

***ASPALATHUS AND PODALYRIA* LEGUMES  
BALANCE ACQUISITION OF PHOSPHORUS AND NITROGEN  
FOR GROWTH IN NUTRIENT POOR FYNBOS SOILS**

by

Pravin Mark Maistry

Thesis submitted for the degree of  
DOCTOR OF PHILOSOPHY

Department of Biological Sciences  
UNIVERSITY OF CAPE TOWN

December 2014

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Main supervisor

Samson BM Chimphango<sup>a</sup>

Cosupervisors

A Muthama Muasya<sup>a</sup>

Alex J Valentine<sup>b</sup>

<sup>a</sup>Department of Biological Sciences, University of Cape Town.

<sup>b</sup>Department of Botany and Zoology, University of Stellenbosch.

## **Declaration**

I Pravin Mark Maistry, know the meaning of plagiarism, and hereby declare that this thesis is my original work, except where properly acknowledged, and that neither the whole nor any part of the thesis has been, is being, or is to be submitted for another degree in this or any other university. I grant the University of Cape Town free license to reproduce the above thesis in whole or in part for the purpose of research.

I am now presenting the thesis for examination for the Degree of PhD.

The thesis contains two papers that were published in collaboration with my supervisors, SBM Chimphango, AM Muasya and AJ Valentine:

- Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2014) Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis*. *Functional Plant Biology* doi.org/10.1071/FP14100 (Chapter 2).
- Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2015) Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume *Podalyria calytrata*. *Journal of Plant Physiology* 174: 16–25 (Chapter 3).

My supervisors have testified that I made substantial contributions to the conceptualisation and design of the papers and that I independently ran experiments and wrote the manuscripts, with their guidance in the form of comments and suggestions (see Appendix A).

**Signature:** .....

**Date:** .....

## ABSTRACT

Legume species nodulate and grow successfully in the Core Cape Subregion, a Mediterranean-climate ecosystem with fynbos vegetation found on infertile soils. The physiological mechanisms enabling tolerance of low availability of phosphorus (P) are yet to be reported in Cape legume species such as *Aspalathus linearis* and *Podalyria calyptрата*; species that demonstrated traits typical of plants from nutrient poor soils. In the three research chapters of the thesis, it was anticipated that low P supply would limit plant growth and increase expression of traits for P acquisition.

In chapter two, the physiological basis for tolerance of limiting P supply and for enhanced growth with simultaneous addition of nitrogen (N) and P in *A. linearis* was investigated. It was hypothesised that increasing N supply would stimulate P acquisition mechanisms associated with greater P demand and enhance plant growth with high P supply. In sand, plants received 100  $\mu\text{M}$ , 300  $\mu\text{M}$ , 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N at a low P level of 10  $\mu\text{M}$  and a high P level of 100  $\mu\text{M}$ . In solution, plants received 200  $\mu\text{M}$  and 500  $\mu\text{M}$  N at a low P level of 5  $\mu\text{M}$  and a high P level of 15  $\mu\text{M}$ . Cluster roots formed only in plants with low P supply. Roots showed greater citrate and malate production and phosphatase activity at 5  $\mu\text{M}$  P than at 15  $\mu\text{M}$  P. At 10  $\mu\text{M}$  P, greater N supply enhanced cluster root formation to 60% of root biomass, and increased phosphatase activity of noncluster roots and succinate release by both root types. At high P supply of 15  $\mu\text{M}$ , greater N supply stimulated phosphatase activity of roots by 50%, increasing P-uptake and plant growth. With increased resource partitioning towards mechanisms for P acquisition due to greater P demand, *A. linearis* is tolerant of low P supply and highly responsive to combined addition of N and P.

In chapter three, the physiological basis for tolerance of low P supply in nodulated *P. calyptрата* was investigated and responses to increased supply of combined-N as  $\text{Ca}(\text{NO}_3)_2$  and P were examined. It was hypothesised that increasing supply of combined-N would stimulate P acquisition mechanisms and enhance plant growth with high P supply. Biomass, leaf [N] and [P], organic acid and phosphatase root exudates, and phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activity in nodules and roots were examined in two N  $\times$  P experiments. Low P supply decreased leaf [P] and limited plant growth, decreasing the nodule:root ratio but increasing nodular PEPC and MDH activity for enhanced P acquisition or P utilisation. Mechanisms for P acquisition such as organic acid root exudates, and PEPC and MDH activity in roots increased in response to an N-induced demand for P. Greater supply of combined-N inhibited nodulation more at low P supply than at high P supply, consistent with balanced acquisition of P and N. With high P supply, the plants nodulated prolifically meeting the P-induced demand for N, and increased supply of combined-N did not enhance plant growth. *P. calyptрата* showed coordinated regulation of P and N acquisition in

response to demand for P and N, by partitioning of resources to roots, nodules, organic acids and through glycolytic enzymes, for tolerating growth at low P supply and responding to greater P supply.

In chapter four it was investigated whether traits such as root:shoot ratio, specific root length, and organic acid exudation by roots, and the ecological niche, are conserved between closely related *Podalyria* species; *P. calytrata* and *P. burchellii* are in a separate clade to *P. leipoldtii* and *P. myrtillifolia*. The hypothesis was that closely related species would differ in their niche, and biomass allocation, specific root length and organic acid exudation responses to P supply. With increasing P supply to each species in the glasshouse, plant biomass, leaf [N], [P], root morphology and release of organic acids were measured. Furthermore, soil and leaf [N] and [P] and climate in field sites of each species were determined. Accumulation and allocation of biomass in *P. leipoldtii* was least responsive to greater P supply. *P. burchellii* allocated more biomass to roots and had fine roots similar to *P. myrtillifolia*; exudation of organic acids by thicker roots in *P. calytrata* may enhance P acquisition. In the field, leaf [P] and climate showed that *P. leipoldtii* occupied the most oligotrophic niche followed by *P. burchellii*, *P. calytrata* and *P. myrtillifolia*. Responses to P supply, and the ecological niche, differed between the closely related N<sub>2</sub>-fixing *Podalyria* species, indicating that the environment overrides phylogeny in determining P acquisition traits for these species, and suggesting that climate regulates nutrient availability, driving distribution and speciation in the fynbos.

Although responsive to increased P supply, contingent upon greater N supply, the Cape legumes have adaptive traits for tolerance of low availability of P. Plant responses were consistent with the theory that plants allocate resources for nutrient acquisition in response to demand, to maximise biomass accumulation and plant fitness. The presence of distinct traits for P acquisition between closely related but geographically separate species, indicates a crucial role for the mechanisms of P acquisition in the distribution and survival of plants in the fynbos.

## List of Abbreviations

AAT	Aspartate aminotransferase
BSA	Bovine serum albumin
C	Carbon
[C]	Concentration of C
CCR	Core Cape Subregion
dm	Diameter
DM	Dry matter
DTT	Dithiothreitol
FM	Fresh matter
IAEA	International Atomic Energy Agency
ICPAES	Inductively coupled plasma atomic emission spectrometry
IRMS	Isotope ratio mass spectrometer
LAR	Leaf area ratio
LWR	Leaf weight ratio
MAP	Mean annual precipitation
MASL	Metres above sea level
Max	Maximum
MDH	Malate dehydrogenase
ME	Malic enzyme
MES	2-Morpholinoethanesulfonic acid
Min	Minimum
N	Nitrogen
[N]	Concentration of N
OAA	Oxaloacetate
P	Phosphorus
[P]	Concentration of P
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PGPR	Plant growth promoting rhizobacteria
P <sub>i</sub>	Inorganic phosphate
PK	Pyruvate kinase
p-NP	para-Nitrophenol
p-NPP	para-Nitrophenylphosphate
PVPP	Polyvinylpolypyrrolidone
RTD	Root tissue density

sH <sub>2</sub> O	Sterile H <sub>2</sub> O
SLA	Specific leaf area
SRL	Specific root length
TCA	Tricarboxylic acid
T	Temperature
UCT	University of Cape Town

## **Acknowledgements**

I am sincerely grateful to Samson Chimphango, my main supervisor, for awarding me this project, sharing his concepts and insights with me, and for his dedicated guidance through completing the thesis. I am thankful to Muthama Muasya and Alex Valentine, my cosupervisors, for their specialist expertise, critique of the work, and use of facilities. Roland Hunter and Marcellous Le Roux kindly provided advice on the enzyme assays. I am also grateful to Simon Power, Mike Cramer and Tony Verboom for their criticism and suggestions. Field collections were made possible with permission from Table Mountain National Park, and CapeNature, permit number 0028-AAA005-00161 (25/01/2010: S. Chimphango). I thank Patrick Lane for his assistance in the field.

Funding was provided by the National Research Foundation, University of Cape Town, and the Oppenheimer Memorial Trust.

## CONTENTS

---

	Page
Declaration	iii
Abstract	iv
List of Abbreviations	vi
Acknowledgments	viii
CHAPTER 1	<b>General Introduction</b>
1.1	Legumes in the oligotrophic soils of the Cape Region 1
1.2	Mechanisms for acquisition of phosphorus 2
1.3	The interaction between N and P supply on nutrient acquisition 4
1.4	Colimitation and balanced acquisition of N and P 6
1.5	Carbon competition 7
1.6	Speciation and distribution of species 8
1.7	Aims and Rationale 9
CHAPTER 2	<b>Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species <i>Aspalathus linearis</i></b>
2.1	Introduction 12
2.2	Materials and Methods 14
2.3	Results 18
2.4	Discussion 24
2.5	Conclusion 27
CHAPTER 3	<b>Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume <i>Podalyria calyprata</i></b>
3.1	Introduction 29
3.2	Materials and Methods 31
3.3	Results 36
3.4	Discussion 42
3.5	Conclusion 44
CHAPTER 4	<b>Mechanisms for acquisition of phosphorus and growth vary in closely related <i>Podalyria</i> legume species with their ecological niche in the Cape fynbos</b>
4.1	Introduction 46
4.2	Materials and Methods 48
4.3	Results 51
4.4	Discussion 60
4.5	Conclusion 64
CHAPTER 5	<b>General Discussion and Conclusion</b> 65
References	70
Appendix A	84

## 1.1 Legumes in the oligotrophic soils of the Cape Region

In the Mediterranean-climate ecosystem of the Core Cape Subregion (CCR), the landscape is dominated by sclerophyllous fynbos vegetation (Cowling and Holmes 1992; Goldblatt and Manning 2012), found on soils of the Table Mountain Sandstone Group (Mucina and Rutherford 2006) that are low in availability of Bray II phosphorus (P) (Mitchell et al. 1984) and total nitrogen (N) (Stock and Lewis 1986). Although the vegetation is mostly mountain and coastal fynbos, the region also contains strandveld and renosterveld, growing on nutrient rich Aeolian sand and shale parent material, respectively (Witkowski and Mitchell 1987; Cowling and Holmes 1992; Richard et al. 1995; Mucina and Rutherford 2006). Witkowski and Mitchell (1987) found that strandveld soils had relatively high available P of 70 mg P kg<sup>-1</sup>. In contrast, the concentration of Bray II P, for undisturbed legumes sites in the fynbos, mostly on soils derived from sandstone, was lower, ranging from 2 to 21 mg P kg<sup>-1</sup> (Spriggs 2004; Maistry et al. 2010). The western CCR is characterised by wet winters and dry summers; in the east there is more year-round rainfall (Cowling et al. 2009). These differences in climate may further influence availability of scarce nutrients. Increased soil moisture can, for instance, increase the rate of diffusion of P to plants roots (Turner and Gilliam 1976), and warmer and wetter soil can also stimulate decomposition and mineralisation of soil nutrients (Lukac et al. 2010). Furthermore, with higher precipitation, greater levels of weathering or leaching may have a positive or negative effect, respectively, on availability of nutrients in the soil (Austin and Vitousek 1998).

Legumes nodulate and grow successfully in the oligotrophic Cape soils (Lamont 1982; Stock et al. 1995; Muofhe and Dakora 1999; Cocks and Stock 2001; Spriggs 2004); with about 805 species of Fabaceae forming the second largest plant family to Asteraceae (Goldblatt and Manning 2012). The legumes possibly benefit from N made available to the plant from a mutualistic symbiosis with rhizobia bacteria that fix atmospheric N<sub>2</sub> (Sprent 1994). In return, the host plant provides the bacteria with carbohydrates. In a glasshouse study (Maistry et al. 2013), the fynbos shrub legumes *Aspalathus linearis* (Burm.f.) R.Dahlgren (Tribe Crotalariae) and *Podalyria calyptata* (Retz.) Willd. (Tribe Podalyrieae) (Goldblatt and Manning 2012) nodulated the best out of 18 Cape legume species at extremely low P supply. These two species also demonstrated traits such as an efficient use of P, poor down-regulation of P-uptake, storage of P in the shoot, and high seed P content. These traits are typical of plants from nutrient poor soils (Hawkins et al. 2007; Ostertag 2010; Lambers et al. 2011). The physiological mechanisms enabling tolerance of low availability of P are yet to be reported in *A. linearis* and *P. calyptata*, and forms the primary objective of this thesis.

## 1.2 Mechanisms for acquisition of phosphorus

Phosphorus is an essential nutrient for plant growth and development due to its essential role in genetic, metabolic, structural and regulatory macromolecules (Marschner 1995; Raghothama and Karthikeyan 2005; White and Hammond 2008). In fynbos soil, however, 60–80% of P may be unavailable to the plant due to either adsorption into highly insoluble metal-P complexes or mineral compounds, or because P exists in the organic form (Mitchell et al. 1984; Witkowski and Mitchell 1987; Schachtman et al. 1998; Hinsinger 2001). As a result, the concentration of soil orthophosphate or inorganic phosphate ( $P_i$ ), the form of P most available to a plant, is often as low as 1 to 10  $\mu\text{M}$  in solution (Schachtman et al. 1998). Consequently, fynbos plants in the old infertile soils of the Cape Region (Hopper 2009), have evolved morphological, physiological and metabolic mechanisms to enhance the availability and acquisition of P (Lambers et al. 2006; Lambers et al. 2011), similar to traits expressed in plants at low P availability in other systems (Lajtha and Harrison 1995; Vance et al. 2003; Gahoonia and Nielsen 2004; Hammond et al. 2004; Lynch and Brown 2006; White and Hammond 2008).

### *Organic acids and phosphatase exudates*

Release of root exudates such as malate and citrate organic acids and acid phosphatase enzymes chemically increase the concentration of P ([P]) in solution, thus increasing the availability of  $P_i$  to the plant (Hinsinger 2001; Lambers et al. 2006). Organic acids solubilise and mobilise sparingly soluble inorganic Ca, Al and Fe-P sources by chelating the cations and reducing the precipitation of P, through competition with the P anion for sorption sites, by displacing sorbed P through ligand exchange, or by the formation of soluble metal-P complexes (Jones and Darrah 1994; Ryan et al. 2001). In studying the rhizosphere extracts of 11 legume species in Australia, Pang et al. (2010) showed that the concentration of organic acids in the extracts increased at low P supply of 6  $\text{mg P kg}^{-1}$  compared to high P supply of 48  $\text{mg P kg}^{-1}$ . Moreover, at low P supply, five of the six Australian species produced a greater concentration of organic acids compared to the five exotic species. In Pearse et al. (2006a), the concentration of organic acids, released per unit dry matter (DM) of roots, increased with low P supply in three *Lupinus* species and in *Pisum sativum* L., but not in *Triticum aestivum* L., *Brassica napus* L., and seven other legume species. In some instances, greater accumulation of biomass and exudation of organic acids by roots with increased supply of P was observed in *T. aestivum* (Pearse et al. 2006b) and in  $\text{N}_2$ -fixing *Lupinus angustifolius* L. (Wang et al. 2008).

Similarly, greater phosphatase activity by *Lupinus albus* L. (white lupin) roots supplied with low amounts of P (Gilbert et al. 1999; Miller et al. 2001; Wasaki et al. 2003) can enhance the availability

of P in soils by hydrolysing organic sources of P (Tarafdar and Claassen 1988; Duff et al. 1994) such as myoinositolhexaphosphate. Accordingly, in nine crop species, phosphatase activity of roots increased under conditions of low P supply (Tadano and Sakai 1991), and was greatest for roots of *L. albus*. With species varying in their organic acid and phosphatase responses to low P supply, as possible mechanisms enabling tolerance of low availability of P, the root exudate responses of Cape legumes warrants investigation.

#### *Cluster roots*

In white lupin, the formation of cluster roots is stimulated by limiting the supply of P (Keerthisinghe et al. 1998; Lambers et al. 2006; Li et al. 2008). Cluster roots are bottlebrush-like clusters of rootlets of determinate growth, that branch off lateral roots (Dinkelaker et al. 1989, 1995). The greater density of root hairs in cluster roots than noncluster lateral roots (Dinkelaker et al. 1989), although excessive for P-uptake due to overlapping zones of depletion, creates a larger surface area of cluster roots, facilitating the release of more citrate and malate (Neumann et al. 1999; Roelofs et al. 2001; Shane and Lambers 2005) and phosphatase enzymes (Reddell et al. 1997; Gilbert et al. 1999; Wasaki et al. 2003) into the rhizosphere compared with noncluster roots. In *Hakea prostrata* R.Br. (Proteaceae), for instance, exudation of citrate and malate was found to be four to fivefold greater in cluster roots than noncluster roots (Shane et al. 2004). Lynch and Brown (2006) note that although white lupin (and Australian Proteaceae) has been examined in detail, the physiology of cluster roots in other species (such as *A. linearis* (Lambers et al. 2006; Maistry et al. 2013)) has received little attention.

#### *Root biomass, morphology, and architecture*

In response to low availability of P, plants may allocate more biomass to roots than shoots, resulting in a higher root:shoot ratio than at high availability of P (Freedden et al. 1989; Nielsen et al. 2001; Poorter et al. 2012). The continued production of root biomass in the soil, by P limited plants, enables access to soil not yet depleted of P. Acquisition of P can also be enhanced by altering the morphology of roots. Specific root length (SRL), the ratio of root length to its mass, can be increased by decreasing root tissue density (RTD) or root diameter or both (Eissenstat 1991). The increase in SRL enables the plant to explore a greater volume of soil per unit DM invested in the root, resulting in more rapid rates of root proliferation and hence greater P-uptake rates than roots with a lower SRL (Eissenstat 1991; Comas et al. 2002). Therefore, SRL was observed to increase with low P supply in the 11 herbaceous legumes (Pang et al. 2010), for enhanced acquisition of nutrients. Roots that have a thin diameter, however, or that are constructed with less dense tissue, also have a faster rate of turnover (Ryser 1996; Eissenstat et al. 2000), resulting in the loss of acquired resources, which may

be a disadvantage in oligotrophic conditions. Hence slow growing species, such as those typically from nutrient poor soils (Chapin 1980), may have a low SRL (Comas et al. 2002; Comas and Eissenstat 2004; Roumet et al. 2006) for persistence of roots and conservation of resources. Increased length and number of root hairs, which results in greater root surface area in contact with a volume of  $P_i$ -containing soil (Foehse and Jungk 1983; Jungk 2001), and enhanced mycorrhizal infection for scavenging larger volumes of soil (Bolan 1991; Brundett 2002; Smith et al. 2003; Lambers et al. 2008) can increase the acquisition of P. Low availability of P can also modify root architecture; plants form shallow basal roots with more adventitious roots and dispersed lateral root formation to enhance foraging in patches of greater P availability such as the topsoil (Lynch and Brown 2001).

