amount of P in the 100 μM P supplied *A. linearis* was 11 times greater than at 10 μM P (Fig. 4E), demonstrating a weak capacity to down-regulate P uptake at higher levels of P supply (Shane *et al.*, 2008). The inability to down-regulate P uptake, also evident in SN_2F *P. calyptrata* plants at 100 μM P supply (Figs. 5A & 5D), *P. pinnata* (Figs. 1A & 1D) and *O. striatum* (Figs. 2A & 2D), is a typical trait of plants indigenous to low P soils that may lead to P toxicity when plants are exposed to relatively higher levels of P supply (Gikaara *et al.*, 2004; Shane *et al.*, 2008).

3.5 CONCLUSION

The results of this study indicated that the P requirement of the N_2-fixing CFR legumes varied with species. The N x P interaction showed that plants of *P. calptrata* have a low P requirement for growth & SN_2F due to the positive interaction observed between 1 and 10 μM P supply. Furthermore, *P. calyptrata* plants showed that nodulation and N_2-fixation does not require more P than the host plant growth because the relative effects of P supply on N_2-fixation parameters and host plant growth was the same. In contrast, the negative interaction in plants of *C. genistoides* indicated a high P requirement for growth & SN_2F, and there was a direct effect of P on nodule functioning rather than indirectly mediated via changes in the host plant growth in this species as well as in *P. pinnata* and *O. striatum*. The observed highest biomass yield and shoot N content at 10 μM P supply indicated that *A. linearis* is adapted to the low P soils of the CFR.

CHAPTER FOUR

THE PHYLOGENETIC RELATIONSHIP OF RHIZOBIA ISOLATES AND SOIL P LEVELS IN THE CAPE FLORISTIC REGION, SA

4.1 INTRODUCTION

Rhizobia is a collective term for the unicellular bacteria that nodulate and fix N_2 in symbiosis only with plants in the family Leguminosae, and *Parasponia* in the family Ulmaceae (Willems, 2006; Sprent, 2007). While the domain Proteobacteria has five subdivisions (α, β, δ, γ, ε) (Moulin *et al.*, 2001), rhizobia were initially restricted to the family Rhizobiaceae (Young & Haukka, 1996) within the α-subclass. Recently, in addition to the traditional Rhizobiaceae genera of *Rhizobium*, *Bradyrhizobium, Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium*, other non-Rhizobiaceae α-Proteobacteria rhizobia genera such as *Methylobacterium*, *Devosia*, *Ochrobactrum* and *Phyllobacterium* have been discovered (Moulin *et al.*, 2001; Willems, 2006) together with the genera *Burkholderia*, *Ralstonia* and *Cupriavidus* in the β-subclass of Proteobacteria (Hung *et al.*, 2005; Willems, 2006). Benhizia *et al.* (2004) have also reported nodulating bacteria from the γ-Proteobacteria lineage isolated from nodules of three wild Mediterranean *Hedysarum* legumes, namely *H. carnosum*, *H. spinosissimum* and *H. pallidum*. The trends in rhizobial studies (Willems, 2006) show that the endosymbionts of only 10% of the 750 legume genera have been fully characterized (Moulin *et al.*, 2001) so more phylogenetically unique rhizobia are expected to be discovered.

Some of the first rhizobia to be isolated from SA soils were *Bradyrhizobium* sp. from the nodules of indigenous CFR *Aspalathus* and *Rafnia* legume spp. (Deschodt & Strijdom, 1976). Recently, an increased number of research projects have investigated the diversity of root nodule bacteria associated with legumes species growing in SA soils. These studies include those of Kock (2004), Spriggs (2004), Le Roux (2003), Phalane (2008) and Joubert (2002) who isolated rhizobia from the nodules of indigenous *Cyclopia*, *Lotonis*, *Lebeckia* and exotic *Acacia* species. The new observations revealed that a diversity of rhizobia symbionts exist in SA soils, including those from the Rhizobiaceae genera found in all studies, and *Burkholderia* rhizobia from *Cyclopia*, *Lotonis* and *Lebeckia* nodules.

Lafay & Burdon (1998) have noted that too few studies aimed to identify the diversity of bacterial symbionts in their natural environment which was surprising given the diversity and dominance of legume plants in their ecosystems. In addition, studies that looked for patterns between the rhizobia phylogeny and their geographic distribution or ecological adaptation are limited. Some studies however, indicate that rhizobia strains do not form phylogenetic affiliations according to their geography (Zhang *et al.*, 1991; Lafay & Burdon, 1998; Gao *et al.*, 2001). In contrast, Barnet & Catt (1991) found that *Rhizobium* and *Bradyrhizobium* strains isolated from Australian *Acacia* species did exhibit a degree of geographic specificity, with the fast growers (*Rhizobium*) found in arid areas of northwestern New South Wales (NSW) and slow growers (*Bradyrhizobium*) in the alpine areas of southeastern NSW. In SA, Kock (2004) associated the acid tolerant *Cyclopia* isolate *Rhizobium tropici* with the very acidic (pH 2.9 to 4.7) CFR soils of their origin. On the other hand, Strijdom (1998) demonstrated the absence of the free living diazotrophic *Beijerinckia* spp. from alkaline soils and further proposed that the absence of *Beijerinckia* spp. from summer hot and dry CFR soils was to due to their sensitivity to high temperature and desiccation. Meanwhile, Cassman *et al.* (1981) found *Rhizobium japonicum* strains to exhibit large differences in growth at low P concentration, leading Graham & Vance (2000) to propose that rhizobia would differ in their adaptation to low soil P. The soils of the CFR vary in soil [P], with those rhizobia strains occurring in low P soils possibly adapted for growth and function at low P similar to the CFR legume species of *P. calyptrata* and *A. linearis* (Chapter 2 & 3). Therefore the strain of rhizobia may be fundamental to the symbiotic effectiveness and the P requirement of the CFR legumes. With this in mind, the objectives of this study were to investigate the phylogenetic diversity of rhizobia in the CFR soils using *Vigna unguiculata* (cowpea) as a traphost, and to identify those isolates from low P sites in the CFR.

Cowpea is a good traphost (Law *et al.*, 2007) because it nodulates promiscuously with various soil rhizobia genera. Phalane (2008) demonstrated 56% inoculation success on cowpea with rhizobia isolated from nodules of *Lebeckia* growing in SA soils. Law *et al.* (2007) demonstrated that

rhizobia in SA and Botswana soils were more effective than the commercial inoculant strain CB756 in nodulating cowpea, corroborating earlier reports by Strijdom (1998) on the wide occurrence of rhizobia strains in soils that inoculate cowpea and outcompete the highly effective CB756 strain. In addition, Mpepereki *et al.* (1996) showed that cowpea had the largest number of effective associations with both fast and slow growing indigenous rhizobia, emphasizing its relative promiscuity.