### *Enzymes for the metabolism of carbon in roots*

At the phosphoenolpyruvate (PEP) branch-point in glycolysis, in plants limited by P supply, modified metabolism of carbon (C) can bypass pyruvate kinase (PK) and the adenylate requiring production of pyruvate. The sequential action of increased PEP carboxylase (PEPC), malate dehydrogenase (MDH), and malic enzyme (ME) activity, can facilitate continued flow of C to the tricarboxylic acid (TCA) cycle with limited demand for P (Duff et al. 1989; Theodorou and Plaxton 1993). In addition, higher PEPC activity in cluster roots than in noncluster roots of *L. albus*, as evidenced by the greater presence of  $^{14}C$ , from PEPC-fixed  $^{14}CO_2$ , in the exuded organic acids of cluster roots than noncluster roots, may enhance the synthesis of citrate and malate (Johnson et al. 1994, 1996; Neumann and Römheld 1999). In contrast, no clear relationship was found between PEPC activity and increased exudation of organic acids in cluster roots (Keerthesinghe et al. 1998; Watt and Evans 1999; Shane et al. 2004). Vance et al. (2003) note that at low P supply, greater exudation of citrate and malate may not depend solely on enhanced expression of PEPC and MDH, but can result from reduced utilisation of the organic acids through lower rates of respiration in cluster roots (Johnson et al. 1994). Phosphoenolpyruvate carboxylase can also recycle  $P_i$  by liberating  $P_i$  from PEP when catalysing its carboxylation to oxaloacetate (OAA) (Theodorou and Plaxton 1993). In  $N_2$ -fixing plants receiving adequate P supply, PEPC also provides C for replenishing TCA cycle intermediates such as citrate, used as C skeletons in the assimilation of N (Cramer et al. 1993; Huppe and Turpin 1994) or in root exudates (Johnson et al. 1994).

### **1.3 The interaction between N and P supply on nutrient acquisition**

In previous studies (Power et al. 2010; Maistry et al. 2013), it was evident that the plants' response to P supply was dependant on the level of N supply so that the effect of N supply on P acquisition mechanisms is of interest. Greater N supply was observed to enhance the biomass of cluster roots in *H. prostrata* (Lamont 1972), and *L. albus* (Sas et al. 2002), but repress the formation of cluster roots

in other *Hakea* spp. (Lamont 1972; Paungfoo-Lonhienne et al. 2009). Some studies have also reported enhanced phosphatase activity of roots and soil with increased availability of N in soils low in P (Olander and Vitousek 2000; Treseder and Vitousek 2001; Houlton et al. 2008). Therefore, the authors predicted that the increased N supply would enhance P availability and increase plant growth in P limited soils. Their studies, however, did not demonstrate an increase in plant biomass with increased phosphatase activity. Nitrogen deposition, to low-P ecosystems such as calcareous grasslands, has been observed to increase the biomass of *Brachypodium pinnatum* L. (Bobbink 1991; Willems et al. 1993). The physiological mechanisms enabling this response, however, were not reported, but speculated to be a result of storage of P and its internal translocation by the grass species. In most instances at low availability of P, there have been no biomass responses to increased N supply (Willems et al. 1993; Carroll et al. 2003; Phoenix et al. 2003) or decreased ecosystem productivity (Bobbink 1991; Willems et al. 1993; Johnson et al. 1999) and plant growth (Lajtha and Klein 1988). It is likely, that with greater N availability at low P supply, the induction of cluster roots (Lamont 1972), and phosphatase activity (Treseder and Vitousek 2001; Johnson et al. 1999; Phoenix et al. 2003) may be plant responses to low availability of P relative to N (i.e. greater demand for P induced by N). This is because a plant can regulate its N:P ratio relative to that of the supply N:P ratio (Bloom et al. 1985; Güsewell 2004; Elser et al. 2010) to compensate for imbalances in availability of the two nutrients. Biological stoichiometry is the study of elemental balance and how living systems maintain relatively constant internal conditions in a changing environment (Elser et al. 2000; Elser et al. 2010). Although the plant N:P ratio usually reflects the soil or solution N:P ratio, it is now acknowledged that vascular plants exert a lot more control over their internal N:P ratios (Güsewell 2004; Elser et al. 2010), as demonstrated by the smaller range of N:P in the plant relative to the supply N:P range (Shaver and Melillo 1984; Lajtha and Klein 1988; Cernusak et al. 2010; Garrisch et al. 2010).

Interactions between N and P can begin at a molecular level. Changing N and P supply both induced the expression of nitrate and phosphate transporter genes in *Solanum lycopersicum* L. (tomato) (Wang et al. 2001), and 19 out of 35 genes in cluster roots of *L. albus* were also up-regulated by both N and P deficiency (Rath et al. 2010). There have been few controlled studies in the literature where plants have been simultaneously grown on a range of N and P concentrations (Lajtha and Klein 1988; Shaver and Melillo 1984). Most of the early work on plant nutrition focussed on the effects of manipulating the concentration of a single nutrient. These studies, however, are still informative in showing the reciprocal relationship between greater supply of one element and greater demand for the other element. Decreased N-uptake was associated with low P supply in tobacco, castor bean and common bean (Rufty et al. 1990; Jeschke et al. 1997; Gniazdowska et al. 1999). Within a framework of low N demand at low P supply (Imsande and Touraine 1994; Gniazdowska et al. 1999; Glass et al. 2002), two hypotheses have been proposed for the down-regulation of N-uptake. Feedback inhibition

occurs when nitrate accumulates in the root due to slow growing shoots (Gniazdowska et al. 1999), whereas amino acids, instead, not used for growth and circulating in the phloem, signal down-regulation of N-uptake (Rufty et al. 1990; Imsande and Touraine 1994; Glass et al. 2002) or N<sub>2</sub>-fixation (Parsons et al. 1993). Accordingly, the concentration of asparagine, which was 17-fold greater in the phloem sap at low P supply than at high P supply, and constituted 28% of total amino acids at low P supply (Sulieman et al. 2013), was responsible for feedback inhibition of N<sub>2</sub>-fixation (Almeida et al. 2000). In nodules, malate derived from PEPC-fixation of CO<sub>2</sub> is the main substrate for respiring bacteroids (Coker and Schubert 1981; Vance et al. 1983; Vance et al. 1985; King et al. 1986; Rosendahl 1990). With low demand for N at low P supply (Hartwig 1998; Almeida et al. 2000; Sulieman et al. 2013), however, the enhanced expression of PEPC and MDH genes, as observed in nodules and cluster roots of *L. albus* at low P supply (Uhde-Stone et al. 2003), most likely would be for allocation of C for acquisition or recycling of P, to balance P and N demand.

Results from studies on *Zea mays* L. showed that increased nitrate supply can also raise P-uptake capacity and assimilation (Cole et al. 1963; Thien and McFee 1972; Smith and Jackson 1987). Furthermore, greater demand for N associated with increased P supply can increase N-uptake (Hills et al. 1970; Jones and Dighton 1993) possibly through greater investment in N-carrier enzymes and root production (Treseder and Vitousek 2001) or in nitrate reductase activity (Gordon et al. 2001). Legumes, in addition, may increase acquisition of N through increased nodulation (Maistry 2013) and N<sub>2</sub>-fixation. A study on *Trifolium pratense* L. (Wall et al. 2000) suggested that acquisition of N was dependant on the level of P supplied. In the nodulated plants, high P supply counteracted the inhibition of nodulation by nitrate (Wall et al. 2000), possibly due to a higher demand for N associated with a low N:P supply ratio at high P supply (Smith 1992). In this regard, the typical observation of increased accumulation of plant and nodule DM (Jakobsen 1985; Israel 1987; Pereira and Bliss 1987; Drevon and Hartwig 1997), nitrogenase activity (acetylene reduction activity) (Crews 1993; Drevon and Hartwig 1997; Sa and Israel 1991) and total N in the plant (Jakobsen 1985; Sa and Israel 1991; Schulze et al. 2006), with greater P supply, may be interpreted as a response to greater demand for N associated with increased P supply, and not due to a high demand for P by the N<sub>2</sub>-fixation process as commonly perceived (Israel 1987; Hellsten and Huss-Danell 2002).

#### **1.4 Colimitation and balanced acquisition of N and P**

The physiological adjustments, described above, for acquisition of P and N in response to supply levels of the other ion, would be to bring the supply N:P ratio closer to the demand N:P ratio for growth under the prevailing conditions (Bloom et al. 1985; Houlton et al. 2008; Vitousek et al. 2010). This may be achieved by responses such as allocating resources away from the acquisition of the highly available nutrient towards acquisition of the limiting one (Bloom et al. 1985; Poorter et al.

2012), by using the nutrient available in excess to get more of the limiting nutrient (Houlton et al. 2008), by internal translocation (Güsewell 2004) and remobilisation (Lambers et al. 2011) of the limiting nutrient, or by down-regulating the uptake system of the nutrient supplied in excess amounts (Shaver and Melillo 1984; Siddiqi et al. 1990). According to the economic analysis of plant growth and resource balance (Bloom et al. 1985; Sinclair and Park 1993), plants allocate resources to maximise “profit” (biomass accumulation or fitness). Therefore, if P, rather than N, for instance, becomes limiting to growth, the plant should adjust allocation of resources towards acquisition of P so that N and P equally limit plant growth. In the book, “Resource Strategies of Wild Plants”, Craine (2009) has noted that the shift in our thinking on resource limitation needs to embrace the concept of colimitation as the rule rather than the exception, because colimitation describes resource limitation of plant growth more accurately than Liebig’s law of the minimum which proposes that plant growth is limited by one resource at any one time (Sinclair and Park 1993). In a glasshouse study (Maistry et al. 2013) and at an ecosystem (Niinemets and Kull 2005; Bishop et al. 2010) and global level (Elser et al. 2007), strong empirical support for colimitation of plant growth has been demonstrated by the prevalence of synergistic N and P colimitation responses to factorial addition of N and P, as inferred from a model (Craine and Jackson 2010; Harpole et al. 2011) that examines possible biomass responses to simultaneous addition of N and P relative to ambient N and P supply. The classic colimitation response of synergistic growth is a result of N and P being supplied in a ratio close to that of plant demand (Davidson and Howarth 2007). In contrast, independent increases in N or P supply result in accumulation of the added nutrient (Craine et al. 2008), and once storage by vacuoles, for example, is exceeded, can induce limitation by the other nutrient as in Liebig’s law of the minimum. According to Davidson and Howarth (2007), however, there is an absence of data from controlled experiments investigating in a more mechanistic way the conditions under which N and P can limit plant growth as outlined in the theoretical analysis of Bloom et al. (1985) and in the meta-analyses of ecosystem productivity (Elser et al. 2007; Harpole et al. 2011).

## **1.5 Carbon competition**

In contrast to the balanced and flexible acquisition of N and P by plants, the mechanisms for enhanced acquisition of P may have a negative effect on N<sub>2</sub>-fixation and assimilation of N. Low demand for N at low P supply (Hartwig 1998; Almeida et al. 2000; Sulieman et al. 2013) may not be responsible for reduced allocation of resources to N<sub>2</sub>-fixation. Responses to meet the greater demand for P may, instead, penalise N<sub>2</sub>-fixation and assimilation of the fixed N<sub>2</sub>, because the diversion of up to 25% of plant C for exudation of citrate and malate (Dinkelaker et al. 1989; Lambers et al. 2006) could compete with energy supply for N<sub>2</sub>-fixation and plant growth. Le Roux et al. (2008) reported that the diversion of C for enhanced synthesis of organic acids in *L. angustifolius* may compete with energy supply for N<sub>2</sub>-fixation and N assimilation and consequently limit plant growth. Conversely, whereas

*L. albus* cluster roots exuded 20 to 40-fold more exudates at low P than high P supply, N<sub>2</sub>-fixation in *L. albus* did not decrease (Vance et al. 2003). The theory that P acquisition could negatively affect N<sub>2</sub>-fixation is based on the assumption that C may be limiting at low P supply. Increased supply of C at low P supply, however, did not increase biomass accumulation (Stöcklin et al. 1998; Almeida et al. 1999). Furthermore, decreased shoot growth in P deficient plants was due to lowered leaf expansion (Radin and Eindenbock 1984; Freeden et al. 1989) and thus photosynthetic capacity was lowered due to reduced sink demand for C (Pieters et al. 2001). Starch accumulation in leaves and roots of low P supplied soybean indicates that more C is assimilated than can be used in growth (Freeden et al. 1989). Hence, it appears that at low P supply, the plant can utilise the excess C assimilated when plant growth is limited by factors other than photosynthesis. It is also thought that N<sub>2</sub>-fixation may incur a high demand for C. Three studies cited in Pate et al. (1979) show a higher respiratory cost for N<sub>2</sub>-fixing than NO<sub>3</sub>-supplied plants, whereas two studies found no greater C cost for the N<sub>2</sub>-fixers. Therefore it is important to assess the effect of P acquisition and N acquisition on plant growth.

## **1.6 Speciation and distribution of species**

Closely related species generally show trait or niche conservatism (phylogenetic signal, Webb 2000; Prinzing et al. 2001; Wiens 2004). Therefore, physiological experiments that compare closely related species are considered to be more rigorous, giving greater confidence that where significant trait or ecological differences are found between species, the differences can be considered functional or adaptive (Fine et al. 2005). Roumet et al. (2006) compared root traits between (fast-growing) annuals and (slow-growing) perennials in three families, Fabaceae, Asteraceae and Poaceae. They found an absence of phylogenetic signal because the life history traits were conserved across the three families; within each family, annuals, compared to perennials, had roots with higher SRL and [N] and lower RTD. Because the closely related annual and perennial species were physiologically different, these results provided strong evidence for distinct strategies of resource acquisition in annuals but resource conservation in perennials, as previously theorised (Grime 1977; Chapin 1980). Similarly, Cavender-Bares et al. (2004) found that closely related oak species occurred in habitats with contrasting soil moisture, suggesting that moisture may influence speciation in the oaks. Several authors have proposed that variation in availability of nutrients in the soil may drive the distribution patterns of species in the CCR (Cowling and Holmes 1992; Richards et al. 1997) because some species could possess traits for enhanced acquisition of nutrients and growth in more oligotrophic soils compared to species lacking these traits (Lamont 1982; Richards et al. 1997; Lambers et al. 2008; Shane et al. 2008). Beadle (1954, 1962) and Ozanne and Specht (1981) have also implicated soil P as a major factor determining species distribution in the heathland vegetation of Australia. This argument for speciation, being due to ecophysiological specialisation and niche segregation, is in contrast to Wiens (2004), who argued that the tendency of closely related species to conserve their ancestral niche can

lead to most speciation. Given the diverse climatic (Cowling et al. 2009) and edaphic (Richards et al. 1997) conditions in the CCR and the effect of climate on nutrient availability (Turner and Gilliam 1976; Austin and Vitousek 1998; Lukac et al. 2010), differences in P acquisition traits between closely related legume species that are geographically separated, would emphasise that these traits play a critical role in the distribution and survival of plants in the Cape.

## 1.7 Aims and Rationale

In this study, observing the plant responses to low availability of P and to ecologically relevant levels of increasing N supply (Glass et al. 2002; Britto and Kronzucker 2005), to induce greater demand for P, were of interest. Colimitation of the growth of legume plants provide an ideal opportunity for clearly observing the effects of changing N and P supply on biomass and biochemical partitioning in the plant. Therefore, a major novelty of the study lies in investigating in a more mechanistic way the conditions under which N and P can limit plant growth. In this regard, the use of species that form cluster roots or nodules was advantageous because formation of cluster roots is a clear indication of inadequate availability of P and formation of nodules clearly increases with N limitation. Closely related species that have an allopatric distribution are also compared, to emphasise the relevance of P acquisition traits for survival in fynbos conditions.

Thus the objectives of the study were to:

- a) investigate the physiological basis for tolerance of limiting P supply in Cape legume species
- b) examine responses of P acquisition mechanisms and biomass to increased supply of combined-N at low P and high P supply in *A. linearis* and *P. calytrata*
- c) assess the effect of N and P acquisition on plant growth at low P supply
- d) investigate whether traits for P acquisition and the ecological niche differed between closely related *Podalyria* species.

Therefore *A. linearis* (Chapter 2) and *P. calytrata* (Chapter 3) received low and high P supply while increasing the supply of combined-N. It was anticipated that low P supply would limit plant growth and stimulate the expression of P acquisition traits. It was hypothesised that increased supply of combined-N would stimulate P acquisition mechanisms associated with greater P demand and result in enhanced plant growth with high P supply. The study on *A. linearis* (Chapter 2) was designed to address objectives (a) and (b); the study on *P. calytrata* (Chapter 3) considered objectives (a–c). Furthermore, four *Podalyria* species (Chapter 4) were studied in the glasshouse and in their natural habitats in the CCR, to address objectives (a) and (d). Again it was anticipated that all four species would increase the expression of traits for acquisition of P with low P supply. It was hypothesised that the two species in each closely related pair of *P. calytrata* and *P. burchellii*, and of *P. leipoldtii*

and *P. myrtilifolia*, would differ in their biomass allocation, SRL and organic acid exudation responses to P supply, and that the two closely related species would occupy a different ecological niche in the fynbos.

---

**2**      **Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis***

---

## 2.1 Introduction

The shrub legume *A. linearis* is found in a Mediterranean-type ecosystem, the CCR (Manning and Goldblatt 2012), where plant-available N and P are scarce resources (Stock and Lewis 1986; Witkowski and Mitchell 1987). Renowned for the commercially important Rooibos herbal tea produced from the shoots of wild or cultivated plants (Morton 1983), *A. linearis* occurs in the northern and western fynbos of the CCR on well drained, oligotrophic, sandstone soils with available P of  $\approx 10 \text{ mg kg}^{-1}$  and total N of  $\approx 1 \text{ g kg}^{-1}$  (Hawkins et al. 2011). In a glasshouse study (Maistry et al. 2013), *A. linearis* demonstrated traits such as an efficient use of P, poor down-regulation of P-uptake, storage of P in the shoot, and high seed P content. These traits are typical of plants from nutrient poor soils (Hawkins et al. 2007; Ostertag 2010; Lambers et al. 2011). Therefore *A. linearis* may be tolerant of growth in soils with low availability of P. The physiological basis for this tolerance of limiting supply of P is yet to be reported.

Cluster roots have been observed in *A. linearis* plants growing in the field and in the glasshouse (Lambers et al. 2006; Hawkins et al. 2011; Maistry et al. 2013). These root structures are bottlebrush-like clusters of densely hirsute rootlets of determinate growth, that branch off lateral roots (Dinkelaker et al. 1989, 1995). Their formation is stimulated by limiting the supply of P (Keerthisinghe et al. 1998; Li et al. 2008). In *L. albus* and *H. prostrata*, the greater surface area of cluster roots (Shane and Lambers 2005) may facilitate the release of more citrate and malate into the rhizosphere compared to noncluster roots (Neumann et al. 1999; Roelofs et al. 2001; Shane et al. 2004). The concentrations of organic acids released by roots were shown to increase with low P supply in studies on wheat, canola and 11 legume species (Pearse et al. 2006; Pang et al. 2010). Organic acids may enhance the availability of P in the rhizosphere by solubilising and mobilising inorganic or sorbed sources of P through chelation of cations or by ligand exchange (Hinsinger 2001; Ryan et al. 2001). In addition, greater phosphatase activity of cluster roots in comparison to noncluster roots may further enhance the availability of P by hydrolysing organic sources of P (Tarafdar and Claassen 1988; Reddell et al. 1997; Gilbert et al. 1999), especially with soil organic matter accounting for 60–80% of P in fynbos soils (Mitchell et al. 1984; Witkowski and Mitchell 1987).

Although low availability of P stimulates the formation of cluster roots, the role of N, however, is not as clear. Application of low levels of N at low P supply enhanced the biomass of cluster roots compared with no N supply (Lamont 1972), whereas higher N supply repressed its formation in *Hakea* spp. (Lamont 1972; Paungfoo-Lonhienne et al. 2009). In contrast, *L. albus* plants with the highest shoot N concentration ([N]) produced more cluster roots than plants with lower shoot [N] (Sas et al. 2002). Some studies have also reported enhanced phosphatase activity of roots and soil with increased availability of N in soils low in P (Olander and Vitousek 2000; Treseder and Vitousek 2001;

Houlton et al. 2008). Lamont et al. (2014) investigated the role of plant growth promoting rhizobacteria (PGPR) on cluster root formation and speculated that addition of N may, through increasing the metabolic activity of the PGPR, stimulate their production of chemical compounds that enhance formation of cluster roots. Although cluster roots do also take up organic N (Hawkins et al. 2005), given the role of cluster roots in P acquisition, it is more likely that the induction of cluster roots and phosphatase activity by greater N supply may be due to low availability of P relative to N (i.e. greater demand for P induced by N (Phoenix et al. 2003)). These effects of N supply on the mechanisms for P acquisition, and observations of N and P colimitation of plant growth (Power et al. 2010; Maistry et al. 2013), imply that plant responses to N and P will depend on the relative supply levels of the two nutrients. Therefore, in *A. linearis*, the interactive effects of N and P supply on P acquisition responses such as cluster root biomass and phosphatase enzymes, needs to be closely examined, and may provide an ideal opportunity for clearly observing the effects of changing N and P supply on biomass and biochemical partitioning in the plant.

Although a plant's N:P ratio usually reflects the soil or solution N:P ratio, a plant can regulate its N:P ratio relative to that of the supply N:P ratio (Güsewell 2004; Elser et al. 2010). In *S. lycopersicum*, the supply of N and P both induced the expression of both nitrate and phosphate transporter genes (Wang et al. 2001), indicating that interactions of N and P begin at a molecular level. Furthermore, the P-induced demand for N associated with increased P supply increased N-uptake possibly through greater investment in N-carrier enzymes and root production (Hills et al. 1970; Rufty et al. 1990; Treseder and Vitousek 2001), while increased nitrate supply raised P-uptake capacity and assimilation (Thien and McFee 1972; Smith and Jackson 1987). A plant can regulate its N:P ratio by allocating resources away from the acquisition of the highly available nutrient towards acquisition of the limiting one (Bloom et al. 1985; Vitousek et al. 2010; Poorter et al. 2012). This may also be achieved by using the nutrient available in excess to get more of the limiting nutrient (Houlton et al. 2008), by internal translocation of the limiting nutrient (Güsewell 2004), or by down-regulating the uptake system of the nutrient supplied in excess amounts (Shaver and Melillo 1984; Siddiqi et al. 1990). Ideally, these adjustments for acquisition of N and P would bring the supply N:P ratio closer to the demand N:P ratio for growth under the prevailing conditions (Bloom et al. 1985). Some plants from the CCR, however, show a weak ability to down-regulate uptake of P when supplied excess P (Shane et al. 2008; Power et al. 2010; Maistry et al. 2013). Although the accumulation of P may be adaptive in environments where nutrient supply is uncertain (Ostertag 2010), with increases in P supply giving lower shoot N:P ratios than expected (Matzek and Vitousek 2009), further accumulation beyond the storage capacity of vacuoles (Ryan et al. 2009) results in nutrient imbalances and decreased growth (Fujita et al. 2010; Maistry et al. 2013).

According to the optimal resource allocation strategy of Bloom et al. (1985), if P, rather than N, becomes limiting to growth for instance, the plant should adjust allocation of resources towards acquisition of P so that N and P equally limit plant growth. This theory of multiple resource limitation is said to describe resource limitation of plant growth more accurately than Liebig's law of the minimum which posits that plant growth is limited by one resource at any one time (Bloom et al. 1985; Vitousek et al. 2010). At an ecosystem and global level, strong empirical support for colimitation of plant growth has been demonstrated by the prevalence of synergistic N and P colimitation responses to factorial addition of N and P (Elser et al. 2007; Craine and Jackson 2010; Harpole et al. 2011). A synergistic colimitation effect is said to occur when the biomass increase to simultaneous addition of N and P, relative to ambient N and P supply, is superadditive (i.e. it is greater than the sum of the responses to independent addition of N and independent addition of P), and emphasises the positive interactive effect of multiple resources on plant growth (Fageria 2001; Harpole et al. 2011). As suggested by Davidson and Howarth (2007), however, there is an absence of data from controlled experiments investigating in a more mechanistic way the conditions under which N and P can limit plant growth as outlined in the theoretical analysis of Bloom et al. (1985) and in the meta-analyses of ecosystem productivity (Elser et al. 2007; Harpole et al. 2011).

The aim of this study was to report on the physiological basis for tolerance of limiting supply of P and for synergistic growth responses to greater N and P supply in *A. linearis*. This was investigated by studying the P acquisition mechanisms as N supply increased at low and high availability of P. It was anticipated that low P supply would limit plant growth and increase expression of traits for P acquisition. It was hypothesised that increasing N supply would stimulate mechanisms for P acquisition due to greater demand for P and result in enhanced plant growth with high P supply. The responses of plant biomass, shoot or leaf [N], [P], and N:P ratio, and phosphatase and organic acid production by cluster and noncluster roots to N and P supply were examined in two N × P experiments.

## **2.2 Materials and methods**

### *Growth conditions for sand culture*

Scarified seeds of *Aspalathus linearis* (Burm.f.) R.Dahlgren ( $232 \pm 17 \mu\text{g N seed}^{-1}$ ,  $38 \pm 1 \mu\text{g P seed}^{-1}$ ) were obtained from Heiveld Cooperative, Nieuwoudtville, Northern Cape, South Africa. Seeds were soaked overnight in water then sown in trays containing acid-washed sand and kept moist until emergence. Four weeks after emergence, seedlings of similar size were transplanted into plastic pots filled with 3 kg of acid-washed sand and thinned to one seedling per pot after a further 4 weeks. The seedlings were watered three times a week with 200 mL of nutrient solution (pH 6.2). The

concentration of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{KH}_2\text{PO}_4$  in the nutrient solution was adjusted to supply 100  $\mu\text{M}$ , 300  $\mu\text{M}$ , 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N at a low P level of 10  $\mu\text{M}$  and a high P level of 100  $\mu\text{M}$ . Therefore, the experimentally administered N:P supply ratios (by mass) at low P supply were 9, 27, 45 and 63 with increasing N supply; at high P supply the ratios were 1, 3, 5 and 6. The appropriate volume of  $\text{CaSO}_4$  solution was added to the nutrient solution to compensate for the adjustments in  $\text{Ca}(\text{NO}_3)_2$  so that all plants received 200  $\mu\text{M}$  Ca. The complete nutrient solution also contained: 200  $\mu\text{M}$   $\text{K}_2\text{SO}_4$ ; 54  $\mu\text{M}$   $\text{MgSO}_4$ ; 0.24  $\mu\text{M}$   $\text{MnSO}_4$ ; 0.10  $\mu\text{M}$   $\text{ZnSO}_4$ ; 0.02  $\mu\text{M}$   $\text{CuSO}_4$ ; 2.4  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ ; 0.03  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ ; 10  $\mu\text{M}$  Fe-EDTA. Pots were flushed with 1 L of tap water once a week to prevent salt accumulation. Plants were grown for 22 weeks from December 2010 to June 2011 in a glasshouse at the University of Cape Town (UCT) (33°57.353'S 18°27.742'E) with an average daytime temperature of 27°C controlled in the range of 20–30°C. There were five pots as replicates ( $n=5$ ) for each of the eight treatments. Pots were placed on trolleys that were rearranged weekly.

#### *Growth conditions for solution culture*

Root physiology responses to nutrient deficiency may be different between plants cultured in sand substrate and nutrient solution, possibly due to differences in zones of depletion around the root and buffering capacity in the two growth media. Many studies, however, report results on plants grown in nutrient solution (Neumann et al. 1999; Roelofs et al. 2001; Shane et al. 2004). Without sand as a buffer, hydroponic culture has the advantages of greater control and certainty over the supply N:P ratio, and less handling of roots during harvesting. Therefore, in addition to the plants cultivated in sand, *A. linearis* plants were also grown in aerated nutrient solution. Four weeks after emergence, seedlings of similar size were transplanted into individual seedling trays containing acid-washed sand and grown for 8 weeks until of a suitable size and vigour to enable the seedling to be transferred to a hydroponic system. The plant was gently removed from the sand and inserted through a hole in the lid of a 5-L black plastic bucket and supported by a loop of black foam around the base of the stem. The lid, holding four seedlings, was placed over the 5-L bucket containing aerated water which was changed to nutrient solution (pH 6.2) after 3 d. After 4 weeks, seedlings were thinned to one per bucket. The N and P treatments, were a factorial design with 200  $\mu\text{M}$  and 500  $\mu\text{M}$  N in a low P level of 5  $\mu\text{M}$  and a high P level of 15  $\mu\text{M}$ . These concentrations of 5  $\mu\text{M}$  and 15  $\mu\text{M}$  P were selected to represent low and high P supply because in preliminary experiments conducted in hydroponic culture, a higher level of P such as 100  $\mu\text{M}$  P was found to be extremely toxic to plant growth. With the levels of N and P supplied, the experimentally administered N:P supply ratio (by mass) at a low P supply increased from 36 to 90 and increased from 12 to 30 at a high P supply. Nitrogen was supplied as  $\text{Ca}(\text{NO}_3)_2$  and P was supplied in a 1:1 ratio of  $\text{KH}_2\text{PO}_4$ :phytic acid (inositolhexaphosphate). The nutrient solution was changed once a week. Plants were grown for 19 weeks from October 2010 to

February 2011 in the glasshouse at UCT. There were four buckets as replicates ( $n=4$ ) for each of the four treatments.

### *Plant biomass*

At harvest, roots were gently washed in water to remove sand. Each plant was separated into shoots or leaves and stems, and noncluster roots and cluster roots. Cluster roots were rinsed meticulously over a 1000- $\mu\text{m}$  sieve to remove sand. Plants did not form nodules in either experiment. Roots were not examined for arbuscular mycorrhizal structures as the plants were not provided with mycorrhiza inoculum during culture. Root death was evident in the plants receiving the combined supply of 100  $\mu\text{M}$  P and 100  $\mu\text{M}$  N, with roots appearing brown in colour, limp and flimsy. Plant material was dabbed dry with paper towels prior to determining weight of fresh matter (FM). Plant material was dried at 60°C for 3 d and reweighed for DM. Prior to weighing, dried cluster roots were inspected in a plastic container for any remaining trapped sand granules. Samples were milled in a Wiley Mill using a 0.5 mm mesh (Arthur H. Thomas Co. Philadelphia, CA, USA).

### *[N] and [P] analysis of plant tissue*

For [N] analysis, between 1.9 and 2.1 mg of milled plant sample was weighed into 8 mm  $\times$  5 mm tin foil capsules (Elemental Microanalysis Ltd, Okehampton, UK) on a micro balance (Sartorius AG, Göttingen, Germany). The capsules were folded to enclose the samples which were combusted in a Flash EA 1112 series elemental analyser (Thermo Electron, Milan, Italy). The resulting gases were fed into a Delta Plus XP IRMS (isotope ratio mass spectrometer) (Thermo Finnigan, Bremen, Germany), via a Conflo III gas control unit (Thermo Finnigan, Germany). The in-house standards used were Merck Gel, a proteinaceous gel produced by Merck (Darmstadt, Germany), and dried leaves of *Tropaeolum majus* L. (common nasturtium), collected from Woodbine Lane on UCT campus. The in-house standards have been calibrated against International Atomic Energy Agency standards. [P] was analysed using inductively coupled plasma atomic emission spectrometry (ICPAES) (Varian Vista MPX ICP-AES, Varian, Mulgrave, Australia) after dry-ashing pulverised plant material at 480°C for 8 h and dissolving in 16% HCl (Kalra 1998).

### *Acid phosphatase assay of roots*

The phosphatase assay was performed on excised roots, as in the determination of phosphatase activities in root exudates (Gilbert et al. 1999) and in the determination of extracellular phosphatase activity of roots (Treseder and Vitousek 2001). The cluster roots and noncluster fine roots were gently blotted dry on tissue paper and then weighed for a FM of between 300 and 600 mg. For cluster

roots, only the cluster roots that appeared cream in colour and that were considered to be mature developed cluster roots were selected to be assayed. Roots were kept on ice in a sterile 20-mL plastic pill vial during harvesting and then stored at  $-20^{\circ}\text{C}$  before analysis. Following an assay procedure adapted from Tabatabai and Bremner (1969), the roots were incubated in the dark in 4 mL of 15 mM 2-morpholinoethanesulfonic acid (MES) and 500  $\mu\text{M}$   $\text{CaCl}_2$  (pH 5.5) and 1 mL of 10 mM para-nitrophenylphosphate (p-NPP) at  $28^{\circ}\text{C}$  for 30 min. The reaction was terminated with 4 mL of 0.5 M NaOH and 1 mL of 0.2 M  $\text{CaCl}_2$  and the assayed root material was recovered and dried in an oven, and DM recorded. For the blank, roots were excluded from the reaction mixture. As a control, a composite sample of cluster or noncluster fine root material was incubated in 4 mL of MES and  $\text{CaCl}_2$  as above. The reaction was terminated with NaOH and  $\text{CaCl}_2$  and then 1 mL of 10 mM p-NPP was added to the test tube. Acid phosphatase activity of roots was based on the amount of para-nitrophenol (p-NP) released as measured spectrophotometrically at 412 nm relative to known p-NP standards and expressed in terms of phosphatase activity per unit of DM ( $\mu\text{mol p-NP g}^{-1} \text{DM h}^{-1}$ ). The assay reading was corrected by subtracting the control absorbance reading from experimental assay readings.

#### *Collection and analysis of organic acid root exudates*

Excised cluster root and noncluster fine roots were gently blotted dry on tissue paper, and then weighed for between 300 and 600 mg FM. Root material was kept on ice in a sterile 20-mL pill vial during harvesting. A volume of 15 mL of sterile  $\text{H}_2\text{O}$  ( $\text{sH}_2\text{O}$ ), containing 200  $\mu\text{M}$   $\text{CaCl}_2$  for electrolytic conductivity and maintaining cellular integrity (Pearse et al. 2006) was then added to each vial, submerging the roots. Organic acids were collected by agitating vials on an orbital shaker (160 rpm; Laboratory Marketing Services, Roodepoort, South Africa) for 1 h at  $25^{\circ}\text{C}$ . The solution was filtered through filter paper and the root material recovered, dried in an oven and its DM recorded. The filtrate was filtered through a 20- $\mu\text{m}$  Acrodisc syringe filter (GVS Filter Technology, Indianapolis, IN, USA) and stored at  $-20^{\circ}\text{C}$  until freeze-dried and resuspended in 1 mL of  $\text{sH}_2\text{O}$  for analysis.

Based on organic acids included in previous studies (Pearse et al. 2006; Pang et al. 2010), root exudates were assayed for the concentration of citrate, malate, succinate, lactate and acetate. Oxalate was omitted from the study but has been reported in root exudates of forest tree species (Smith 1976); in pine seedlings associated with ectomycorrhiza that release oxalate, P availability may be enhanced (Gadd 1999; Casarin et al. 2004). The organic acids were analysed at the Central Analytical Facility, Stellenbosch University, using enzymatic test kits in an Arena 20XT Enzyme Robot (Thermo Electron Oy, Vantaa, Finland). The concentration of malate, lactate and acetate in the sample was determined photometrically by measuring the increase in absorbance at 340 nm (Enzytec™ Fluid

enzyme kits, Thermo Fisher Scientific Oy, Vantaa, Finland), while the decrease in absorbance associated with the oxidation of NADH was used to determine the concentration of citrate and succinate (Yellow line enzyme kits, Roche, R-Biopharm AG, Darmstadt, Germany).

### *Statistical analysis*

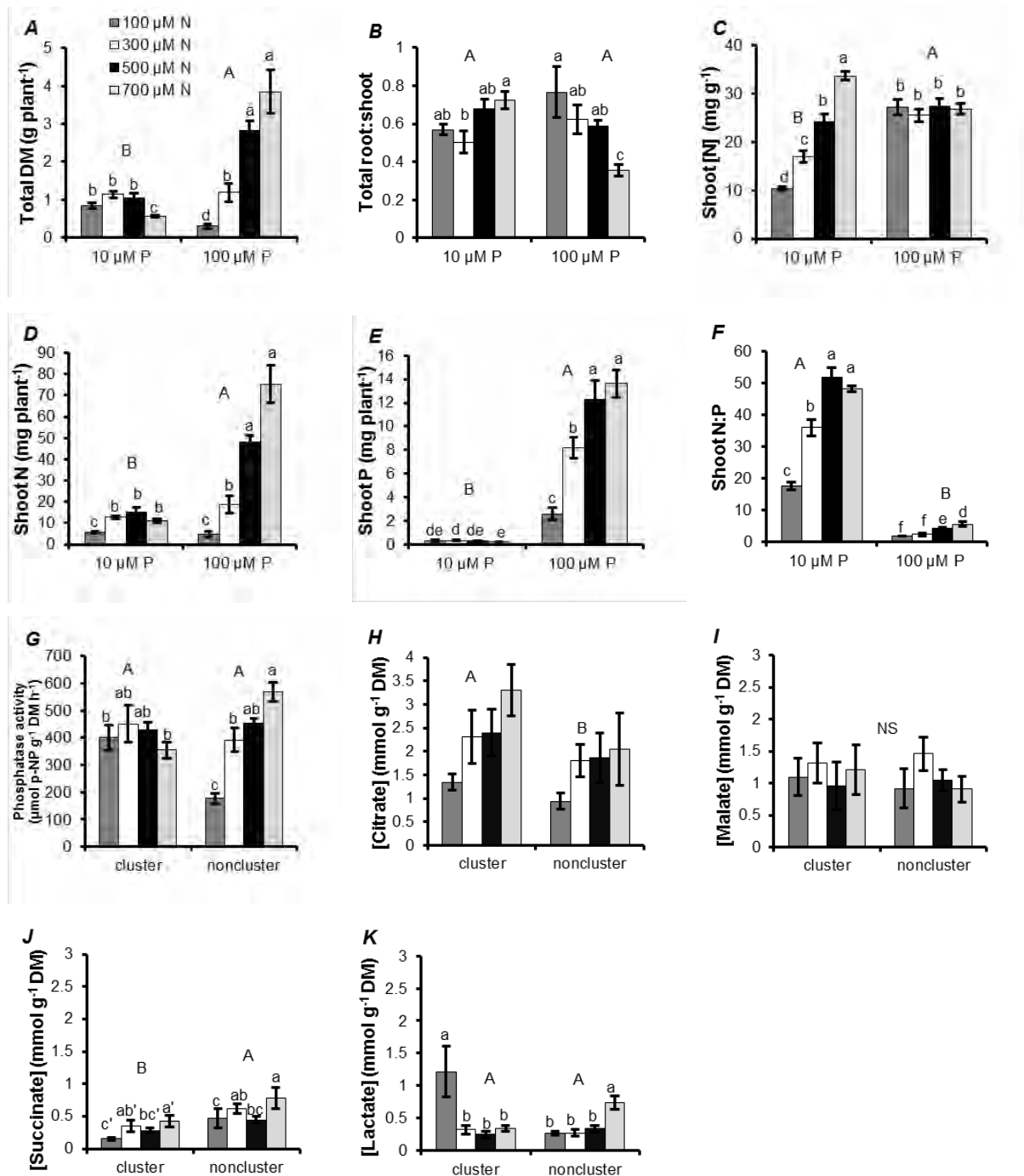
To reduce heteroscedasticity, all measurements were  $\log_e$  transformed before statistical analysis. To test the  $N \times P$  interaction, a factorial ANOVA was performed (STATISTICA version 11, StatSoft, Tulsa, OK, USA). Means that were significantly different at  $P < 0.05$  were separated by Duncan's multiple range test. The effect of N supply on the percent cluster roots of total root DM in plants receiving low P supply was assessed using one-way ANOVA. Pearson's correlation coefficients were used to test statistical relationships between phosphatase activity of noncluster roots and shoot [N] and shoot N:P ratio.