It was hypothesized that the rhizobia isolates in the soils of the CFR would cluster phylogenetically according to soil P levels. Consequently, rhizobia were isolated from CFR soil using cowpea as a traphost and subjected to 16S rRNA phylogenetic analysis to determine the phylogeny of the rhizobia. Another objective of the study was to determine the fertility levels of the soil from the different CFR sites using cowpea as a bioassay plant (Olsvig-Whittaker & Morris, 1982; Richards *et al.*, 1995). The biomass data of the cowpea was used to assess the fertility of the soil samples.

4.2 MATERIALS AND METHODS

Soil collection and analysis

Soil was collected from 34 sites in the CFR. At each site, four replicates of soil were taken within stands of legume species. Surface litter was cleared away and soil was sampled to a depth of 20 cm. Each replicate soil sample was put into a sterile clear plastic bag and sealed tightly and labelled. At the laboratory a sub-sample was air-dried and sieved (1 mm mesh) for measurement of soil P and pH at BemLab (Pty) Ltd. Soil pH was determined by shaking 2 g of soil in 20 ml 1 M KCl at 180 rpm for 60 min, centrifuging at 10 000g for 10 min and measuring pH of the supernatant. Soil was prepared for P analysis by extracting 6.6 g soil in Bray II solution (Bray & Kurtz, 1945) before filtering and analyzing using inductively coupled plasma atomic emission spectrometry (Varian Vista MPX ICP-AES; Varian, Mulgrave

Australia). The remaining soil was stored in a 10˚C fridge for isolation of rhizobia.

Plant growth

In a glasshouse experiment cowpea was sown in 18 cm diameter plastic pots containing 300 g of composite soil per site over 2.5 kg of silica sand. Four seeds per pot were sown in the soil and then covered with 2 cm of silica sand. There were three replicate pots per site. Controls were non-inoculated cowpea plants supplied with 2 mM NH_4NO_3 (N-fed) and cowpea plants inoculated with commercial strain *Bradyrhizobium*-sp(*Vigna*) (Comm. rhizobia). All plants were grown under natural light and daytime temperature and watered twice a week with 400 ml ½ strength N free Hoagland solution (except for the control that received 2 mM NH_4NO_3). Seedlings were thinned to two per pot after appearance of the first set of trifoliate leaves. Thirty days after emergence all the plants in the soil treatments started receiving 1 mM NH_4NO_3 in the Hoagland solution as some of the plants showed signs of N deficiency.

Plant harvest and analysis

Plants were harvested 85 days after sowing. The soil around the roots was removed by gently shaking and washing the roots in water. Nodules were separated from the roots, counted, weighed and frozen in 30% glycerol in labelled 20 ml pill vials. Root and shoot FW were measured and the plant material was dried at 60˚C for 72 hours in a forced draught oven, and then reweighed for DM weight. The dried shoots were milled in a Wiley Mill using a 0.5 mm mesh (Arthur H. Thomas Co. Philadelphia, CA, USA) and then analyzed for N concentration using the procedure presented in section 2.2 of this thesis.

Isolation of rhizobia bacteria from nodules

Four large pink nodules where selected from each vial. Nodules were rinsed in 95% ethanol then submerged in 0.1% acidified $HgCl_2$ for three minutes. Nodules were then rinsed in six changes of distilled water. Each nodule was then aseptically dissected and crushed in sterile saline solution (0.85% NaCl). A drop of nodule squash was transferred on to a YEM agar plate (Vincent, 1970) and streaked with a sterile wire loop. Plates were incubated at 28˚C for up to 10 days. In addition to the cowpea nodule isolates, rhizobia obtained from nodules of *O. striatum*, *A. linearis*, and *C. subternata* plants that were inoculated with CFR soil (Chapter 1) were similarly isolated.

Each day the plates were observed for colony morphology, contraindications and number of days for growth to occur. Plates with white, watery, colorless growth that appeared only after two to three days and up to 10 days (Vincent, 1970) were selected as rhizobia. The bacteria from each plate were again restreaked in a manner to get single colonies and these single colonies were picked and restreaked for multiple colonies. Again, plates were incubated at 28˚C for up to 10 days. Selected rhizobia plates were then stored at 0˚C for subsequent PCR analysis.

16S rRNA PCR

Amplification of the 16S rRNA gene of the selected strains was performed with the forward primer (16f27 5´-AGAGTTTGATCCTGGCTCAG-3´) and the reverse primer (16r1485 5´-TACCTTGTTACGACTTCACCCCA-3´) (Lane, 1991). PCR reactions were prepared to a final volume of 36 µl, containing 10 x PCR Buffer (3.0 µl), $MgCl_2$ (4.2 µl), dNTPs (1.2 µl), forward primer (1.0 µl), reverse primer (1.0 µl), Kapa-Taq (0.2 µl), and sH_2O (25.4 µl). In a laminar flow, rhizobia DNA was added to the PCR mixture straight from a single colony of the agar plate (Thies *et al.*, 2001) by touching a sterile pipette tip to bacterial growth and then agitating the tip in the eppendorf tube containing the PCR mixture to release the bacteria into the solution. The PCR reactions

were done in a GeneAmp® PCR system 9700 (Applied Biosystems, California USA). The PCR thermal cycling profile cycle (Laguerre *et al.*, 1994) was set at an initial denaturation of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final elongation step of 72°C for 7 min was followed by a final holding temperature of 4°C. A 1.0 µl aliquot of the amplified DNA product was separated by 1% Agarose Gel electrophoresis in the presence of 1 X TAE buffer at 160v for 13 min. The DNA was stained in the gel using EtBr and visualized using UV light and recorded using CCD camera. A 100 bp DNA marker (O' Gene Ladder Plus, Fermentas) was used to view the results of the PCR. The complete PCR product mixture was sent for sequencing to Stellenbosch Sequencing Facility, Stellenbosch University, Stellenbosch.

Constructing a dataset

The website: "List of Prokaryotic names with standing in Nomenclature" (http://www.bacterio.cict.fr/) was consulted to obtain the culture collection number (accession number) of the different rhizobia type strains used in the phylogenetic analysis. This information was then used to obtain the 16S rRNA sequence of the type strains from the Nucleotide database in the GenBank database (http://www.ncbi.nlm.nih.gov/). The 16S rRNA sequence of the type strains were then saved in BioEdit version 7.0 (Hall, 1999).