## **2.3 Results**

### *Sand culture*

#### Biomass

There was a significant interaction (Fig. 1A,  $F_{3,32}=47.79$ ,  $P < 0.001$ ) between N and P supply on biomass accumulation. Increasing P supply to the plants receiving 100  $\mu\text{M}$  N, from 10 to 100  $\mu\text{M}$  P, reduced biomass accumulation by threefold, but had no effect on biomass at 300  $\mu\text{M}$  N supply, and increased the biomass formed in plants supplied with 500 and 700  $\mu\text{M}$  N. At low P supply, increasing N supply to 500  $\mu\text{M}$  had no effect on biomass, whereas 700  $\mu\text{M}$  N supply decreased biomass by 50%. At high P supply, however, greater supply of 300  $\mu\text{M}$  N increased biomass accumulation by fourfold, and by a further twofold with 500  $\mu\text{M}$  N, with no significant increase thereafter. There was also a significant  $N \times P$  interaction (Fig. 1B,  $F_{1,32}=10.78$ ,  $P < 0.001$ ) on total root:shoot ratio. Increasing P supply decreased the total root:shoot ratio at 700  $\mu\text{M}$  N supply but had no effect on the ratio at the lower levels of N supply. Furthermore, increasing N supply increased the total root:shoot ratio at low P supply but decreased the ratio at high P supply. Cluster roots did not form in plants receiving high P supply. At low P supply, however, increasing N supply from 100  $\mu\text{M}$  to 300  $\mu\text{M}$  dramatically increased the proportion of cluster roots from 25% to nearly 60% of total root DM (Fig. 2A,  $F_{3,16}=6.64$ ,  $P < 0.01$ ); this proportion remained the same at 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N supply.



**Fig. 1.** Effect of P and N supply on (A) total dry matter (DM), (B) total root:shoot ratio, (C) shoot N concentration ([N]), (D) shoot N content, (E) shoot P content, and (F) shoot N:P ratio in *Aspalathus linearis* grown in sand culture. Effect of N supply and root type on (G) phosphatase activity, and (H) citrate, (I) malate, (J) succinate and (K) lactate concentrations in plants receiving 10 μM P. Means ± s.e. ( $n=5$ , except H–K, where  $n=4$ ). Different lowercase letters indicate significantly different means between P and N levels at  $P < 0.05$  from a factorial ANOVA. Different lowercase letters with a prime symbol (') indicate significantly different means where no N × P interaction occurred; different uppercase letters indicate significantly different means between P levels.

## Shoot concentration and amount of N and P, and N:P ratio

A significant N × P interaction was evident on shoot [N] (Fig. 1C,  $F_{3,32}=43.17$ ,  $P<0.001$ ) and shoot N content (Fig. 1D,  $F_{3,32}=19.99$ ,  $P<0.001$ ). Increasing P supply at 100  $\mu\text{M}$  and 300  $\mu\text{M}$  N supply increased shoot [N]. With greater P supply at 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N, however, shoot [N] was similar or lower than the respective plants receiving low P supply (Fig. 1C), possibly due to a dilution effect because the plants with greater P supply accumulated more N in their shoots (Fig. 1D). Increasing N supply increased shoot [N] at low P supply but had no effect on shoot [N] at high P supply (Fig. 1C), possibly due to the diluting effect of greater biomass (Fig. 1D). A significant N × P interaction also occurred on shoot [P] (Table 1,  $F_{3,32}=13.36$ ,  $P<0.001$ ) and shoot P content (Fig. 1E,  $F_{3,32}=21.32$ ,  $P<0.001$ ). Increasing P supply increased shoot [P] at all levels of N supply. Increasing N supply did not change shoot [P] at low P supply, but decreased shoot [P] at high P supply (Table 1) possibly as a dilution effect since the plants accumulated more P with greater N supply (Fig. 1E). With greater P supply, the shoot N:P ratio decreased (Fig. 1F,  $F_{3,32}=4.01$ ,  $P<0.05$ ) at all levels of N supply. At low P supply, the shoot N:P ratio of 18 increased with 300  $\mu\text{M}$  N supply, up to 52 at 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N supply; at high P supply, the shoot N:P ratio of 2 increased to 4 and 6 with application of 500  $\mu\text{M}$  and 700  $\mu\text{M}$  N respectively.

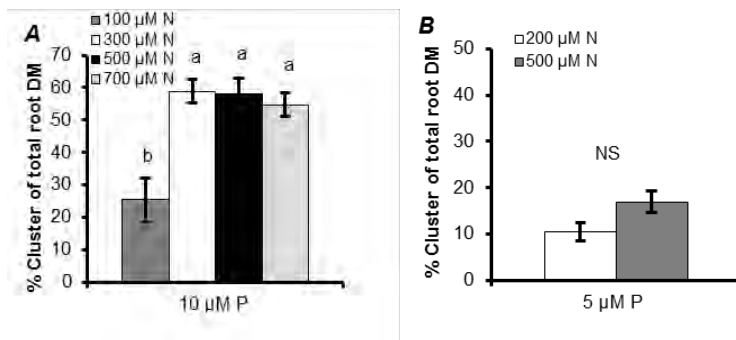
**Table 1.** Effect of P and N supply on shoot P concentration ([P]) in *Aspalathus linearis* grown in sand culture. Means  $\pm$  s.e. ( $n=5$ ). Different lowercase letters indicate significantly different means between P and N levels ( $F_{3,32}=13.36$ ,  $P<0.001$ ) from a factorial ANOVA.

N ( $\mu\text{M}$ )	Shoot [P] ( $\text{mg g}^{-1}$ )	
	10 $\mu\text{M}$ P	100 $\mu\text{M}$ P
100	0.60 $\pm$ 0.04 <sup>cd</sup>	15.08 $\pm$ 1.18 <sup>a</sup>
300	0.48 $\pm$ 0.04 <sup>d</sup>	12.54 $\pm$ 1.81 <sup>a</sup>
500	0.48 $\pm$ 0.05 <sup>d</sup>	7.24 $\pm$ 1.44 <sup>b</sup>
700	0.70 $\pm$ 0.03 <sup>c</sup>	5.12 $\pm$ 0.62 <sup>b</sup>

## Phosphatase activity and organic acid exudation of roots

At low P supply, the phosphatase activity and organic acid exudation of cluster roots and noncluster roots was compared. Overall, there was no difference in phosphatase activity of cluster roots and noncluster roots when expressed on a DM basis (Fig. 1G). Increasing N supply, however, increased ( $F_{3,32}=15.10$ ,  $P<0.001$ ) the phosphatase activity of noncluster roots more than threefold but not that of cluster roots. The phosphatase activity of noncluster roots showed a strong positive correlation with

shoot [N] ( $R^2=0.76$ ,  $P<0.01$ ) and shoot N:P ratio ( $R^2=0.66$ ,  $P<0.01$ ). On a DM basis, cluster roots released greater amounts of citrate (Fig. 1H,  $F_{1,24}=4.3$ ,  $P<0.05$ ) than noncluster roots, whereas the increased exudation of citrate with greater N supply was not significant ( $P<0.05$ ). The exudation of malate (Fig. 1I) was similar between both root types. In contrast, noncluster roots released more succinate (Fig. 1J,  $F_{1,24}=22.19$ ,  $P<0.001$ ) than cluster roots, and increasing N supply stimulated greater release of succinate in both types of roots ( $F_{3,24}=5.43$ ,  $P<0.01$ ). With greater N supply, lactate exudation by cluster and noncluster roots decreased and increased (Fig. 1K,  $F_{3,24}=7.89$ ,  $P<0.001$ ) respectively. Acetate was not detected in the analysis.

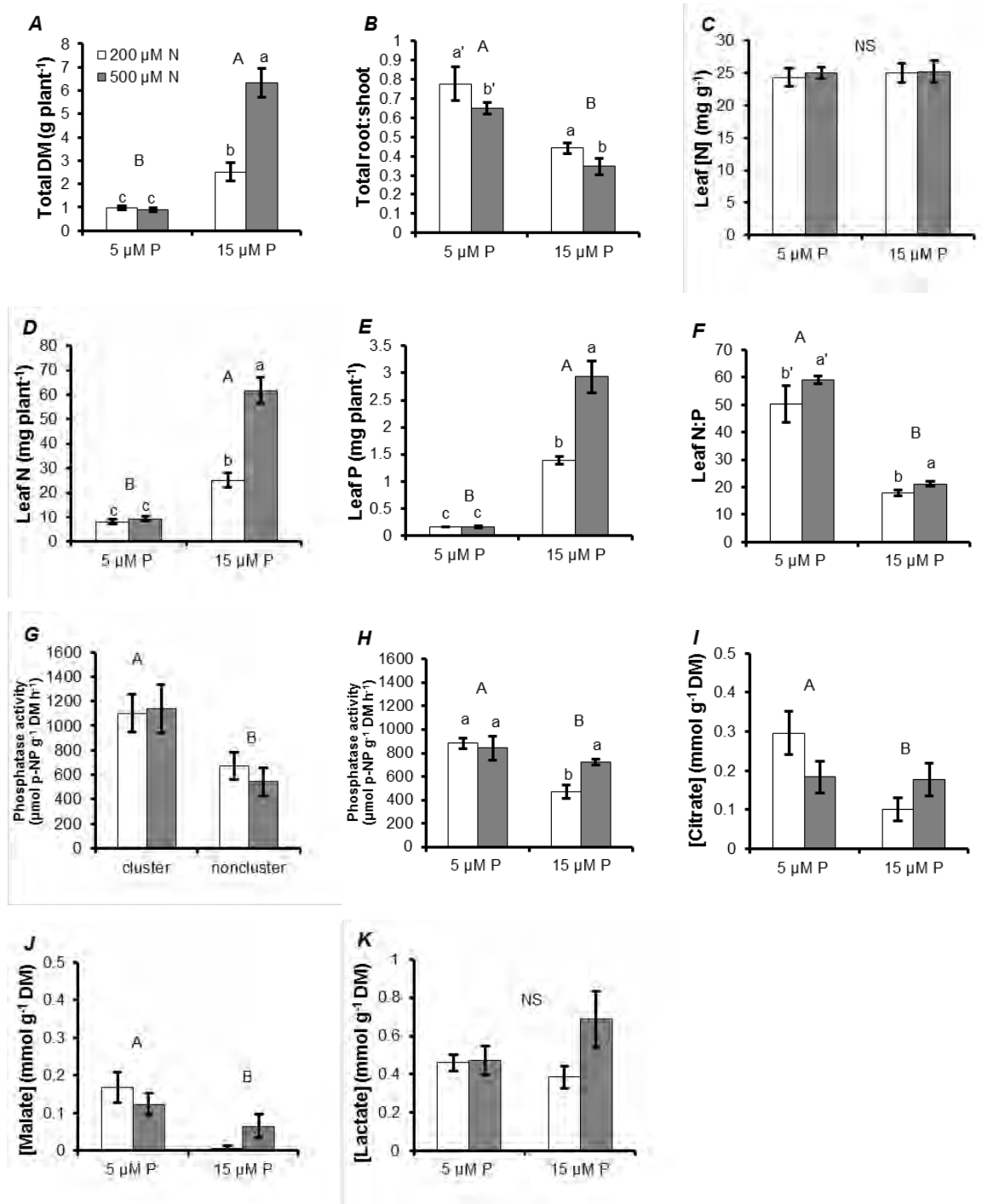


**Fig. 2.** Effect of N supply on the percentage of cluster roots of total root dry matter (DM) in *Aspalathus linearis* plants receiving low P supply, grown in (A) sand with 10 μM P and (B) solution with 5 μM P. Different lowercase letters indicate significantly different means between N levels at  $P<0.05$  from a one-way ANOVA.

### Solution culture

#### Biomass

A significant  $N \times P$  interaction (Fig. 3A,  $F_{1,12}=19.78$ ,  $P<0.001$ ) was observed because increasing P supply from 5 μM to 15 μM increased biomass accumulation by 2.6-fold at 200 μM N supply, but by sevenfold in the plants receiving 500 μM N supply. Furthermore, at low P supply, increasing N supply had no effect on biomass accumulation but increased the mass of plants by 2.5-fold at high P supply. The proportion of total root DM to shoot DM decreased (Fig. 3B,  $F_{1,12}=40.86$ ,  $P<0.001$ ) from  $\approx 70\%$  to 40% with increasing P supply. In addition, at both low and high P supply, increasing N supply decreased the total root:shoot ratio ( $F_{1,12}=5.23$ ,  $P<0.05$ ). Cluster roots did not form in plants at high P supply. At low P supply (Fig. 2B), however, the increased formation of cluster roots with increasing N supply was not significant.



**Fig. 3.** Effect of P and N supply on (A) total dry matter (DM), (B) total root:shoot ratio, (C) leaf N concentration ([N]), (D) leaf N content, (E) leaf P content, (F) leaf N:P ratio, root (H) phosphatase activity, (I) citrate, (J) malate, and (K) lactate concentrations in *Aspalathus linearis* grown in solution culture. Effect of N supply and root type on (G) phosphatase activity in plants receiving 5 μM P. Means ± s.e. ( $n=4$ ). Different lowercase letters indicate significantly different means between P and N levels at  $P<0.05$  from a factorial ANOVA. Different lowercase letters with a prime symbol (') indicate significantly different means where no N × P interaction occurred; different uppercase letters indicate significantly different means between P levels.

## Leaf concentration and amount of N and P, and N:P ratio

Leaf [N] (Fig. 3C) was not affected by N or P supply. This was due possibly to a dilution effect because increasing P supply increased the accumulation of N more at 500 than at 200  $\mu\text{M}$  N supply (Fig. 3D,  $F_{1,12}=13.11$ ,  $P<0.01$ ), whereas increasing N supply had no effect on N accumulation at low P supply but increased the content of foliar N at high P supply. Foliar [P] increased (Table 2,  $F_{1,12}=156.84$ ,  $P<0.001$ ) by threefold with increased P supply and was not influenced by N supply at each level of P supplied. Only at high P supply, increasing N supply increased the amount of P in the leaf (Fig. 3E,  $F_{1,12}=19.60$ ,  $P<0.001$ ). The ratio of leaf N:P decreased (Fig. 3F,  $F_{1,12}=190.15$ ,  $P<0.001$ ) with increasing P supply at both levels of N supply and increased with increasing N supply from 50 to 59 and from 18 to 21 at low and high P supply respectively ( $F_{1,12}=5.99$ ,  $P<0.05$ ).

**Table 2.** Effect of P and N supply on leaf P concentration ([P]) in *Aspalathus linearis* grown in solution culture. Means  $\pm$  s.e. ( $n=4$ ). Different uppercase letters indicate significantly different means between P levels ( $F_{1,12}=156.84$ ,  $P<0.001$ ) from a factorial ANOVA.

N ( $\mu\text{M}$ )	Leaf [P] ( $\text{mg g}^{-1}$ )	
	5 $\mu\text{M}$ P	15 $\mu\text{M}$ P
200	0.50 $\pm$ 0.04 <sup>B</sup>	1.43 $\pm$ 0.14 <sup>A</sup>
500	0.43 $\pm$ 0.03 <sup>B</sup>	1.20 $\pm$ 0.11 <sup>A</sup>

## Phosphatase activity and organic acid exudation of roots

At low P supply, phosphatase activity of cluster and noncluster roots was not influenced by increasing N supply but was twofold greater in cluster roots than in noncluster roots (Fig. 3G,  $F_{1,12}=14.34$ ,  $P<0.01$ ). Low P supply increased the phosphatase activity of roots by 1.5-fold compared with the phosphatase activity of roots at high P supply (Fig. 3H,  $F_{1,12}=19.30$ ,  $P<0.001$ ); at high P supply, increasing N supply from 200  $\mu\text{M}$  to 500  $\mu\text{M}$  increased phosphatase activity of roots by 50% ( $F_{1,12}=8.56$ ,  $P<0.05$ ). Low P supply also increased the exudation of citrate (Fig. 3I,  $F_{1,12}=4.91$ ,  $P<0.05$ ) and malate (Fig. 3J,  $F_{1,12}=15.12$ ,  $P<0.01$ ) by roots, whereas lactate production (Fig. 3K) was not stimulated by low P supply. Acetate was not detected in the analysis and the amount of succinate released by roots was negligible (data not shown).

## 2.4 Discussion

The physiological basis for tolerance of limiting P supply and for synergistic growth responses to simultaneous addition of N and P was investigated in *A. linearis*, by studying the response of its P acquisition mechanisms to increasing supply of N at low and high availability of P. The P content (data not shown) of all plants at low P supply was greater than their initial seed P content, indicating that the plants were growing and taking up P from the growth medium. As anticipated, low P supply of 5  $\mu\text{M}$  P in solution culture or 10  $\mu\text{M}$  P in sand culture limited biomass accumulation in *A. linearis* plants and stimulated P acquisition mechanisms. Notably, in the plants cultured in both sand and solution, the expression of P acquisition mechanisms was shown to be dependent on N supply and was enhanced by the addition of N. Therefore the hypothesis that increasing N supply would stimulate P acquisition mechanisms because of greater demand for P and result in enhanced growth with high P supply was supported.

With low P supply greatly reducing shoot and foliar [P] (Tables 1 and 2), tissue N:P ratios were observed to increase. In plants that received low P supply, these high internal N:P ratios signify a low availability of P relative to N and hence a greater demand for P. In order to balance their N:P ratios plants would need to increase allocation of resources towards acquisition of P, as suggested by Bloom et al. (1985). Hence cluster roots, a key adaptation for P acquisition (Shane and Lambers 2005), which were completely repressed at high P supply, formed at low P supply, similar to *L. albus* (Dinkelaker et al. 1989; Li et al. 2008). Also analogous to responses in *L. albus* (Neumann et al. 1999) and in *H. prostrata* (Shane et al. 2004), the cluster roots of plants cultured in sand released greater concentrations of citrate in comparison to noncluster roots (Fig. 1H). Although organic acids may vary in efficacy based on their anion charge, the tricarboxylated citrate is reported to be the most effective at solubilising P, followed by malate and succinate (Ryan et al. 2001). Therefore increased exudation of citrate by cluster roots at low P supply is a strong mechanism for enhanced acquisition of P (Shane and Lambers 2005). Similar to *Casuarina cunninghamiana* Miq., which is found in soils with low availability of N and P (Reddell et al. 1997), the cluster roots of plants cultured in solution showed greater phosphatase activity than noncluster roots (Fig. 3G). In addition, at low P supply, total root:shoot ratio increased, and phosphatase activity of roots (Fig. 3H) and citrate and malate released by roots was greater than at high P supply of 15  $\mu\text{M}$ . These responses to low P supply are consistent with previous reports of biomass allocation (Nielsen et al. 2001; Poorter et al. 2012) and root biochemistry responses (Tadano and Sakai 1991; Pearse et al. 2006) for enhanced P acquisition.

With low P supply of 10  $\mu\text{M}$  P in sand and with N supply increasing from 100 to 300  $\mu\text{M}$  N, shoot [N] increased but shoot [P] remained at 0.5 mg P g<sup>-1</sup>, resulting in an increase in the shoot N:P ratio from 18 to 36 (Fig. 1F). The increase in shoot [N] and shoot N:P ratio induced a greater demand for P

because the plant responded by stimulating a suite of P acquisition traits. These responses included a substantial increase in the proportion of cluster roots to 60% of root biomass (Fig. 2A), comparable to proportions of 40–65% reported previously (Dinkelaker et al. 1995; Shane and Lambers 2005); phosphatase activity (Fig. 1G), and the concentration of citrate and malate released remained high. With the increase in N supply, the phosphatase activity of noncluster roots (Fig. 1G) and succinate released by cluster roots and noncluster roots (Fig. 1J) also increased. The fact that a dramatic increase in cluster root biomass and the increased release of organic acids did not negatively affect plant biomass (Fig. 1A), similar to *L. albus* (Keerthisinghe et al. 1998), is evidence for a lack of C constraint on plant growth in *A. linearis*, given that cluster roots and organic acids have high metabolic costs (Dinkelaker et al. 1989; Lambers et al. 2006).

At high P supply of 15  $\mu\text{M}$  in solution culture, a synergistic growth response to combined addition of N and P occurred (Fig. 3A) and was associated with an N-induced stimulation of P acquisition. Biomass increased with supply of 15  $\mu\text{M}$  P and 200  $\mu\text{M}$  N until N possibly became limiting to growth, with a supply N:P ratio of 12. With greater N supply of 500  $\mu\text{M}$  N, the satiation of the high demand for N would have contributed to increased accumulation of biomass observed at 500  $\mu\text{M}$  N supply but would also have caused a high demand for P, as also indicated by the increase in the supply N:P ratio from 12 to 30. In response to the high demand for P, the phosphatase activity of roots increased by 50% (Fig. 3H) thereby contributing to the increased uptake of P (Fig. 3E) and ultimately enhancing biomass accumulation. Accordingly, a P limiting supply ratio of 30 was adjusted in the leaf to a more balanced ratio of 21 (Fig. 3F). The *A. linearis* plants showed the capacity to adjust allocation of resources towards acquisition of the more limiting P resource as suggested by the theoretical analysis of plant growth and resource balance in Bloom et al. (1985), thereby balancing supply with demand requirements for growth. With fertilisation by N increasing growth and inducing greater P demand, the limitation of which was then in turn alleviated by enhanced acquisition of P, growth can be stimulated incrementally by N and P (Davidson and Howarth 2007), thereby creating the observed synergistic N and P colimitation response (Fig. 3A), consistent with earlier reports of such an effect (Elser et al. 2007; Harpole et al. 2011). Enhanced phosphatase activity for P acquisition with greater N supply, is also consistent with the suggestion (Houlton et al. 2008) that the nutrient available in excess is used to acquire more of the limiting nutrient, given that phosphatase is an enzyme richer in N compared to C-rich organic acids, for instance.

Although the synergistic growth response at 15  $\mu\text{M}$  P supply occurred with a supply ratio of 30 that resulted in a more balanced foliar N:P ratio of 21, similar to N:P ratios of wild plants from nutrient poor soils (Lambers et al. 2010; Stock and Verboom 2012), enhanced biomass accumulation at 100  $\mu\text{M}$  P and 700  $\mu\text{M}$  N supply (Fig. 1A) occurred with a very low shoot N:P ratio of 6 that was similar

to the N:P supply ratio. The low shoot N:P ratio was due to accumulation of P and not N limitation *per se* as would be suggested by designated threshold N:P ratios for indicating limitation by N or P (Güsewell 2004). Weak down-regulation of P-uptake was suggested in plants that received 100  $\mu\text{M}$  P and 300  $\mu\text{M}$  N, with 26-fold greater shoot [P] (Table 1) and 22-fold greater shoot P content (Fig. 1E) compared with plants that were the same size but supplied 10  $\mu\text{M}$  P and 300  $\mu\text{M}$  N. Similar to *Ptilotus polystachyus* (Gaudich.) F.Muell., a native Australian non-legume herb from nutrient poor soils showing tolerance of high P supply (Ryan et al. 2009), *A. linearis* may accumulate large amounts of P in its vacuoles. Tolerance of high tissue [P], without decreasing plant growth, may be adaptive for species from low P soils with a poor ability to down-regulate P-uptake. In response to seasonal flushes of nutrient availability, for instance, P can be stored for times of low or uncertain nutrient supply (Ostertag 2010). Nevertheless, the stored P would give lower shoot N:P ratios than expected (Matzek and Vitousek 2009), with a shoot N:P ratio of 6 not a true reflection of the plants' demand N:P ratio.

As a possible consequence of a low capacity to down-regulate uptake of the nutrient supplied in excess, *A. linearis* plants grew most poorly in the growth substrate with the most imbalanced N:P supply ratio when one nutrient was supplied in excess amounts and the other limiting to growth. At high P supply when N supply was limiting, the plants receiving 100  $\mu\text{M}$  P and 100  $\mu\text{M}$  N were smaller than the plants supplied 10  $\mu\text{M}$  P and 100  $\mu\text{M}$  N (Fig. 1A) yet accumulated similar N, and showed no greater N constraint (Fig. 1D), but eightfold more P in their shoot (Fig. 1E). The resulting shoot [P] of 15  $\text{mg P g}^{-1}$ , compared with shoot [P] of 5  $\text{mg P g}^{-1}$  (Table 1) in the large plants supplied with 100  $\mu\text{M}$  P and 700  $\mu\text{M}$  N, indicates excessive accumulation of P. Hence the tissue N though present is less available to the plant due to an excess of tissue P. The plants growing with the imbalanced N:P supply ratio of 100  $\mu\text{M}$  P and 100  $\mu\text{M}$  N may have invested at an early stage more heavily to root biomass, to account for this imbalance (Fig. 1B). Under the specific experimental conditions, there was little payoff for investing in root biomass, as no access to additional N could be achieved. On the contrary the P-induced N limitation necessitated reduced investment to photosynthetic tissue, resulting in decreased biomass accumulation (Fig. 1A). Similarly at low P supply of 10  $\mu\text{M}$ , the plants receiving 700  $\mu\text{M}$  N were smaller than the plants supplied with 100  $\mu\text{M}$  N but accumulated similar levels of P and showed no greater P constraint (Fig. 1E) but two times more shoot N (Fig. 1D). The resulting highest shoot [N] of 34  $\text{mg N g}^{-1}$  (Fig. 1C) suggests an imbalance of tissue N and P when P was limiting, given the lower foliar [N] of 24  $\text{mg N g}^{-1}$  for legumes in the CCR (Power et al. 2011) and of 13–19  $\text{mg N g}^{-1}$  for global vegetation from published studies cited in Lambers et al. (2010). Therefore, the poor growth of *A. linearis* plants (Fig. 1A) appears to occur because P, though present, is less available to the plant due to an excess of tissue N. In contrast to the excess supply of 700  $\mu\text{M}$  N at low P supply, it appears likely that more than 700  $\mu\text{M}$  N with 100  $\mu\text{M}$  P supply may elicit a greater biomass accumulation response than that currently

observed, especially since shoot [P] of 5 mg P g<sup>-1</sup> (Table 1) was also high relative to 1.2 mg P g<sup>-1</sup> (Table 2) for good growth. Given the large biomass accumulated with a foliar N:P of 21 at 500 μM N and 15 μM P supply (Figs. 3A, 3F), it is predicted that supplies of 2.3 mM N and 100 μM P or 700 μM N and 30 μM P in sand culture, which would give a supply N:P ratio of 21, would produce greater biomass than was observed with 700 μM N and 100 μM P supply and a shoot N:P ratio of 6.

## 2.5 Conclusion

In *A. linearis* plants cultured in both sand and solution, addition of nitrogen induced a greater demand for P and stimulated P acquisition responses. *A. linearis* is tolerant of low P supply and highly responsive to simultaneous addition of N and P through increased biomass and biochemical partitioning to roots, cluster roots, organic acids and phosphatase enzymes for P acquisition. The adaptations for enhanced acquisition of P at low P supply concur with the distribution of *A. linearis* in oligotrophic CCR soils. Furthermore, tolerance of high shoot [P] and synergistic growth is consistent with an ecological role as a post-fire coloniser when fast growth rates during times of greater resource availability would be favoured.

---

**3    Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume *Podalyria calytrata***

---

### 3.1 Introduction

The CCR is a Mediterranean-type ecosystem dominated by sclerophyllous fynbos vegetation (Manning and Goldblatt 2012) found on oligotrophic soils of the Table Mountain Sandstone Group with low availability of Bray II P of about 4 mg kg<sup>-1</sup> and total N of 1–2 g kg<sup>-1</sup> (Mitchell et al. 1984; Stock and Lewis 1986). The shrub legume *P. calytrata* is found in the south-west CCR especially in mountain fynbos on the Cape Peninsula (Schutte-Vlok and van Wyk 2011). The species is favoured for horticulture with its attractive silver-green foliage and fragrant purple-white blossoms that attract bees, butterflies and birds. In a glasshouse study (Maistry et al. 2013), *P. calytrata* showed superior nodulation at low P supply compared with 16 other fynbos legumes, and also demonstrated traits such as high P-use efficiency, poor down-regulation of P-uptake, storage of P in the shoot and a high seed P content. These traits are typical of plants from oligotrophic soils (Hawkins et al. 2007; Ostertag 2010; Lambers et al. 2011). The physiological basis for *P. calytrata* to tolerate low availability of P is yet to be reported. Increased exudation of organic acids and phosphatase activity by roots may enhance acquisition of P from soils with low availability of P (Lambers et al. 2006). The exudation of organic acids by roots was shown to increase with limited P supply (Pearse et al. 2006; Pang et al. 2010) and may enhance the availability of P in the rhizosphere by chelating cations that precipitate P, or through ligand exchange by displacing sorbed P (Hinsinger 2001; Ryan et al. 2001). In addition, soil organic matter accounts for 60–80% of P in fynbos soils (Mitchell et al. 1984; Witkowski and Mitchell 1987), and root-associated acid phosphatases may further enhance the availability of P for plants by hydrolysing these organic sources of P (Tarafdar and Claassen 1988; Gilbert et al. 1999).

The addition of N to soils with low availability of P was also observed to increase the extracellular phosphatase activity of roots and soil (Olander and Vitousek 2000; Treseder and Vitousek 2001). It is possible that the induction of the P acquisition mechanism by greater supply of combined-N may be in response to lower availability of P relative to N, i.e., a greater demand for P induced by N (Phoenix et al. 2003). Colimitation of the growth of legume plants by N and P (Power et al. 2010; Maistry et al. 2013), implies that plant responses to N and P will depend on the relative supply levels of the two nutrients, so that the interactive effects of N and P supply on the mechanisms for acquisition of P and N and growth need to be closely examined. Plants can regulate their N:P ratios relative to that of the supply N:P ratio (Güsewell 2004; Elser et al. 2010). Thus, with increasing supply of combined-N under P limiting conditions legume plants would need to allocate resources away from the acquisition of N towards acquisition of the more limiting P (Bloom et al. 1985) through decreasing nodulation (Streeter 1988) but increasing phosphatase activity or release of organic acids. On the other hand, stimulation of growth in the fynbos legume *Cyclopia genistoides* (L.) R.Br. by adding P, was observed to decrease biomass allocation to roots for reduced investment in acquisition of P, but induce nodulation so as to enhance N supply to meet the P-induced demand for N (Maistry et al.

2013). These physiological adjustments for acquisition of P and N would be to bring the supply N:P ratio closer to the demand N:P ratio for growth (Bloom et al. 1985; Houlton et al. 2008; Vitousek et al. 2010).

Consistent with the resource allocation theory (Bloom et al. 1985) for balanced acquisition of P and N, decreased nodulation with low P supply has been attributed to reduced demand for N due to limitation of plant growth by deficiency of P (Almeida et al. 2000; Sulieman et al. 2013), often accompanied by greater investment of resources for P utilisation (Theodorou and Plaxton 1993; Araujo et al. 2008) or P acquisition such as greater root biomass, phosphatase activity and exudation of organic acids (Lambers et al. 2006). At the biochemical level, increased PEPC activity at low P supply may enhance P acquisition through greater synthesis of citrate and malate as in cluster roots of *L. albus* (Johnson et al. 1996; Neumann and Römheld 1999), or recycle  $P_i$  by liberating  $P_i$  from PEP when catalysing its carboxylation to OAA (Theodorou and Plaxton 1993). In addition, C from the sequential action of increased PEPC, MDH and ME activity can facilitate continued mitochondrial respiration in the TCA cycle with limited demand for P, by bypassing the adenylate requiring production of pyruvate through PK (Duff et al. 1989; Theodorou and Plaxton 1993). In  $N_2$ -fixing plants receiving adequate P supply, PEPC also provides C for replenishing TCA cycle intermediates such as citrate, used in the assimilation of N (Cramer et al. 1993) or in root exudates (Johnson et al. 1994). Furthermore, OAA that is derived from PEPC may be transaminated to aspartate via aspartate aminotransferase (AAT) for assimilation of N (Coker and Schubert 1981), or converted by MDH to malate for respiring bacteroids (Vance et al. 1985; Rosendahl et al. 1990; Fischinger and Schulze 2010). Therefore PEPC, MDH and AAT play a critical role in coordinating the flow of C between N and P pools in nodules (Vance and Heichel 1991; Smith et al. 2000; Colebatch et al. 2004; Fischinger and Schulze 2010).

Inhibition of nitrogenase activity by factors such as low P supply (Sa and Israel 1991) or  $O_2$  (Laing et al. 1979), reduces synthesis and concentration of organic acids in nodules (Rosendahl et al. 1990; Vance and Heichel 1991) possibly due to low demand for malate by bacteroids or for C skeletons in the assimilation of N. Given that tissue [P] and [N] correlate positively (Garten 1976) and that low P supply decreased nodulation in several studies (Olivera et al. 2004; Araujo et al. 2008; Maistry et al. 2013), P limited  $N_2$ -fixing plants have, therefore, low demand for N (Almeida et al. 2000; Sulieman et al. 2013). Thus, the enhanced expression of PEPC and MDH genes as observed in nodules and cluster roots of *L. albus* at low P supply (Uhde-Stone et al. 2003) most likely is for allocation of C for acquisition or recycling of P. Alternatively, low demand for N at low P supply may not be responsible for reduced allocation of resources to  $N_2$ -fixation. Responses to meet the greater demand for P instead may negatively affect  $N_2$ -fixation and assimilation of the fixed  $N_2$ , because the diversion of up to 25% of plant C for exudation of citrate and malate (Dinkelaker et al. 1989; Lambers et al.

2006) could compete with energy supply for N<sub>2</sub>-fixation and plant growth. It has recently been reported that the diversion of C for enhanced synthesis of organic acids in *L. angustifolius* may compete with energy supply for N<sub>2</sub>-fixation and N assimilation (Le Roux et al. 2008).

The physiological basis for tolerance of limiting P supply in nodulated *P. calytrata* was investigated, and responses to increased supply of combined-N and P were examined. This was achieved by studying growth responses and the mechanisms for acquisition of P and N and assimilation of N at low and high P supply while increasing the supply of combined-N. It was anticipated that low P supply would limit plant growth and increase the expression of mechanisms for P acquisition. It was hypothesised that increased supply of combined-N would also stimulate P acquisition mechanisms associated with greater P demand and result in enhanced plant growth with high P supply. The responses of plant biomass, leaf [N] and [P] and leaf N:P ratio, extracellular phosphatase activity and organic acid exudates of roots, and PEPC, PK, MDH, ME, and AAT activity in nodules and roots to N and P supply were examined in two N × P experiments.

### 3.2 Materials and methods

#### *Growth conditions for sand culture*

Seeds of *Podalyria calytrata* ( $1.75 \pm 0.54$  mg N seed<sup>-1</sup>,  $0.149 \pm 0.003$  mg P seed<sup>-1</sup>) were obtained from a natural population growing in Table Mountain National Park. The seeds were soaked overnight in boiled water then sown in seedling trays containing acid-washed sand. Immediately after emergence, each seedling was inoculated with rhizobia isolated from nodules harvested from plants of the same population in Table Mountain National Park and inoculum prepared according to Vincent (1970). The rhizobia isolated from nodules were fast growing and creamy-white to watery in appearance (personal observation), typical of the *Burkholderia tuberum* strain that nodulates *P. calytrata* (Sprent et al. 2013). Four weeks after emergence, seedlings of similar size were transplanted into plastic pots filled with 3 kg of acid-washed sand and inoculated with rhizobia. Seedlings were thinned to one per pot after a further four weeks, and there were five pots as replicates ( $n=5$ ) for each of the eight treatments. The concentration of Ca(NO<sub>3</sub>)<sub>2</sub> and KH<sub>2</sub>PO<sub>4</sub> in the solution was adjusted to supply the appropriate nitrate concentrations of 100 μM, 300 μM, 500 μM and 700 μM and P concentrations of 10 μM and 100 μM, respectively. Therefore with increasing provision of combined-N the experimentally administered N:P supply ratios (by mass) at a low P level was 9, 27, 45 and 63, and at a high P levels the ratios were 1, 3, 5 and 6. To compensate for the adjustments in Ca(NO<sub>3</sub>)<sub>2</sub>, CaSO<sub>4</sub> was added to the nutrient solution so that all plants received 200 μM Ca. In addition to Ca(NO<sub>3</sub>)<sub>2</sub>, KH<sub>2</sub>PO<sub>4</sub> and CaSO<sub>4</sub>, the complete nutrient solution also contained: 200 μM K<sub>2</sub>SO<sub>4</sub>, 54 μM MgSO<sub>4</sub>, 0.24 μM MnSO<sub>4</sub>, 0.10 μM ZnSO<sub>4</sub>, 0.02 μM CuSO<sub>4</sub>, 2.4 μM H<sub>3</sub>BO<sub>3</sub>, 0.03 μM

Na<sub>2</sub>MoO<sub>4</sub>, and 10 μM Fe-EDTA at pH 6.2, as used in the culture of legumes and Proteaceae from the CCR (Power et al. 2010; Maistry et al. 2013) and South West Australia (Shane et al. 2004). The plants were watered three times a week with 200 mL of nutrient solution. Pots were flushed with 1 L tap water once a week to prevent salt accumulation. Plants were grown for 21 weeks from April 2011 to August 2011 in a glasshouse at UCT (S 33° 57.353; E 18° 27.742) with an average daytime temperature of 21°C controlled in the range of 20–30°C inside the glasshouse. Pots were placed on trolleys that were rearranged weekly.