Sequence alignment and phylogenetic analysis

The sequences of the isolates obtained from the Stellenbosch Sequencing Facility were edited using Staden package version 1.60 (Staden *et. al.*, 1998) and the consensus sequences were imported into BioEdit version 7.0 (Hall, 1999). The strains were not authenticated by the Koch's postulate procedure. However, all 16S rRNA sequences of the isolates were compared to those in the GenBank database using BLAST (Altschul *et al.*, 1990) and confirmed that the isolates were rhizobia. The consensus sequences of the rhizobia isolates and the 16S rRNA type strain sequences were first electronically aligned

using ClustalW multiple alignment and then the remaining residues were aligned manually. Once aligned, simple indel coding was performed using Gap Coder (Young & Healy, 2003). Phylogenetic analysis and reconstruction was done using maximum parsimony methods in PAUP version 4.0b10 (Swofford, 2002). The parsimony settings were: 1000 random taxon addition sequences, with tree bisection and reconnection (TBR) branch swapping and maxtrees set to increase without limit. To assess support on branches, 1000 bootstrap replicates using TBR branch swapping, with simple addition and maxtrees set to 100 were performed.

4.3 RESULTS

Rhizobia isolation

Phenotypically all the isolates were fast growers with moderate to abundant growth after two to three days of incubation at 28˚C (showing no contraindication of growth in the first few days (Vincent, 1970)). Colony morphology of the isolates on YEM agar medium was mostly high, gummy and semi-transparent in appearance.

Twenty one rhizobia isolates were obtained. Seventeen of the isolates were from the nodules of cowpea grown in 13 different CFR soil sites. These isolates were MBOT2, MBOT7, MBOT37, MBOT10, MBOT12A, MBOT12B, MBOT14, MBOT15, MBOT16, MBOT18, MBOT20, MBOT25, MBOT39, MBOT26, MBOT35, MBOT44A and MBOT44B as indicated in Table 1. Isolates in the table are named by an abbreviation "MBOT" followed by a number designating the CFR site of their origin. Three isolates were from nodules of *O. striatum*, *A. linearis*, and *C. subternata* named MBOT-Os, MBOT-Al and MBOT-Cs respectively. The final isolate MBOT-X was from the cowpea inoculated with the commercial inoculant for cowpea, *Bradyrhizobium*-sp(*Vigna*). Therefore a total of 21 isolates were subjected to PCR analysis.

PCR and phylogenetic analysis

The genes from all the isolates possessed 98 – 100% sequence similarity with a rhizobia type strain species already described in the GenBank nucleotide database except MBOT2 which had 95% sequence similarity with a rhizobia strain in Genbank. The isolates were found to be in the α-rhizobia genera *Rhizobium* and *Mesorhizobium* (Fig. 1) and the β-rhizobia genus *Burkholderia* (Fig. 2). Two phylogenetic trees were constructed for each of the two prominent α- and β-rhizobia lineages representing 16 and five isolates respectively.

Table 1. Rhizobia isolates and soil data for the different sites in the CFR. Means with similar letters are not significantly different at ***P < 0.001.

Rhizobia Isolates	**CFR sites**	**Parent material** (Mucina & Rutherford 2006)	**P Bray II (mg/kg)**	**pH**
MBOT2	Gydo Pass, Ceres	Shale	21.00a	5.23b
MBOT7/ 37	Grootberg, Khamieskroon	Granite	11.25ab	5.00b
MBOT10	Jonaskop Valley, Worcester	Alluvial sand	13.00ab	6.13a
MBOT12A/ 12B	Vogelgat Nature Reserve-1	Sandstone	5.25bc	3.65ef
MBOT14	Bettys Bay	Dune sands	2.25d	4.33c
MBOT15	Vogelgat Nature Reserve-2	Sandstone	2.75cd	3.25f
MBOT16	Camps Bay, Cape Town	Granite	7.25b	4.70bc
MBOT18	Mitchells Pass, Ceres	Sandstone	10.50ab	4.20cd
MBOT20	Lions Head, Cape Town	Granite	17.25a	4.95b
MBOT25/ 39	Tierfontein Farm, Elim	Sandstone	2.00d	3.83de
MBOT26	Bainskloof Pass, Wellington	Sandstone	2.50cd	3.38ef
MBOT35	Van Rhyns Pass, Nieuwoudtville	Shale	-	-
MBOT44A/ 44B	Biedouw River, Clanwilliam	Shale	-	-
	CFR legume species	F-statistic $_{(10,33)}$	9.17***	24.20***
MBOT-Os	*O.striatum*			
MBOT-Al	*A. linearis*			
MBOT-Cs	*C.subternata*			
MBOT-X	Cowpea inoculated with *Bradyrhizobium*-sp(*Vigna)*			

In Figure 1, the isolates MBOT2, MBOT-Os, MBOT18, MBOT-Al, MBOT35, MBOT25, MBOT20, MBOT10, MBOT44A, MBOT7, MBOT14, MBOT15, MBOT16, MBOT39, MBOT26, and MBOT37 are all grouped as α-rhizobia. The Gydo Pass isolate MBOT2 formed a separate branch, while isolates MBOT-Os and MBOT18 from the Mitchells Pass soils clustered close to the

M. loti and *M. ciceri* group but formed a separate branch. The MBOT-AI strain isolated from *A. linearis* nodules formed a separate branch in a *Mesorhizobium* cluster consisting of *M. amorphae*, *M. septentrionale*, *M. haukuii*, *M. plurifarium* and *M. thiogangeticum*. In contrast to these *Mesorhizobium* isolates a distinct clade of a highly related group of isolates sharing high 16S rRNA sequence similarity was formed by eight isolates namely MBOT44A, MBOT7, MBOT14, MBOT15, MBOT16, MBOT39, MBOT26 and MBOT37. The isolates MBOT15, MBOT16, MBOT39, MBOT26 and MBOT37 were all closely related to each other and formed a separate branch that was close to the *R. multihospitium*, *R. tropici*, *R. hainanense*, *and R. miluonense* cluster. MBOT7 and MBOT14 also formed a part of this cluster with *R. tropici*. On the other hand MBOT44A formed a separate branch in this cluster and was closest neighbour to MBOT7. Isolate MBOT35 that was also in the *Rhizobium* lineage showed high relatedness to *R. giardinii* and formed a separate branch with this species. The strains MBOT25, and MBOT20 formed a separate group within an *Agrobacterium* cluster showing 100% and 99% respective similarity to their nearest neighbour *A. radiobacter*. The Jonaskop isolate MBOT10 formed a separate clade with *R. hautlense*, *R. galagae* and *R. cellulosilyticus* and a separate branch in this grouping but was closest neighbour to *R. cellulosilyticus*.