#### *Growth conditions for solution culture*

Root physiology responses to nutrient supply may be different between plants cultured in sand substrate and nutrient solution, possibly due to differences in zones of depletion around the root and buffering capacity in the two growth media. Some studies, however, report results on plants grown in nutrient solution (Neumann et al. 1999; Roelofs et al. 2001; Shane et al. 2004). Without sand as a buffer, hydroponic culture has the advantages of greater control and certainty over the supply N:P ratio, and less handling of nodules and roots during harvesting. Therefore, in addition to the plants cultivated in sand, *P. calypttrata* plants were also grown concurrently in solution. Seeds were germinated, and inoculated early, similar to the procedure for plants cultured in sand. Four weeks after emergence, seedlings of similar size were transferred to a hydroponic system. The roots of each seedling were inoculated with rhizobia, and the plant was inserted through a hole in the lid of a 5-L black plastic bucket and supported by a loop of black foam around the base of the stem. The lid, holding four seedlings, was placed over the 5-L bucket containing aerated water which was changed to the nutrient solution (pH 6.2) after 3 d. Seedlings were provided a start-up N of 100 μM NO<sub>3</sub> with 4 μM P during the next four weeks and were then thinned to one per bucket. There were four buckets as replicates (*n*=4) for each of the four treatments. The N and P treatments were a factorial design with N<sub>2</sub>-fixing only (N<sub>2</sub>-fixing) and 500 μM NO<sub>3</sub> in a low P level of 5 μM and a higher P level of 15 μM. These concentrations of 5 μM and 15 μM P were selected to represent low and high P supply because in preliminary experiments conducted in hydroponic culture, a higher level of P such as 100 μM P was found to be extremely toxic to plant growth. The inoculated plants that received 500 μM NO<sub>3</sub> did not form nodules and are hereafter referred to as “NO<sub>3</sub>-supplied” plants. The experimentally administered N:P supply ratio (by mass) for NO<sub>3</sub>-supplied plants was 90 at low P supply and 30 at high P supply. The nutrient solution was changed once a week. Plants were grown for 23 weeks from May to October in the glasshouse at UCT.

### *Plant biomass*

At harvest, roots were gently washed in water to remove sand and each plant was separated into leaves, stems, roots and nodules. Plant material was dabbed dry with paper towels prior to determining FM. Only the NO<sub>3</sub>-supplied plants grown in solution did not form nodules. Seven of the youngest fully opened leaves of each plant were selected for leaf area measurements on a LI-3100 Area Meter (LI-COR, Lincoln, NE, USA). Plant material was dried at 60°C for 3 d and after DM recorded the material was milled in a Wiley Mill using a 0.5 mm mesh (Arthur H. Thomas Co. Philadelphia, CA, USA).

### *Plant tissue [N] and [P] analysis*

Leaf [N] was determined using mass spectrometry. Between 1.9 and 2.1 mg of milled plant sample was weighed into 8 mm × 5 mm tin foil capsules (Elemental Microanalysis Ltd., Okehampton, UK) on a Sartorius microbalance. The capsules were folded to enclose the samples which were combusted in a Flash EA 1112 series elemental analyser (Thermo Electron, Italy). The resulting gases were fed into a Delta Plus XP IRMS (Thermo Finnigan, Germany), via a Conflo III gas control unit (Thermo Finnigan). The in-house standards used were Merck Gel, a proteinaceous gel produced by Merck, and dried leaves of *T. majus* collected from Woodbine Lane, UCT campus. The in-house standards have been calibrated against International Atomic Energy Agency standards. Leaf [P] was analysed using ICPAES (Varian Vista MPX ICP-AES; Varian, Mulgrave, Australia) after dry-ashing pulverised plant material at 480°C for 8 h and dissolving in 16% HCl (Kalra, 1998).

### *Root extracellular phosphatase assay*

The phosphatase assay was performed on excised roots as in the determination of phosphatase activities in root exudates (Gilbert et al. 1999) and in the determination of extracellular phosphatase activity of roots (Treseder and Vitousek 2001). During harvesting between 300 and 600 mg FM of excised fine roots were kept on ice in a sterile 20-mL pill vial and then stored at -20°C before analysis. Following a procedure adapted from Tabatabai and Bremner (1969), roots were assayed in the dark in a solution of 4 mL of 15 mM MES and 500 μM CaCl<sub>2</sub> (pH 5.5) and 1 mL of 10 mM p-NPP at 28°C for 30 min. The reaction was terminated with 4 mL of 0.5 M NaOH and 1 mL of 0.2 M CaCl<sub>2</sub>, the assayed root material was recovered and dried in the oven at 60°C for 3 d and DM recorded. For the blank, root matter was excluded from the reaction mixture. For the control, a composite sample of fine root material from each treatment was incubated in 4 mL of MES and CaCl<sub>2</sub> as above, the reaction terminated with NaOH and CaCl<sub>2</sub> and then 1 mL of 10 mM p-NPP was added to the test tube. Acid phosphatase activity of roots was based on the amount of p-NP released as

measured spectrophotometrically at 412 nm relative to known p-NP standards and expressed in terms of phosphatase activity per unit of DM ( $\mu\text{mol p-NP g}^{-1} \text{ DM h}^{-1}$ ). The assay reading was corrected by subtracting the control absorbance reading from experimental assay readings.

#### *Collection and analysis of organic acid root exudates*

Between 300 and 600 mg FM of excised fine roots were kept on ice in a sterile 20-mL pill vial during harvesting. A volume of 15 mL of  $\text{sH}_2\text{O}$ , containing 200  $\mu\text{M}$   $\text{CaCl}_2$  for electrolytic conductivity and maintaining cellular integrity (Pearse et al. 2006) was then added to each vial, submerging the roots. Organic acids were collected by agitating vials on an orbital shaker (160 rpm) for 1 h at 25°C. The solution was filtered through filter paper and the root material was recovered, dried at 60°C for 3 d and DM recorded. The filtrate was filtered through a 20- $\mu\text{m}$  Acrodisc syringe filter and stored at -20°C until freeze dried and resuspended in 1 mL of  $\text{sH}_2\text{O}$ . Analysis of citrate, malate, succinate, and acetate concentration was performed at the Central Analytical Facility, Stellenbosch University, with enzymatic test kits in an Arena 20XT Enzyme Robot (Thermo Electron Oy, Finland). Concentration of malate and acetate in the sample was determined photometrically by measuring the increase in absorbance at 340 nm (Enzytec™ *Fluid* enzyme kits, Thermo Fisher Scientific Oy, Finland). The decrease in absorbance associated with the oxidation of NADH was used to determine the concentration of citrate and succinate (Yellow line enzyme kits, Roche, R-Biopharm AG, Darmstadt, Germany).

#### *Protein extraction and determination in nodules and roots*

At harvest, nodules and a sample of fine roots were weighed for FM then frozen in liquid nitrogen and stored at -80°C. For PEPC, PK, MDH and ME, between 300 and 600 mg of nodule or root tissue was ground in liquid nitrogen and extracted in 1.5–2 mL of buffer solution consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, 2% (m/v) insoluble polyvinylpyrrolidone (PVPP) (Ocaña et al. 1996) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 mL of buffer (Le Roux et al. 2008). For AAT, extract was obtained from 300 to 600 mg of tissue ground in 3–6 mL of buffer made with 100 mM maleic acid-KOH (pH 6.8), 100 mM sucrose, 2% (v/v)  $\beta$ -mercaptoethanol, 15% (v/v) ethylene glycol and 10% (m/v) PVPP (Olivera et al. 2004). The tissue protein concentration was determined using Bradford reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard (Bradford 1976).

### *In vitro enzyme assays on nodule and root extracts*

The activity of PEPC, PK, MDH and AAT was determined spectrophotometrically (Multiskan Spectrum, Thermo Electron Corporation) by measuring the decrease in absorbance accompanying NADH oxidation at 340 nm and 20°C, whereas ME activity was based on the absorbance of NADPH reduction (Le Roux et al. 2008). All the reactions were initiated by adding 30 µL of crude extract to 220 µL reaction mixture giving a total volume of 250 µL. Initial reaction rates have been shown to be proportional to the concentration of enzyme under the specified conditions. *In vitro* specific enzyme activity was expressed as nmol min<sup>-1</sup> mg<sup>-1</sup> protein to represent potential *in vivo* enzyme activity.

PEPC (EC 4.1.1.31) activity was determined in an assay mixture containing 100 mM Tris (pH 8.5), 5 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 4 mM PEP, 0.20 mM NADH and 5 U of MDH (Ocaña et al. 1996). The blanks consisted of a reaction medium without PEP and one with sH<sub>2</sub>O instead of enzyme.

PK (EC 2.7.1.40) activity was assayed in a buffer containing 75 mM Tris-HCl (pH 7.0), 5 mM MgCl<sub>2</sub>, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and 3 U lactate dehydrogenase (Smith 1985). For two blanks, PEP was omitted in one reaction while the other reaction was minus enzyme.

NADH-MDH (EC 1.1.1.37) activity was measured in a pH 7.5 solution containing 25 mM KH<sub>2</sub>PO<sub>4</sub> buffer, 0.2 mM NADH and 0.4 mM OAA (Appels and Haaker 1988). The blanks consisted of a reaction medium without OAA and one without enzyme.

NADP-ME (EC 1.1.1.40) was assayed in a mixture that contained 80 mM Tris-HCl (pH 7.5), 2 mM MnCl<sub>2</sub>, 1 mM malate and 0.4 mM NADP (Smith 1985). The blanks consisted of a reaction medium without malate, and without enzyme.

AAT (EC 2.6.1.1) activity was assayed in 50 mM Tris-HCl buffer (pH 8.0) with 4 mM MgCl<sub>2</sub>, 10 mM aspartic acid, 0.2 mM NADH and 1 mM 2-oxoglutarate (Olivera et al. 2004). One blank did not contain aspartate and 2-oxoglutarate and the other was without enzyme.

### *Statistical analysis*

To reduce heteroscedasticity, all measurements were log<sub>e</sub> transformed before statistical analysis. To test the N × P interaction, a factorial ANOVA was performed (STATISTICA version 11, StatSoft, Tulsa, OK, USA). The effect of P supply on nodule:root ratio and on nodule enzyme activity in N<sub>2</sub>-fixing plants was assessed using one-way ANOVA. Means that were significantly different at *P*<0.05 were separated by Duncan's multiple range test.

### 3.3 Results

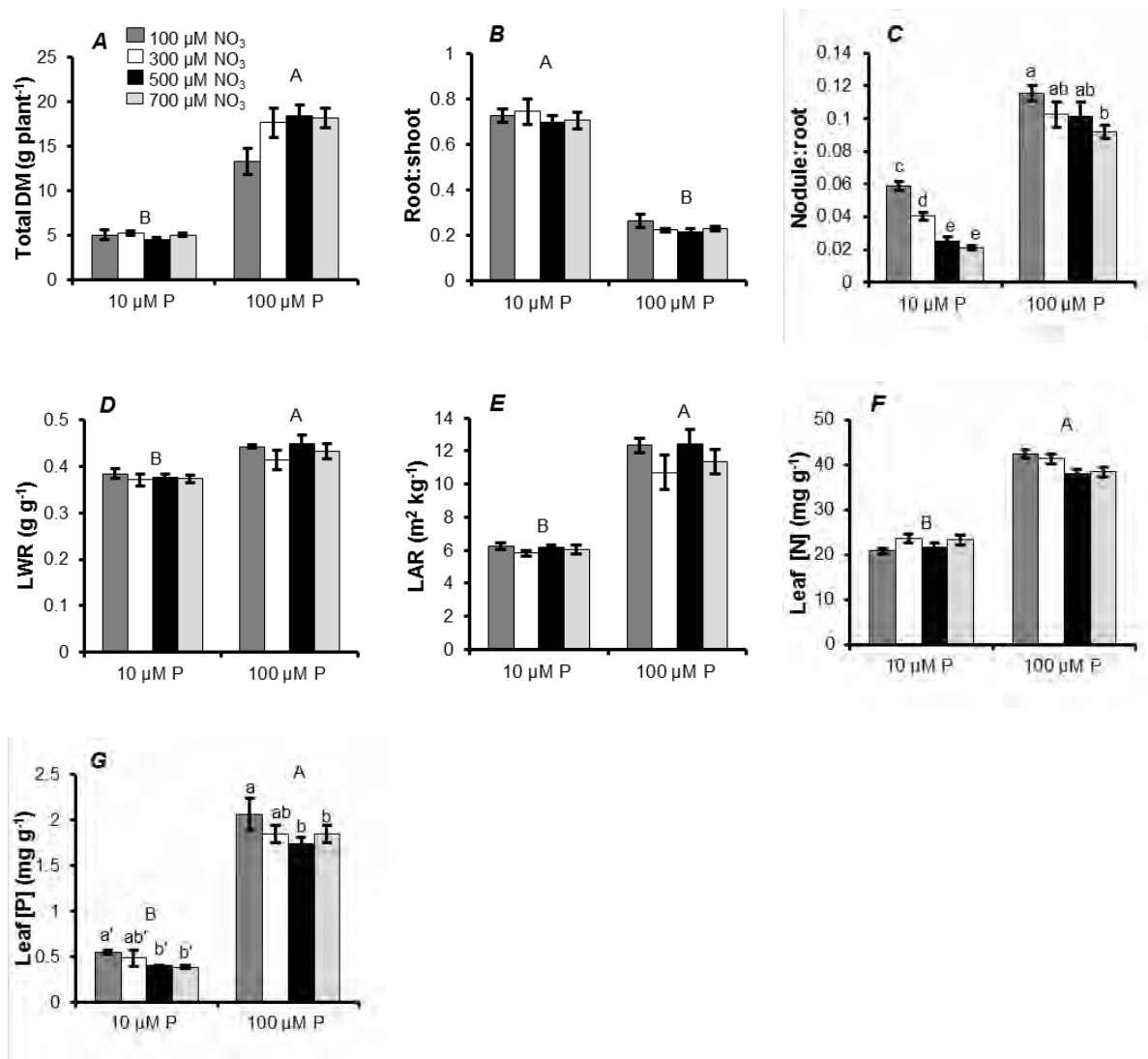
#### *Sand culture*

##### Biomass accumulation

Low P supply decreased biomass accumulation more than threefold (Fig. 1A,  $F_{1,32}=435.98$ ,  $P<0.001$ ), while increasing nitrate supply had no effect on the biomass formed at both low and high P supply. Decreasing the amount of P supply, however, increased the allocation of biomass to roots by threefold (Fig. 1B,  $F_{1,32}=690.30$ ,  $P<0.001$ ), from just above 20% of the shoot DM at high P supply to approximately 70% of shoot DM at low P supply, but increasing nitrate supply did not influence biomass allocation between root and shoot. In contrast, low P supply decreased the proportion of roots that were nodules by nearly twofold from approximately 12% of root mass at 100  $\mu\text{M}$  P to 6% at 10  $\mu\text{M}$  P supply (Fig. 1C,  $F_{1,32}=489.82$ ,  $P<0.001$ ). There was, however, a significant interaction ( $F_{3,32}=14.55$ ,  $P<0.001$ ) between nitrate and P supply on the ratio of nodule:root, because increasing nitrate supply decreased nodulation by 60% at low P supply compared with a 20% reduction at 100  $\mu\text{M}$  P supply. Specifically, 300  $\mu\text{M}$   $\text{NO}_3$  induced a lower level of nodulation at 10  $\mu\text{M}$  P supply, while a higher level of nitrate of 700  $\mu\text{M}$  was necessary to reduce nodulation with supply of 100  $\mu\text{M}$  P. Leaf weight ratio (LWR), the fraction of total plant DM allocated to leaves, decreased by 6% (Fig. 1D,  $F_{1,32}=38.44$ ,  $P<0.001$ ), and the ratio of total leaf area to total plant DM (LAR) decreased twofold with low P supply (Fig. 1E,  $F_{1,32}=259.34$ ,  $P<0.001$ ), but the ratios were not changed by increasing nitrate supply at either level of P.

##### Leaf [N] and [P] and N:P ratio

Decreasing P supply reduced leaf [N] from ca. 40  $\text{mg N g}^{-1}$  at 100  $\mu\text{M}$  P supply to ca. 22  $\text{mg N g}^{-1}$  in plants receiving 10  $\mu\text{M}$  P (Fig. 1F,  $F_{1,32}=523.02$ ,  $P<0.001$ ). On the other hand, increasing nitrate supply had no effect on foliar [N]. Low P supply decreased leaf [P] from 2.06  $\text{mg P g}^{-1}$  to 0.54  $\text{mg P g}^{-1}$  in plants receiving 100  $\mu\text{M}$   $\text{NO}_3$  (Fig. 1G,  $F_{1,32}=722.13$ ,  $P<0.001$ ). Furthermore, increasing nitrate supply decreased leaf [P] to 0.38  $\text{mg P g}^{-1}$  at low P supply and to 1.74  $\text{mg P g}^{-1}$  at high P supply ( $F_{3,32}=4.00$ ,  $P<0.05$ ). The leaf N:P ratio was higher at low P supply than at high P supply (Table 1,  $F_{1,32}=305.35$ ,  $P<0.001$ ). There was a significant ( $F_{3,32}=3.68$ ,  $P<0.05$ )  $\text{N} \times \text{P}$  interaction on the leaf N:P ratio because at low P supply increasing nitrate supply increased the ratio from 39 to 62, whereas at high P supply, increasing nitrate supply did not change the leaf N:P ratio of ca. 22.