In Figure 2, the β-rhizobia isolates MBOT-Cs and MBOT44B, MBOT12A and MBOT12B grouped on two separate branches in a single clade. On one branch MBOT-Cs, the isolate from a *C. subternata* nodule was closely related to *B. phytofirmans*. The isolates MBOT44B, MBOT12A and MBOT12B formed a separate branch and appear closely related to each other. MBOT-X, the rhizobia isolated from the cowpea inoculated with commercial inoculant grouped in a separate clade whose nearest neighbour was *B. caribiensis*.

Figure 1. Phylogenetic analysis of rhizobia isolates using parsimony, based on 16S rRNA gene sequences of *Rhizobium* and *Mesorhizobium* type strains (T) and α-rhizobia CFR soil isolates in bold. Bootstrap replicates (1000) are listed as percentages (only those higher than 60%) at the branching points. Type strain accession numbers are indicated in parenthesis. The scale bar represents nucleotide substitutions per site.

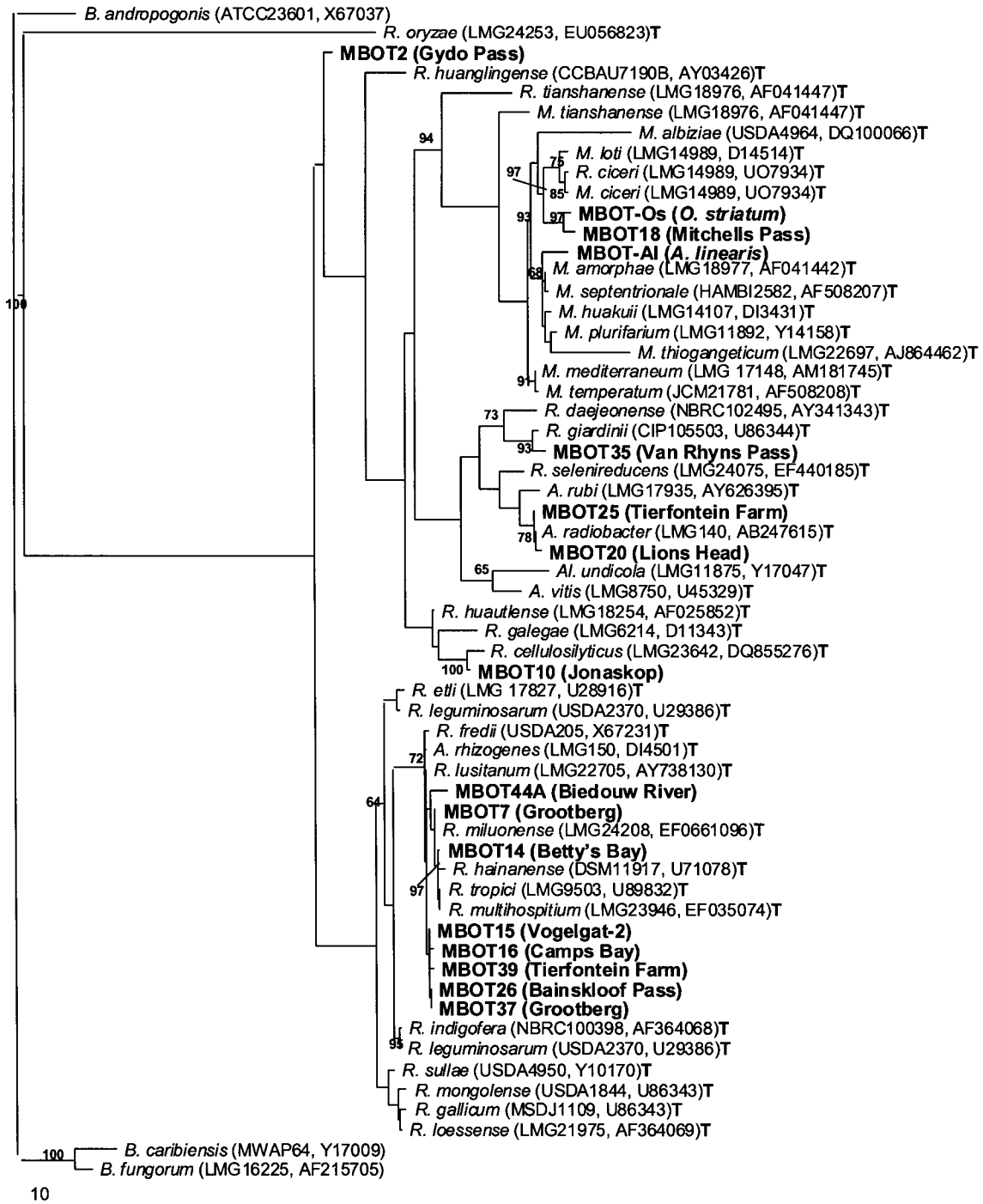
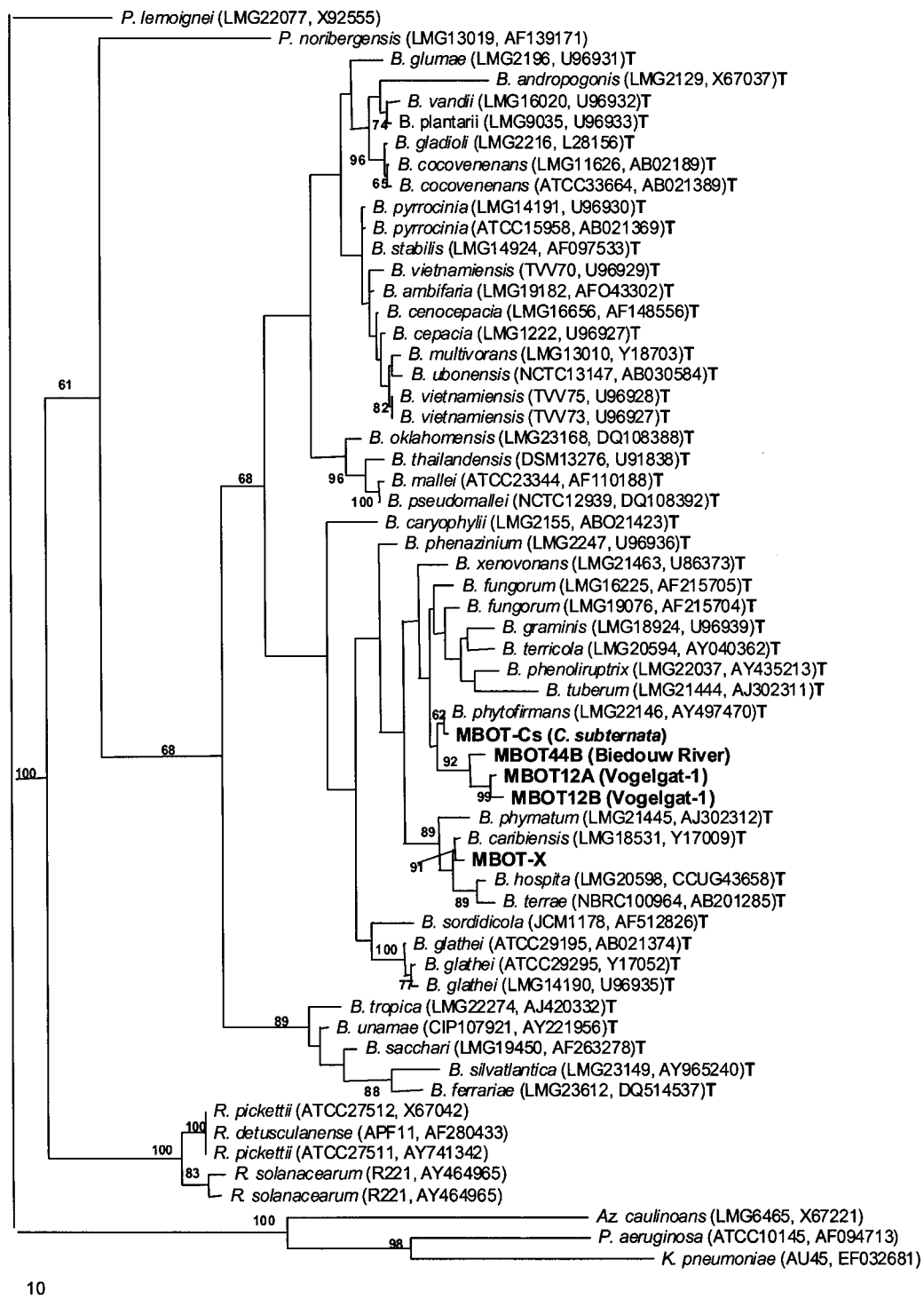