**Fig.1.** Effect of phosphorus (P) and nitrate (NO<sub>3</sub>) on (A) total dry matter (DM), (B) root:shoot, (C) nodule:root, (D) leaf weight ratio (LWR), (E) leaf area ratio (LAR), (F) leaf [N], and (G) leaf [P] in nodulated *Podalyria calyprata* grown in sand culture. Means  $\pm$  s.e. ( $n=5$ ). Different lowercase letters indicate significantly different means between levels of P and NO<sub>3</sub> at  $P<0.05$  from a factorial ANOVA. Different lowercase letters with a prime symbol (') indicate significantly different means where no  $N \times P$  interaction occurred; different uppercase letters indicate significantly different means between P levels.

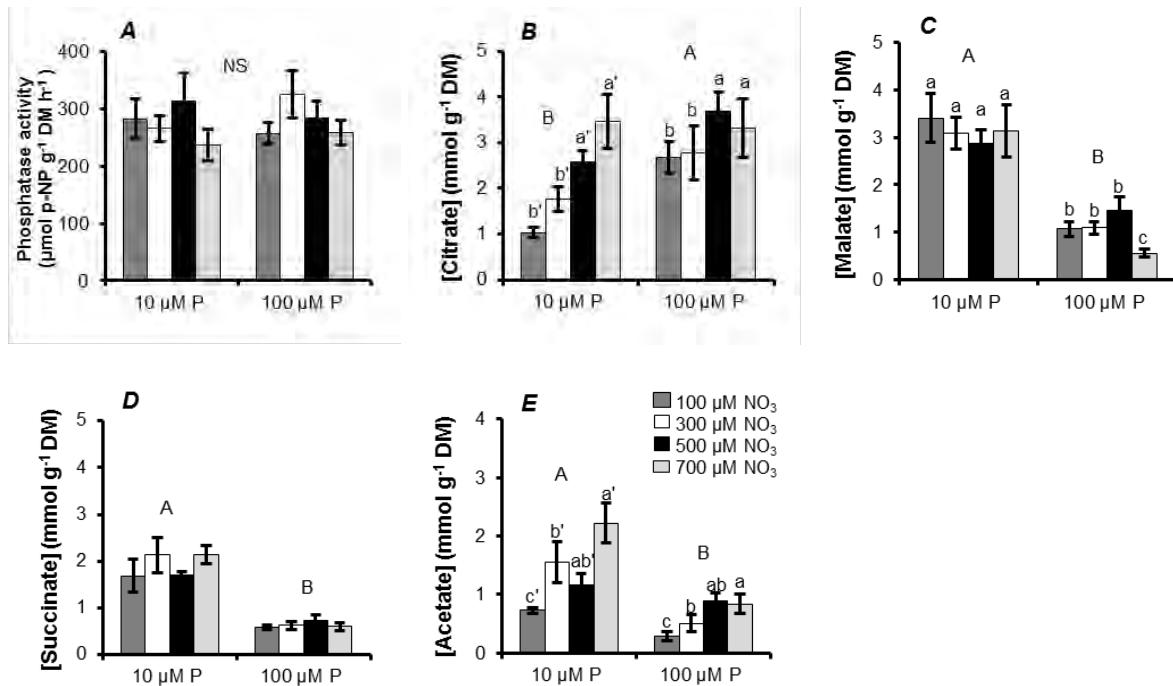
#### Extracellular phosphatase activity and organic acid exudates of roots

Phosphatase activity of roots of approximately 300  $\mu\text{mol p-NP g}^{-1} \text{ DM h}^{-1}$  (Fig. 2A) was not influenced by nitrate or P supply. The amount of citrate released by roots, however, decreased (Fig. 2B,  $F_{1,24}=10.36$ ,  $P<0.01$ ) with low P supply but increased ( $F_{3,24}=6.38$ ,  $P<0.01$ ) with greater nitrate supply at both 10  $\mu\text{M}$  and 100  $\mu\text{M}$  P. In contrast, the exudation of malate (Fig. 2C), succinate (Fig. 2D) and acetate (Fig. 2E) by roots increased ( $F_{1,24}=107.36$ , 100.14, and 34.86 respectively,  $P<0.001$ )

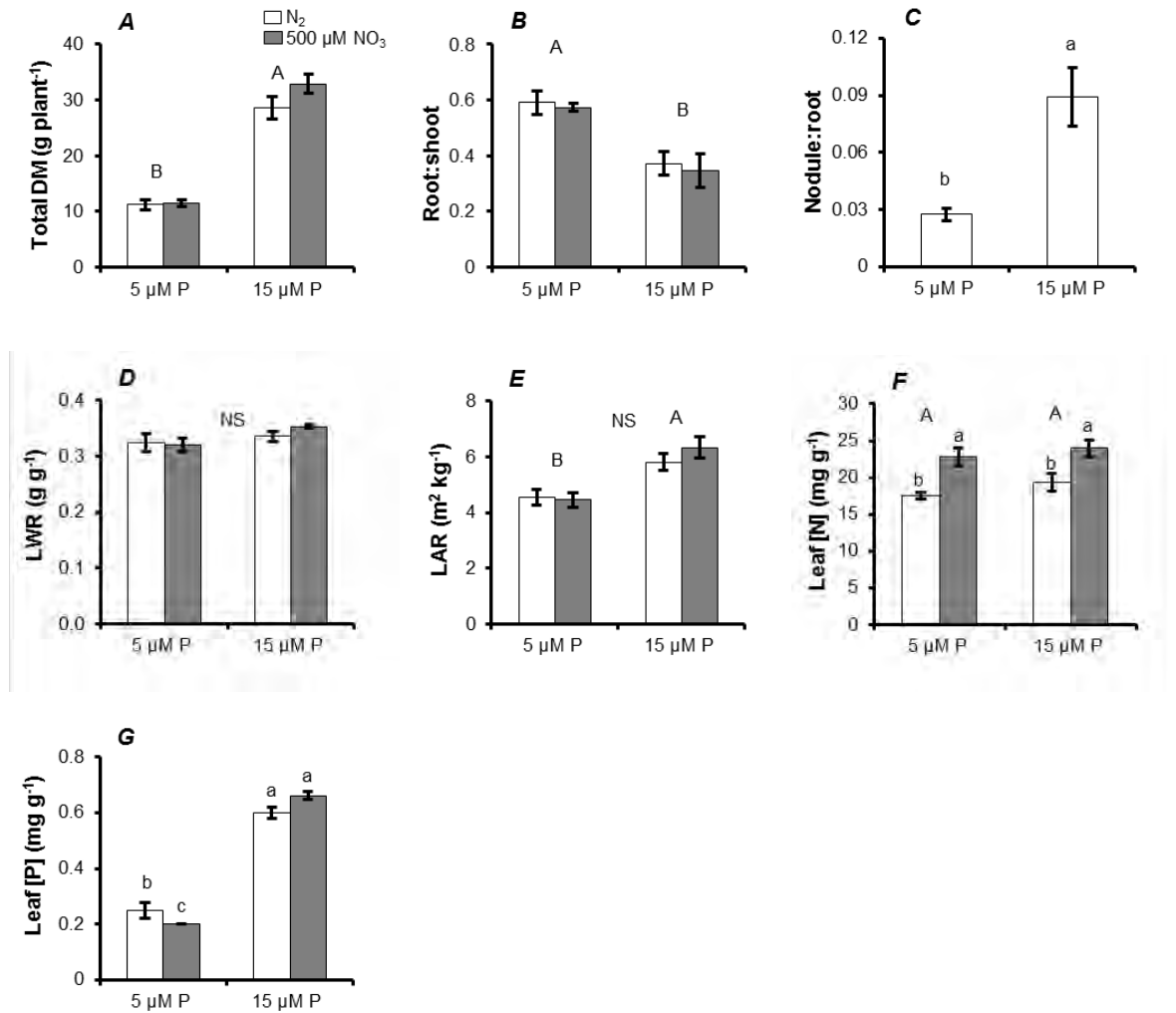
with 10  $\mu\text{M}$  P supply. Increasing nitrate supply also increased the amount of acetate released by roots receiving both 10 and 100  $\mu\text{M}$  P ( $F_{3,24}=10.72$ ,  $P<0.001$ ).

**Table 1.** Effect of P and nitrate ( $\text{NO}_3$ ) supply on leaf N:P ratio of nodulated *Podalyria calyprata* grown in sand culture. Means  $\pm$  s.e. ( $n=5$ ). Different lowercase letters indicate significantly different means between P and  $\text{NO}_3$  levels ( $F_{3,32}=3.68$ ,  $P<0.05$ ) from a factorial ANOVA. The experimentally administered N:P supply ratio is indicated in brackets preceding the leaf N:P ratio.

$\text{NO}_3$ ( $\mu\text{M}$ )	Leaf N:P ratio	
	10 $\mu\text{M}$ P	100 $\mu\text{M}$ P
100	(9) $39 \pm 1^b$	(1) $21 \pm 1^c$
300	(27) $53 \pm 6^a$	(3) $23 \pm 1^c$
500	(45) $54 \pm 3^a$	(5) $22 \pm 1^c$
700	(63) $62 \pm 5^a$	(6) $21 \pm 1^c$



**Fig. 2.** Effect of phosphorus (P) and nitrate ( $\text{NO}_3$ ) on (A) extracellular phosphatase activity and (B) citrate, (C) malate, (D) succinate, and (E) acetate concentration of root exudates in nodulated *Podalyria calyprata* grown in sand culture. Means  $\pm$  s.e. (A,  $n=5$ ; B–E,  $n=4$ ). Different lowercase letters indicate significantly different means between P levels and  $\text{NO}_3$  levels at  $P<0.05$  from a factorial ANOVA. Different lowercase letters with a prime symbol (') indicate significantly different means where no  $\text{N} \times \text{P}$  interaction occurred; different uppercase letters indicate significantly different means between P levels. NS=not significant.



**Fig. 3.** Effect of phosphorus (P) and source of nitrogen (N) on (A) total dry matter (DM), (B) root:shoot, (D) leaf weight ratio (LWR), (E) leaf area ratio (LAR), (F) leaf [N] and (G) leaf [P], and effect of P on (C) nodule:root in *Podalyria calytrata* grown in solution culture. Means  $\pm$  s.e. ( $n=4$ ). Different lowercase letters indicate significantly different means between P levels and source of N at  $P<0.05$  from a factorial ANOVA. Different uppercase letters indicate significantly different means between P levels, with (C) assessed from a one-way ANOVA.

### Solution culture

#### Biomass accumulation

Plants of *P. calytrata* receiving low P supply of 5  $\mu\text{M}$  accumulated nearly threefold less biomass compared to plants supplied with 15  $\mu\text{M}$  P and this biomass was not changed in either the N<sub>2</sub>-fixing or NO<sub>3</sub>-supplied plants (Fig. 3A,  $F_{1,12}=238.09$ ,  $P<0.001$ ). The proportion of the root relative to the shoot, however, increased with low P supply, from 35% to 60% of the shoot DM, and was also not

influenced by the source of N (Fig. 3B,  $F_{1,12}=22.52$ ,  $P<0.001$ ). Similar to the response of plant biomass, the nodule:root ratio in the  $N_2$ -fixing plants decreased threefold (Fig. 3C,  $F_{1,6}=30.63$ ,  $P<0.001$ ) with low P supply. Nodules did not form on the roots of  $NO_3$ -supplied plants. Supply of 5  $\mu M$  P did not change the LWR (Fig. 3D) but decreased the LAR by 25% (Fig. 3E,  $F_{1,12}=25.89$ ,  $P<0.001$ ). The LWR and LAR were not changed by the provision of combined-N at both levels of P supply.

#### Leaf [N] and [P] and N:P ratio

Decreasing P supply did not decrease leaf [N] (Fig. 3F), possibly due to a dilution effect in the plants supplied with 15  $\mu M$  P, which accumulated more biomass (Fig. 3A) and, therefore, more N. At both low P and high P supply, the  $NO_3$ -supplied plants, with ca. 24 mg N  $g^{-1}$ , had about 25% greater leaf [N] than the  $N_2$ -fixing plants ( $F_{1,12}=24.60$ ,  $P<0.001$ ). Low P supply decreased leaf [P] from ca. 0.66 mg P  $g^{-1}$  to 0.25 mg P  $g^{-1}$  in the  $N_2$ -fixing plants (Fig. 3G,  $F_{1,12}=287.60$ ,  $P<0.001$ ). Furthermore, increasing nitrate supply of 500  $\mu M$  decreased leaf [P] ( $F_{1,12}=6.04$ ,  $P<0.05$ ) to 0.20 mg P  $g^{-1}$  at low P supply but leaf [P] was similar between the two N treatments at high P supply. The leaf N:P ratio was higher at 5  $\mu M$  than 15  $\mu M$  P supply (Table 2,  $F_{1,12}=200.99$ ,  $P<0.001$ ), however, there was a significant ( $F_{1,12}=5.45$ ,  $P<0.05$ ) N  $\times$  P interaction on the leaf N:P ratio because at low P supply addition of 500  $\mu M$   $NO_3$  increased the ratio from 72 to 114, whereas at high P supply nitrate supply did not change the leaf N:P ratio.

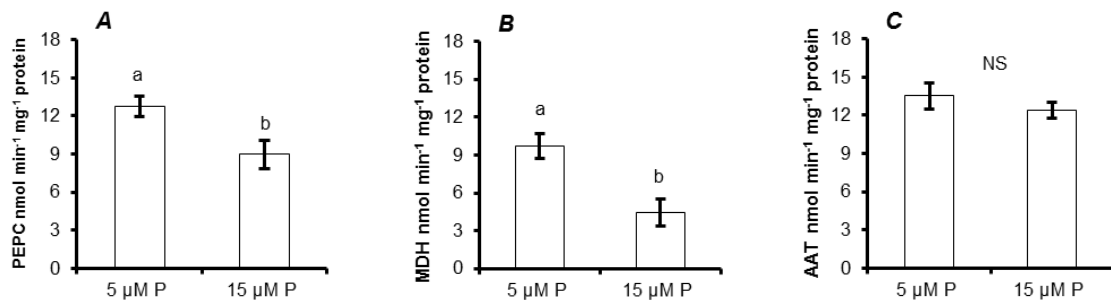
**Table 2.** Effect of P and source of N on leaf N:P ratio of *Podalyria calyptata* grown in solution culture. Means  $\pm$  s.e. ( $n=4$ ). Different lowercase letters indicate significantly different means between P levels and N source ( $F_{1,12}=5.45$ ,  $P<0.05$ ) from a factorial ANOVA. The N:P supply ratio is indicated in brackets preceding the leaf N:P ratio.

N source	Leaf N:P ratio	
	5 $\mu M$ P	15 $\mu M$ P
$N_2$	72 $\pm$ 7 <sup>b</sup>	31 $\pm$ 1 <sup>c</sup>
500 $\mu M$ N	(90) 114 $\pm$ 6 <sup>a</sup>	(30) 38 $\pm$ 2 <sup>c</sup>

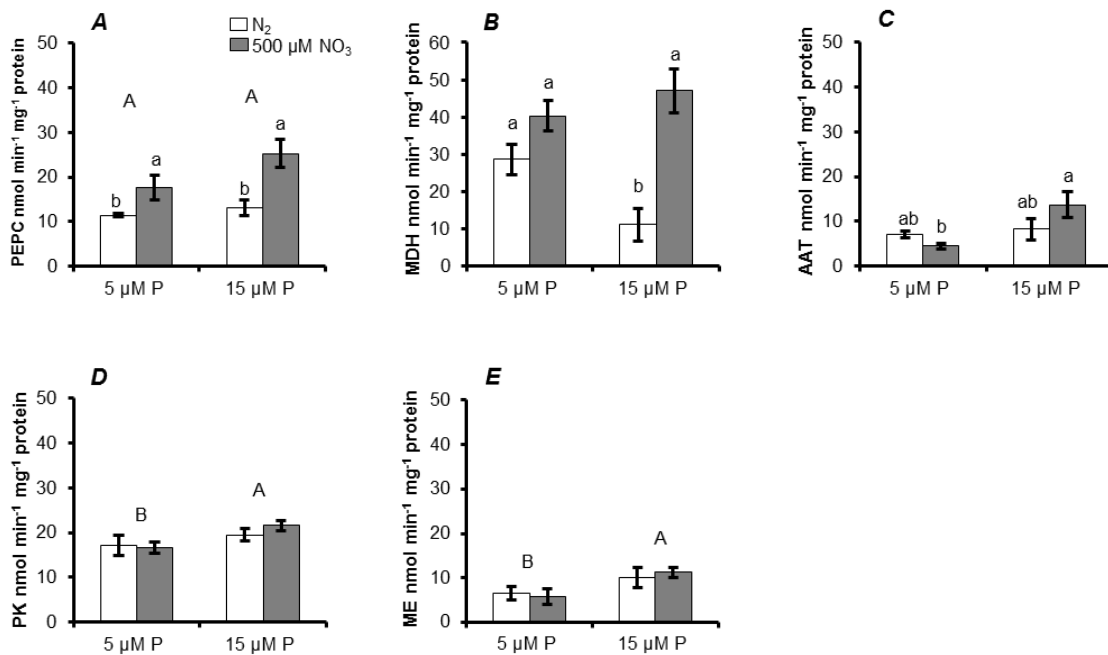
#### Nodule extract enzyme activity

In the nodules of plants receiving low P supply, PEPC activity increased by 40% (Fig. 4A,  $F_{1,6}=5.45$ ,  $P<0.05$ ) and MDH activity increased twofold (Fig. 4B,  $F_{1,6}=6.92$ ,  $P<0.05$ ) relative to the respective

enzyme activity in nodules of N<sub>2</sub>-fixing plants receiving 15 μM P. Nodular AAT (Fig. 4C), PK and ME (data not shown) activity, however, were not changed by P supply.



**Fig. 4.** Effect of phosphorus (P) on *in vitro* specific activity of (A) phosphoenolpyruvate carboxylase (PEPC), (B) malate dehydrogenase (MDH) and (C) aspartate aminotransferase (AAT) in nodules of N<sub>2</sub>-fixing *Podalyria calytrata* grown in solution culture. Means ± s.e. (*n*=4). Different lowercase letters indicate significantly different means between P levels at *P*<0.05 from a one-way ANOVA. NS=not significant.



**Fig. 5.** Effect of phosphorus (P) and source of nitrogen (N) on *in vitro* specific activity of (A) phosphoenolpyruvate carboxylase (PEPC), (B) malate dehydrogenase (MDH), (C) aspartate aminotransferase (AAT), (D) pyruvate kinase (PK) and (E) malic enzyme (ME) in roots of *Podalyria calytrata* grown in solution culture. Means ± s.e. (*n*=4). Different lowercase letters indicate significantly different means between P levels and source of N at *P*<0.05 from a factorial ANOVA; different uppercase letters indicate significant means between P levels.