Figure 2. Phylogenetic analysis of rhizobia isolates using parsimony, based on 16S rRNA gene sequences of *Burkholderia* type strains (T) and β-rhizobia CFR soil isolates in bold. Bootstrap replicates (1000) are listed as percentages (only those higher than 60%) at the branching points. Type strain accession numbers are indicated in parenthesis. The scale bar represents nucleotide substitutions per site.



Soil characteristics

The soil P analysis (Table 1) indicates that available P in some of the CFR sites were significantly different from each other with P ranging from a low 2 mg P kg soil^{-1} from the Tierfontein Farm, Betty's Bay, Bainskloof and Vogelgat-2 sites to a high of 17 and 21 mg P kg soil^{-1} at Lionshead and Gydo Pass. These levels of P are comparable with those reported by Spriggs (2004) who measured a range of available soil P from 1.3 to 17.6 mg P kg soil^{-1} for 19 undisturbed *Cyclopia* habitats in the CFR. The pH analysis revealed that all the sites were acidic. Four sites, namely Vogelgat-1, Vogelgat-2, Bainskloof and Tierfontein Farm had very acidic soils with pH < 4.0. The Jonaskop Valley soil was moderately acidic with a pH of 6.1, typical of alluvial sands found in the CFR floodplains and valleys (Mucina & Rutherford, 2006). Soil analysis was not done for the Van Rhyns Pass and Biedouw River sites because of unreplicated sample collection.

Soil fertility and N_2-supply indices

The cowpea inoculated with the Comm. rhizobia and the N-fed cowpea accumulated the highest biomass (Table 2). Among the cowpea plants grown in the CFR soil, the Camps Bay and Grootberg soils produced the highest biomass of 18.4 and 17.3 g plant^{-1} respectively though this was almost 80% less than the Comm. rhizobia and N-fed controls. The least biomass was accumulated in the Vogelgat-2 soil with plants weighing only 5.6 g plant^{-1}. The amount of N in the shoot was highest in the Comm. rhizobia cowpea plants with the 142 mg N plant^{-1}, more than 2.5 times greater than the Camps Bay cowpea which accumulated the next highest amount of N in the shoot (Table 2). Both the Vogelgat-2 and Gydo Pass cowpea had the lowest shoot N contents of the CFR soil with 7.3 and 10.9 mg N plant^{-1} respectively. Similarly, the Comm. rhizobia plants with 2.5 g nodule FW and 79 nodules plant^{-1} produced more than twice the nodule FW and nodule number than the highest nodulating CFR soil sample (Table 2). Of the CFR soil the Camps Bay, Jonaskop and Lionshead soils had the highest nodule FW and nodule

numbers plant^{-1} while the Vogelgat-2, Bainskloof and Gydo Pass CFR cowpea produced the lowest nodule FW and nodule number plant^{-1} (Table 2).

Table 2. Biomass, nodule and tissue nutrient data for cowpea plants grown in CFR soil and receiving 1mM NH_4NO_3. Cowpea plants grown in sand either inoculated with *Bradyrhizobium*-sp(*Vigna)* or not nodulated and receiving 2mM NH_4NO_3 were used as controls. Means with similar letters are not significantly different at ***P < 0.001.

Soil treatment	Total fresh biomass (g) plant^{-1}	Shoot N content (mg) plant^{-1}	Nodule fresh weight (g) plant^{-1}	Nodule number plant^{-1}
Gydo Pass	8.10d	10.92gh	0.10e	1.67de
Grootberg	17.34b	38.88bc	0.80bc	11.33cd
Jonaskop	14.36bc	31.81bcd	1.02abc	42.50b
Vogelgat-1	8.78d	20.99def	0.37cde	2.83de
Bettys Bay	10.36cd	14.93efg	0.52bcd	15.67bcd
Vogelgat-2	5.55e	7.28h	0.01f	0.17e
Camps Bay	18.42b	56.32b	1.17ab	39.33b
Mitchells Pass	10.98cd	30.11cde	0.75bc	25.33bc
Lions Head	13.88bc	28.76cd	0.89abc	20.50bc
Tierfontein Farm	8.63d	11.87fgh	0.20de	3.67de
Bainskloof Pass	8.24d	11.75fgh	0.05e	0.67de
2mM N	29.53a	43.17bc	-	-
Comm. rhizobia	33.08a	142.38a	2.52a	78.83a
F-statistic $_{(12,26)}$	18.52***	19.17***		
F-statistic $_{(11,19)}$			9.65***	12.11***

The soil fertility index applies the principle of using total biomass of a crop as a bioassay to determine soil fertility (Olsvig-Whittaker & Morris, 1982; Richards *et al.*, 1995). This index showed that the Camps Bay and Grootberg sites which had the highest cowpea biomass were therefore the most fertile soil relative to Tierfontein Farm, Bainskloof, Gydo Pass and Vogelgat-2 which had the lowest cowpea biomass and therefore least fertile soil (Table 3). The N_2-supply index in Table 3 ranked sites in descending order of performance according to the total score, which was a sum of the component scores of N supply, namely shoot N, nodule FW, and nodule number. The N_2-supply indices of Camps Bay and Jonaskop were the highest while those of Tierfontein Farm, Gydo Pass, Bainskloof and Vogelgat-2 were the lowest (Table 3).

Table 3. Soil Fertility Index and N_2-supply Index of CFR sites and cowpea controls ranked in descending order (shaded area). For the Soil Fertility Index, cowpea plants were ranked using only total biomass accumulated (Table 2). The N_2-supply Index is an overall rank based on the total score of N supply which was the sum of the component ranks (in brackets) based on shoot N, nodule FW and nodule number (Table 2).

		Components of N_2-supply Index			
Soil Fertility Index	**N_2-supply Index**	Shoot N	Nodule FW	Nodule number	Total score
Comm. rhizobia 2mM Nitrate	*Comm. rhizobia*	*Comm. rhizobia*	*Comm. rhizobia*	*Comm. rhizobia*	
1Camps Bay	**1**Camps Bay	Camps Bay (1)	Camps Bay (1)	Camps Bay (2)	4
2Grootberg	**2**Jonaskop	Jonaskop (3)	Jonaskop (2)	Jonaskop (1)	6
3Jonaskop	**3**Grootberg	Grootberg (2)	Grootberg (4)	Grootberg (6)	12
4Lions Head	**3**Mitchells Pass	Mitchells Pass (4)	Mitchells Pass (5)	Mitchells Pass (3)	12
5Mitchells Pass	**3**Lions Head	Lions Head (5)	Lions Head (3)	Lions Head (4)	12
6Bettys Bay	**6**Bettys Bay	Bettys Bay (7)	Bettys Bay (6)	Bettys Bay (5)	18
7Vogelgat-1	**7**Vogelgat-1	Vogelgat-1 (6)	Vogelgat-1 (7)	Vogelgat-1 (8)	21
8Tierfontein Farm	**8**Tierfontein Farm	Tierfontein Farm (8)	Tierfontein Farm (8)	Tierfontein Farm (7)	23
9Bainskloof Pass	**9**Gydo Pass	Gydo Pass (10)	Gydo Pass (9)	Gydo Pass (9)	28
10Gydo Pass	**10**Bainskloof Pass	Bainskloof Pass (9)	Bainskloof Pass (10)	Bainskloof Pass (10)	29
11Vogelgat-2	**11**Vogelgat-2	Vogelgat-2 (11)	Vogelgat-2 (11)	Vogelgat-2 (11)	33

4.4 DISCUSSION

Phylogenetic analysis and soil characteristics

Phylogenetic analysis of the 16S rRNA gene sequences of rhizobia isolated from CFR soils using cowpea traphosts confirmed the existence of phylogenetically distinct subclasses of α-rhizobia and β-rhizobia. Among the α-rhizobia, three of the isolates grouped as *Mesorhizobium* and 13 as *Rhizobium* while the other five where β-rhizobia in the genus *Burkholderia*. This diversity of rhizobia in the CFR was similarly reported by recent studies investigating the diversity of root nodule bacteria from field samples of indigenous and exotic SA legumes. For instance, in 39 isolates from nodules of *Lebeckia*, a CFR legume genus, Phalane (2008) found *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Burkholderia* strains. Kock (2004) also found *Burkholderia*, *Rhizobium*, and *Bradyrhizobium* genera in 55 rhizobia isolates from *Cyclopia* species. Twenty six of these isolates were from CFR *Cyclopia* nodules obtained from field sampled plants (Spriggs, 2004) and three namely UCT50, UCT42 and UCT53, were most closely related to *Rhizobium tropici*, *Bradyrhizobium liaoningense* and *Bradyrhizobium japonicum* while the other 23 isolates were all *Burkholderia* strains. The eight isolates from nodules of *Lotonis* sp. growing in SA soils in Le Roux (2003) were *Sinorhizobium* and *Burkholderia* (from CFR soils), and *Rhizobium* and *Bradyrhizobium* (from soils in Gauteng and Mpumulanga provinces, SA).

Half of the α-rhizobia isolates (38% of the total number of isolates in this study) including MBOT44A, MBOT7, MBOT14, MBOT15, MBOT16, MBOT39, MBOT26 and MBOT37 formed one clade together with the rhizobia type strains *R. multihospitium*, *R. tropici*, *R. hiananense*, *R. miluonense*, *R. lusitanum* and *R. fredii* (Fig. 1). These type strains were isolated from nodules of different legume species including *P. vulgaris*, *G. max*, *Leucaena* sp., *Lespedeza* sp. and native legumes from China (Martinez-Romero *et al.*, 1991; Jarvis *et al.*, 1992; Valverde *et al.*, 2006; Gu *et al.*, 2008; Han *et al.*, 2008). Isolate MBOT14 from the low P Betty's Bay site (Table 1), with a high

bootstrap value of 97% and sequence similarity of 100% to *R. tropici* is highly supported as a *R. tropici* strain, similar to MBOT7 from Grootberg and MBOT16 from Camps Bay that shared 100% and 98% respective similarity with *R. tropici*. According to BLAST results (Altschul *et al.*, 1990) the other Grootberg isolate MBOT37 was also found to share 100% similarity with UCT50, an isolate from the Kock (2004) study who grouped the UCT50 strain as *R. tropici*. Therefore it is suggested that MBOT14, MBOT7, MBOT16 and MBOT37 and the other closely related isolates in the clade (Fig. 1) such as the low P isolates MBOT15, MBOT26 and MBOT39 (Table 1) are possible strains of *R. tropici*. Strains of *R. tropici* have been identified to be acid tolerant compared to other *Rhizobium* species (Graham *et al.*, 1994) because they are able to grow in medium with pH less than 5.0. The optimum pH for the growth of root nodule bacteria falls between 6.0 and 7.0 (Vincent, 1970; Jordan, 1984). In this study, the pH of the soil of the seven isolates that are in close association with *R. tropici* (Grootberg, Betty's Bay, Vogelgat-2, Camps Bay, Tierfontein Farm and Bainskloof Pass) were in the range of 3.3 to 5.0 (Table 1). Therefore, the proliferation of rhizobia isolates similar to *R. tropici* could be attributed to the acidic nature (pH $\leq$5.0) of the soils.

Four of the other α-rhizobia isolates also grouped as *Rhizobium* strains, with both MBOT25 from the lowest P site of Tierfontein Farm and MBOT20 from Lions Head closely related to *A. radiobacter*. The *Agrobacterium* genus is reported to be phylogenetically intertwined with *Rhizobium* (Young *et al.*, 2001; Willems, 2006) and is currently the subject of much confusion and debate. While Young *et al.* (2001) have proposed merging the genus *Agrobacterium* into *Rhizobium*, with *A. radiobacter* which is a non-pathogenic bacterial strain becoming *R. radiobacter*, this proposal has not been supported by other scientists such as Farrand *et al.* (2003) because of observed unique phenotypic traits that differentiate the two genera. With 93% branching support, the isolate MBOT35 from the Van Rhyns Pass soil is possibly a strain of *R. giardinii* which has been previously isolated from *P. vulgaris* nodules (Amarger *et al.*, 1997). Isolate MBOT10 from Jonaskop Valley soil that formed a separate clade with three other *Rhizobium* species

had a 100% bootstrap support for its close relatedness to *R. cellulosilyticus*, a species reported by Garcia-Fraile *et al.* (2007).

The three isolates MBOT-Os, MBOT18, MBOT-Al belonged to *Mesorhizobium*, occurring in two distinct clusters in the *Mesorhizobium* lineage (Fig. 1). Based on the high bootstrap support (97%) in the comparative sequence analysis, the isolates MBOT-Os from *O. striatum* nodules and MBOT18 from Mitchells Pass soil appear to be strains of the same species, closely related to either *M. loti* or *M. ciceri*. *M. loti* was previously isolated from nodules of *Lotus japonicum*, *Lupinus* and *Leuceana* spp. (Jarvis *et al.*, 1982) while *M. ciceri* has been found in nodules of *Cicer arietinum* plants growing in Spain, Morocco, Syria and Turkey (Nour *et al.*, 1994). *M. ciceri* strains have been reported to be saline tolerant of up to 12% NaCl in solution (Soussi *et al.*, 2001). Therefore it would be interesting to test strains MBOT-Os and MBOT18 for symbiotic effectiveness under conditions of salt or osmotic stress and relate their response to the summer dry soils of the CFR. The *A. linearis* nodule isolate MBOT-Al, although in a grouping with low (68%) bootstrap support, showed high sequence similarity (97%) using BLAST (Altschul *et al.*, 1990) with *M. haukuii* which has previously been isolated from *Astragalus sinicus* (Chen *et al.*, 1991). It was not possible to associate the Gydo Pass isolate MBOT2 with other known rhizobia type strains because it formed a separate branch from the other isolates and types strains.

The five isolates namely MBOT-CS, MBOT44B, MBOT12A, MBOT12B and MBOT-X that grouped in the β-rhizobia lineage were all associated as *Burkholderia* rhizobia (Fig. 2). The isolates MBOT12A and MBOT12B from Vogelgat-1 and MBOT44B from Biedouw River are closely related to each other with 92% and 99% bootstrap support for the respective branches. MBOT-Cs formed a separate branch with *B. phytofirmans*, however the branching pattern has low bootstrap support of 62%. Notably, only four *Burkholderia* species are known symbiotic N_2-fixing strains (Willems, 2006) with *B. tuberum* reported to nodulate *Mimosa* spp. and *A. carnosa* (Chen *et*

al., 2001; Moulin *et al.*, 2001), *B. phymatum* nodulates legumes of *Machaerium* (Vandamme *et al.*, 2003), and *B. caribiensis* first isolated from soils in Martinique, was also isolated from *Mimosa* nodules, while *B. cepacia* comes from *Alysicarpus* nodules (Vermis *et al.*, 2004). Therefore it is suggested that the four *Burkholderia* isolates MBOT-CS, MBOT44B, MBOT12A and MBOT12B are either a new species or they could be a strain of *B. tuberum*, the closest related N_2-fixing strain. Similarly, Kock (2004) found that of the 23 *Burkholderia* isolates obtained from CFR *Cyclopia* spp., 13 formed a highly homogenous group with *B. tuberum* and where therefore also proposed as strains of this species. Phalane (2008) reported that seven of the nine *Burkholderia* isolates from *Lebeckia* spp. also formed a single clade only with *B. tuberum*. The isolate MBOT-X with 91% bootstrap support and 99% sequence similarity is most probably a *B. caribiensis* strain. Although the isolation of a β-rhizobia strain from a nodule of a cowpea plant that was inoculated with commercial strain *Bradyrhizobium*-sp(*Vigna*) appears as an anomaly, it suggests that different rhizobia strains can nodulate the same host plant, also reported by Hung *et al.* (2005) who isolated a *B. caribiensis* and a *Rhizobium* strain from different nodules of the same legume plant *Catenaria caudatum*. Several bacterial strains are often isolated from a single host legume (Young & Haukka, 1996), therefore it is most probable that the nodules of the cowpea were infected by *Bradyrhizobium*-sp(*Vigna*) and *Burkholderia*, but the latter species was selected in the isolation process.

It was apparent that the seven isolates that formed part of a *Rhizobium* clade related to *R. tropici* (Fig. 1) were also from different sites with significantly different available soil P ranging from 2 to 11 mg P kg soil^{-1} (Table 1). In addition, two closely related strains MBOT25 and MBOT20, came from the Tierfontein and Lions Head sites respectively, with significantly different ($P < 0.001$) available P levels of 2 and 17 mg P kg soil^{-1} and with significantly ($P < 0.001$) different pH levels of 3.8 and 4.9 (Fig. 1, Table 1). Furthermore, isolates MBOT25 and MBOT39 were both from the lowest ($P < 0.001$) P site of Tierfontein Farm but were phylogenetically separate, while rhizobia from the α-rhizobia (Vogelgat-2) and β-rhizobia (Vogelgat-1) lineages were isolated

from the same location, Vogelgat Nature Reserve (Figs. 1 & 2). These observations indicate that the CFR rhizobial strains did not form phylogenetic affiliations according to soil P or geography, similar to the results of Zhang *et al.* (1991), Lafay & Burdon (1998) and Gao *et al.* (2001). For example, Gao *et al.* (2001) obtained 54 isolates from diverse geographical regions in China and observed that in each of the *Mesorhizobium*, *Sinorhizobium* and *Rhizobium* clusters formed, isolates were from several different regions.

Soil fertility and N_2-supply indices

Cowpea grown in the CFR soil and sand mixture produced significantly less biomass than the cowpea inoculated with the Comm. rhizobia or fed 2 mM NH_4NO_3 (Table 2). However, among the CFR soils, cowpea grown in Camps Bay and Grootberg soils accumulated the highest biomass while those from Tierfontein Farm, Bainskloof, Gydo Pass and Vogelgat-2 had the lowest biomass. Therefore according to the soil fertility index, Camps Bay and Grootberg demonstrated to have highly fertile soil relative to the latter four sites (Table 3). Apparently soils from Camps Bay and Grootberg are granite derived while those from Vogelgat-2, Bainskloof Pass and Tierfontein Farm are from sandstone parent material (Mucina & Rutherford, 2006). Thus, as expected, granite derived soils showed higher fertility than sandstone derived soil.

The total cowpea biomass significantly correlated (R^2 = 0.73) with shoot N content indicating that the N supply of the soil played a major role in plant growth. The N was obtained from both the soil and SN_2F. Therefore a separate index was developed to determine the N supply from the sites by assessing shoot N, nodule FW and nodule number as these parameters are indicative of the symbiotic N_2-effectiveness of the rhizobia isolate from the site. Clearly the N_2-supply index of Camps Bay soil was the highest while the N_2-supply index of Tierfontein Farm, Gydo Pass, Bainskloof Pass and Vogelgat-2 was the lowest (Table 3). Although the N_2-supply index for Grootberg, Mitchells Pass and Lions Head were the same, their soil fertility index using the total biomass yield was different. This suggests that factors

(not assessed in this study) other than N impacted on the growth of the bioassay crop, supporting the view that total biomass of a plant is a measure integrating all edaphic factors affecting growth (Richards *et al.*, 1995).

4.5 CONCLUSION

This study found that there is a diversity of rhizobia, belonging to the genera *Rhizobium*, *Mezorhizobium* and *Burkholderia*, in the low P CFR soil. However, the phylogenetic relatedness of rhizobia isolates was independant of geography, soil fertility or available soil P with a clade consisting of isolates from different sites in the CFR while distantly related isolates were from soils with similar [P]. The rhizobia isolates from the low P sites were associated with rhizobia type strains. Follow up studies will test the symbiotic effectiveness of the isolates.

CHAPTER FIVE
GENERAL DISCUSSION AND CONCLUSION

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In this study it was hypothesized that N_2-fixing legumes indigenous to low P soils of the CFR have a low P requirement for growth & SN_2F. From an initial 18 indigenous CFR legumes (Chapter 2), four species namely *A. linearis*, *V. oroboides*, *P. calyptrata* and *C. genistoides* were the highest nodulating species at 0.1 μM P, demonstrating possible low P requirement for growth and SN_2F. The variation in growth and SN_2F evident amongst the CFR legumes at 0.1 μM P thus formed the basis for selection of low P genotypes. The superior nodulation of certain wild legumes such as *A. linearis* and *P. calyptrata* further demonstrates the efficacy of studying wild legumes when aiming to identify superior N_2-fixers at low P (Sprent, 1999). Subsequently (Chapter 3), *P. calyptrata* was found to have a low P requirement for growth & SN_2F because of a positive interaction between N and P when P supply was increased from 1 to 10 μM P. The species *A. linearis* not only showed the greatest capacity to nodulate and fix N_2 at 0.1 μM P (Chapter 2), but the N-fed plants also showed the greatest biomass and N accumulation response to 10 μM P with no increase thereafter (Chapter 3). Taken together these results demonstrate low P adaptation for growth & SN_2F in *A. linearis* and *P. calyptrata*. These two species are therefore promising plants for investigating the molecular mechanisms and genes involved in growth & SN_2F at low P, with potential applications in agriculture and agroforestry using genetic engineering. Similarly, in *P. pinnata*, the high N_2-fixing efficiency in nodules that declined with P fertilization can be considered an adaptation to the generally low P conditions of the CFR. Therefore, *P. pinnata* also makes a promising subject for studying the molecular basis of the low P trait, also with potential applications for crop plants.

Uncovering certain traits of the unique and largely understudied legume biodiversity in the nutrient poor CFR was compelling motivation for scientific study. However, investigating the growth responses of wild legumes with no previous history on adequate nutrient requirements posed a challenge to the N X P interaction study. For example, there is a need to establish the critical

concentration of combined-N for supply to CFR legumes for future interaction studies, so that N-fed plants are supplied adequate levels of N, allowing for suitable comparisons to SN_2F plants.

Both *A. linearis* and *P. calyptrata* showed a low P requirement for growth & SN_2F. However, variation in P levels also affects rhizobia growth and functioning so that rhizobia isolates from the low P CFR sites may also be adapted to low P. Therefore future research should examine the P nutrition of all the isolates and test the symbiotic N_2-effectiveness between the low P adapted isolates and the low P plants such *A. linearis* and *P. calyptrata*. Although the CFR rhizobia isolates did not cluster according to available soil P as hypothesized, isolates from low P soils and from relatively high P or more fertile soils were identified (Chapter 4). For instance, those isolates from the low P sites may be adapted to fix N_2 at low P, while the soil fertility results suggested that the closely related *R. tropici* strain from the Camps Bay soil may be an effective N_2-fixing strain. In addition, isolates that are adapted for N_2-fixation at low P would have potential for use in infertile agricultural soils, and with low input small-scale farmers such as CFR rooibos (*A. linearis*) and honeybush (*Cyclopia*) tea farmers and fynbos nurseries.

The overall purpose of this study was to identify N_2-fixing legumes indigenous to the low P areas of the CFR that have a low P requirement for growth & SN_2F. The P nutrition of the wild legumes from the CFR indicated that there are exceptions to the dogma of a high P requirement for growth & SN_2F typical of crop legumes. Thus, plants of *P. calyptrata* and *A. linearis* showed a low P requirement for growth & SN_2F and *P. pinnata* nodules showed maximum N_2-fixing efficiency at low nodule [P]. In terms of the microsymbiont, the rhizobia isolates from the low P sites were associated with rhizobia type strains.

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