## Root extract enzyme activity

Phosphoenolpyruvate carboxylase activity in the roots of N<sub>2</sub>-fixing and NO<sub>3</sub>-supplied plants was not changed by low P supply, but it was greater in the NO<sub>3</sub>-supplied plants than in the N<sub>2</sub>-fixing plants at both 5 and 15 μM P (Fig. 5A,  $F_{1,12}=19.67$ ,  $P<0.001$ ). Malate dehydrogenase activity recorded significant interactive effects of N and P supply (Fig. 5B,  $F_{1,12}=5.92$ ,  $P<0.05$ ). At 15 μM P supply, MDH activity in the roots of N<sub>2</sub>-fixing plants was low relative to that of the NO<sub>3</sub>-supplied plants and the plants at low P supply. There was also a significant N × P interaction on AAT activity in roots because the application of low P supply decreased root AAT activity in NO<sub>3</sub>-supplied plants but not in the N<sub>2</sub>-fixing plants (Fig. 5C,  $F_{1,12}=5.84$ ,  $P<0.05$ ). With low P supply both PK (Fig. 5D,  $F_{1,12}=4.81$ ,  $P<0.05$ ) and ME (Fig. 5E,  $F_{1,12}=6.72$ ,  $P<0.05$ ) activity in the NO<sub>3</sub>-supplied and N<sub>2</sub>-fixing plants decreased.

## 3.4 Discussion

In the plants cultured in both sand and solution, the hypothesis that increased supply of combined-N would stimulate mechanisms for P acquisition and result in enhanced plant growth with high P supply was partly supported. In response to a greater demand for P induced by the addition of combined-N, the mechanisms for P acquisition were enhanced at both low and high P supply. With greater P supply, however, high nodulation levels in plants receiving low nitrate supply of 100 μM in sand, or without nitrate supply in solution, possibly increased the supply of N in these plants, thus meeting the P-induced demand for N and preventing the increased supply of combined-N from having a significant effect on accumulation of more biomass.

The plants receiving low P supply in sand and solution culture showed low foliar [P], but adequate levels of N that were within the range of foliar [N] of 24 mg N g<sup>-1</sup> for legumes in the CCR (Power et al. 2011) and of 13–19 mg N g<sup>-1</sup> for global vegetation from published studies (Lambers et al. 2010). Therefore with low P supply the low tissue levels of P relative to available N indicate high demand for P (or low demand for N). The plant would be expected to balance levels of tissue P and N (Güsewell 2004; Elser et al. 2010) by allocating resources away from the acquisition of N towards mechanisms for the acquisition, conservation or recycling of the more limiting P (Bloom et al. 1985). Hence, with low P supply, two to threefold lower investment of resources into nodule biomass (Figs. 1C and 3C) was accompanied by stimulation of P acquisition traits such as greater exudation of malate, succinate and acetate by roots of plants grown in sand (Fig. 2C–E), and greater allocation of biomass to roots, as mechanisms for enhanced acquisition of P (Hinsinger 2001; Nielsen et al. 2001; Lambers et al. 2006; Poorter et al. 2012). Based on anion charge, the tricarboxylated citrate is reported to be the most effective at solubilising P, followed by malate and succinate, and then acetate

(Ryan et al. 2001). Furthermore, with low P supply of 5  $\mu\text{M}$  in solution culture, the activity of PK in roots also decreased (Fig. 5D). This finding is consistent with reduced rates of phosphorylation of ADP to ATP (Theodorou and Plaxton 1993), implying more recycling of  $\text{P}_i$ . Lower cytosolic ME activity (Fig. 5E) can decrease the internal use of malate (Copeland et al. 1989), and facilitate greater exudation levels by roots, as was observed in plants grown in sand at low P supply. Low P supply decreased nodule biomass but increased nodular PEPC and MDH activity. It is proposed, that due to the high tissue N:P ratio of 72 (Table 2), greater PEPC and MDH activity in nodules, similar to enhanced expression of PEPC and MDH genes in low-P nodules and cluster roots of *L. albus* (Uhde-Stone et al. 2003), most likely is for acquisition of P (Johnson et al. 1996; Al-niemi et al. 1998) or for greater recycling of  $\text{P}_i$  (Theodorou and Plaxton 1993) and not for increased production of malate for respiration by bacteroids (Vance et al. 1985; Rosendahl et al. 1990; Fischinger and Schulze 2010). Notably, with the greater PEPC or MDH activity in nodules and roots (Fig. 5B) at low P supply, AAT activity in  $\text{N}_2$ -fixing plants (Figs. 4C and 5C) was unaffected, indicating that the investment of C for acquisition or recycling of P did not negatively affect assimilation of the fixed  $\text{N}_2$  in nodules and roots.

With increased supply of combined-N, plant responses of reduced leaf [P] or increases in leaf [N] or both, together with an increasing N:P supply ratio, may have induced a greater demand for P. It is known for instance, that increased amounts of N in the solution or in the plant tissue increased the demand and capacity for uptake of P (Cole et al. 1963; Chapin 1980; Fageria 2001). As a result, the plant roots showed greater citrate and acetate exudation and greater PEPC and MDH activity for enhanced acquisition of P, similar to *L. angustifolius* where high N supply also stimulated citrate exudation at high P supply (Hocking and Jeffrey 2004). Consistent with balanced acquisition of P and N, increased supply of combined-N decreased the nodule:root ratio by 60% at low P supply but by 20% at high P supply of 100  $\mu\text{M}$  (Fig. 1C). The lower inhibition of nodulation by nitrate at high P supply, as demonstrated previously (Wall et al. 2000), is clear evidence for a coordinated response to lower N demand associated with low P supply, but higher N demand associated with high P supply. Moreover, such colimitation of nodule growth by N and P indicates that the level of nodulation or  $\text{N}_2$ -fixation is dependent on the relative supply levels of N and P. As was also observed in other studies (Leidi and Rodriguez-Navarro 2000; Gentili and Huss-Danell 2003), however, high P supply did not counteract the nitrate inhibition of nodulation in solution culture, possibly because of the combined effect of a high leaf [N] and a relatively high N:P supply ratio of 30 in the  $\text{NO}_3$ -supplied plants.

It was also evident that neither the increasing investments in C for greater synthesis and exudation of organic acids nor the decreasing biomass allocation to nodules, associated with greater supply of combined-N, altered the total plant biomass accumulated, root:shoot ratio, and LWR or LAR. The amount of biomass allocated to roots and leaves was not affected by nodule growth because the plants

instead acquired N from the nitrate supplied. Therefore, N acquisition, and organic acid metabolism for nutrient acquisition, appears to have a demand for C that is not limiting total biomass accumulation. Given that low P supply to the plants decreased the nodule:root ratio and increased P acquisition traits, it may be interpreted that C for nodule biomass was instead invested into acquisition of P, implying that nodule growth was C limited and hence low N supply was also limiting plant growth. Biomass, however, did not increase with increasing nitrate supply of 300  $\mu\text{M}$  (Fig. 1A) or 500  $\mu\text{M}$  (Fig. 3A) to the nodulated plants at low P supply, confirming that plant growth was not limited by N supply. It is clear that with low P supply, the allocation of C for acquisition of P is a regulated response to P limitation (Bloom et al. 1985), and is consistent with balanced acquisition of P and N for high P demand and low N demand, thereby tolerating growth at limiting P supply. In addition, with increasing nitrate supply, P limited plants were able to maintain biomass accumulation despite the high and different N:P supply and foliar ratios (Tables 1 and 2), showing tolerance of N:P ratio imbalances.

At high P supply in sand and solution, the plants with low nitrate supply of 100  $\mu\text{M}$  or without nitrate supply nodulated prolifically. Given that N limitation makes plants unresponsive to P fertilisation (Power et al. 2010; Maistry et al. 2013), the investment in nodulation to increase the supply of N to meet the P-induced demand for N, may explain the increased biomass accumulation in the plants supplied with low or no nitrate, that led to similar biomass accumulation with plants receiving greater supply of 700  $\mu\text{M}$  or 500  $\mu\text{M}$   $\text{NO}_3^-$ . The ability to nodulate and fix  $\text{N}_2$  to meet the N demand of increased availability of P may also explain the proliferation of *P. calytrata* seedlings in post-fire fynbos soils (Manders 1990), a time when levels of P in the soil are noted to be higher (Brown and Mitchell 1986).

### **3.5 Conclusion**

*Podalyria calytrata* plants cultured in sand and solution increased expression of traits for P acquisition in response to a greater demand for P induced by the addition of combined-N, and reduced nodulation due to low demand for N at low P supply. Within the framework of a coherent theory of nutrient balance, *P. calytrata* showed co-ordinated regulation of P and N acquisition in response to demand for P and N by partitioning resources to roots, nodules, organic acids and through glycolytic enzymes, for tolerating growth at low P supply and responding to greater P supply. By averting N-limitation at high P supply, growth of  $\text{N}_2$ -fixing *P. calytrata* may be enhanced with anthropogenic additions of P to N limited Mediterranean ecosystems.

---

**4 Mechanisms for acquisition of phosphorus and growth vary in closely related *Podalyria* species (Fabaceae) with their ecological niche in the Cape fynbos**

---

## 4.1 Introduction

The CCR is a Mediterranean-type ecosystem dominated by sclerophyllous fynbos vegetation (Manning and Goldblatt 2012) occurring on mostly sandstone parent rock with low availability of P and N (Stock and Lewis 1986; Witkowski and Mitchell 1987). The shrub legume *P. calytrata*, favoured in horticulture for its attractive silver-green foliage and fragrant purple-white blossoms, is found in the CCR on sandstone mountain fynbos soils (Manders 1990; Schutte-Vlok and van Wyk 2011). In a glasshouse study, N<sub>2</sub>-fixing *P. calytrata* showed traits that are typical of plants from oligotrophic soils (Cocks and Stock 2001; Hawkins et al. 2007; Ostertag 2010; Lambers et al. 2011) through superior nodulation at low P supply compared with 16 other fynbos legume species, high P-use efficiency, storage of P in the shoot and high seed P content (Maistry et al. 2013). The physiological and morphological basis for this tolerance of low availability of P is yet to be reported.

Increased exudation of organic acids by roots may increase the availability of P from sparingly soluble sources through chelating cations that precipitate P or by displacing sorbed P through ligand exchange (Hinsinger 2001; Ryan et al. 2001; Lambers et al. 2006). Therefore, with limited provision of P supplied as KH<sub>2</sub>PO<sub>4</sub>, the concentration of organic acids effluxed by roots increased in *P. sativum* and in *Lupinus* species supplied with 1 mM NO<sub>3</sub> (Pearse et al. 2006a), and in 11 inoculated Australian native and exotic perennial legume species (Pang et al. 2010). In some instances, however, greater accumulation of biomass and exudation of organic acids by roots with increased supply of P was observed in *T. aestivum* (Pearse et al. 2006b) and in N<sub>2</sub>-fixing *L. angustifolius* (Wang et al. 2008), while *B. napus* plants with the highest foliar [P] also released more organic acids per root DM than plants with lower foliar [P] (Pearse et al. 2007). In response to low availability of P, plants may adjust allocation between organs, partitioning more biomass to roots than shoots, resulting in a higher root:shoot ratio than at high P availability (Nielsen et al. 2001; Poorter et al. 2012). Acquisition of P can also be enhanced by altering organ morphology through increasing SRL. By increasing root length more than root mass through making roots with low RTD or a small diameter or both (Eissenstat 1991), a greater SRL allows a plant to increase the volume of soil explored per unit DM invested in the root, resulting in more rapid rates of root proliferation and hence greater P-uptake rates than roots with lower SRL (Eissenstat 1991; Comas et al. 2002). In a glasshouse experiment (Pang et al. 2010), SRL was observed to increase with low P supply in the 11 herbaceous legumes being studied for their potential as new forage pasture.

The monophyletic genus *Podalyria* (Boatwright et al. 2008) consists of 17 species occurring in the CCR. Based on a Bayesian analysis of molecular data (Boatwright et al. 2008; Schnitzler et al. 2011), and an analysis of morphological and chemical characters (Schutte-Vlok and van Wyk 2011), *Podalyria* species form two clades, with *P. calytrata* and *P. burchellii* DC. in a separate clade to that

of *P. leipoldtii* L. Bolus ex A. L. Schutte and *P. myrtillifolia* (Retz.) Willd. Given the pervasiveness of oligotrophic soils in the CCR, the superior nodulation and growth of *P. calytrata* at low P supply (Maistry et al. 2013), and with a high N-demanding lifestyle in legumes (McKey 1994) possibly inducing a greater demand for P (Maistry et al. 2014), the other three *Podalyria* species may also possess mechanisms for enhanced acquisition of N and P. Although not observed for all functional traits (Cavender-Bares et al. 2004; Losos 2008), a pattern of phylogenetic signal in which the closely related species pair of *P. calytrata* and *P. burchellii*, and the pair of *P. leipoldtii* and *P. myrtillifolia* should show trait or niche conservatism (Webb 2000; Prinzing et al. 2001; Wiens 2004) is a widely accepted null expectation. The four species are distributed in different regions of the CCR (Schutte-Vlok and van Wyk 2011), with *P. calytrata* found in the south-west Cape Peninsula and allopatric with *P. burchellii* which occurs in the eastern CCR; *P. leipoldtii* is restricted to north-west Cape regions and also allopatric, with its non-overlapping distribution, to *P. myrtillifolia* which is found mainly in the south-east. Wiens (2004) has argued that phylogenetic niche conservatism can lead to such allopatric speciation. Several authors, however, have proposed that variation in availability of nutrients in the soil drives the distribution patterns of species in the CCR (Cowling and Holmes 1992; Richards et al. 1997) because some species possess traits for enhanced acquisition of nutrients and growth in more oligotrophic soils compared to species lacking these traits (Lamont 1982; Richards et al. 1997; Lambers et al. 2008; Shane et al. 2008). The Cape fynbos also experiences a latitudinal gradient of summer drought and wet winters in the west but less seasonal climate in the east (Cowling et al. 2009), and variation in temperature and precipitation may cause changes in nutrient availability (Austin and Vitousek 1998; Lukac et al. 2010). For instance, decomposition and mineralisation of soil nutrients are stimulated in warmer and wetter soil (Lukac et al. 2010), but with higher precipitation, increased leaching can also negate the positive effects of greater weathering on nutrient availability (Austin and Vitousek 1998). With their discrete distribution, and the associated climatic (Cowling et al. 2009) and edaphic (Richards et al. 1997) diversity in CCR habitats, the biophysical conditions in which each species is able to survive and maintain a stable population size (niche, Wiens and Graham 2005) may therefore differ.

In this study it was investigated whether traits for P acquisition and the ecological niche differed between closely related *Podalyria* species. In the glasshouse the responses of accumulation and allocation of biomass and root morphology and physiology traits for acquisition of N and P to increasing P supply were studied in the four nodulated *Podalyria* species. Soil, leaf, and climate parameters for the four *Podalyria* species in their natural habitats in the CCR were then examined. It was anticipated that all four species would increase expression of traits for acquisition of P with low P supply. It was hypothesised that the two species in each closely related pair of *P. calytrata* and *P. burchellii*, and of *P. leipoldtii* and *P. myrtillifolia*, would differ in their biomass allocation, SRL and organic acid exudation responses to P supply, and that the two closely related species would occupy a

different ecological niche in the fynbos. In the pot experiment, each *Podalyria* species was supplied 10, 25, 50 and 100 mg P kg<sup>-1</sup>, and plant biomass, leaf [N], leaf [P], root morphology and organic acids released by fine roots were measured; soil and leaf [N] and [P] and climate data were obtained for each species in the field.

## 4.2 Materials and methods

### *Growth conditions for sand culture in the glasshouse*

Seeds of *P. calytrata*, *P. burchellii*, *P. leipoldtii*, and *P. myrtillifolia* were obtained from Silverhill Seeds and Books, Cape Town, South Africa, who harvest seeds from wild plants in the fynbos. The seeds were soaked overnight in boiled water then sown in seedling trays containing acid-washed sand. Four weeks after emergence, seedlings of similar size of each species were transplanted into plastic pots filled with 3 kg of acid-washed sand. The sand was previously mixed in a cement mixer for 20 min with 10, 25, 50, or 100 mg P kg sand<sup>-1</sup> of insoluble P. The insoluble P consisted of a 3:1 ratio of FePO<sub>4</sub>:Ca<sub>5</sub>(PO<sub>4</sub>)<sub>3</sub> similar to the ratio of Fe-P:Ca-P in fynbos soils (Witkowski and Mitchell 1987; Power et al. 2010). During transplanting, 10 g pot<sup>-1</sup> of soil, collected from legume habitats in the CCR, was placed around the stem base and roots of each seedling as rhizobia inoculum. Each pot contained four seedlings, which were thinned to one seedling per pot after a further four weeks. Thus the experimental design was four *Podalyria* species that each received four levels of P supply, and there were six pots as replicates ( $n=6$ ) for each treatment. The plants were watered three times a week with 200 mL of nutrient solution (pH 6) that contained: 400 μM Ca(NO<sub>3</sub>)<sub>2</sub>, 200 μM K<sub>2</sub>SO<sub>4</sub>, 54 μM MgSO<sub>4</sub>, 0.24 μM MnSO<sub>4</sub>, 0.10 μM ZnSO<sub>4</sub>, 0.02 μM CuSO<sub>4</sub>, 2.4 μM H<sub>3</sub>BO<sub>3</sub>, 0.03 μM Na<sub>2</sub>MoO<sub>4</sub>, and 10 μM Fe-EDTA. However, for the first four weeks only, N was provided at a concentration of 200 μM. Plants were grown for 20 weeks from March to July 2011 in a glasshouse at UCT (33° 57.353' S 18° 27.742' E) with an average daytime temperature of 21°C controlled in the range of 20–30°C inside the glasshouse. All pots were randomly arranged on trolleys that were rearranged once a week. In addition, two species, namely *P. calytrata* and *P. myrtillifolia* were cultured, similar to the plants above, for 18 weeks from April to August 2013 ( $n=5$ ), to investigate whether increasing supply of Fe could stimulate organic acid production by roots (Emmanuel Delhaize, personal communication), similar to the mechanism for Al-induced efflux of malate and citrate for tolerance of Al (Ryan et al. 1995; Yang et al. 2000). All plants were inoculated with the CCR soil and supplied 25 mg P kg<sup>-1</sup> but with three Fe supply levels of 47, 95 or 190 mg Fe kg<sup>-1</sup> which were analogous to the levels of Fe in 25, 50 and 100 mg P kg<sup>-1</sup> when the P was applied in a 3:1 ratio of FePO<sub>4</sub>:Ca<sub>5</sub>(PO<sub>4</sub>)<sub>3</sub>. The additional Fe was supplied as FeCl<sub>3</sub> mixed into the sand, and the nutrient solution was adjusted to pH 5 to avoid precipitation of Fe.

### *Plant biomass and analysis of leaf nutrients for sand culture*

At harvest, roots were gently washed in water to remove sand and each plant was separated into leaves, stems, roots and nodules. Plant material was dabbed dry with paper towels prior to determining FM. Roots were not examined for arbuscular mycorrhizal structures because it was not possible to identify arbuscules in the roots of *P. calytrata* examined for mycorrhizal infection during preliminary investigations. Six of the youngest fully opened leaves of each plant were selected for leaf area measurements on a LI-3100 Area Meter (LI-COR, Lincoln, NE, USA). Plant material was dried at 60°C for 3 d and after DM recorded, leaf material was milled in a Wiley Mill using a 0.5 mm mesh (Arthur H. Thomas Co., Philadelphia, CA, USA). Foliar [N] was determined by digesting the milled leaf material in a LECO FP-528 nitrogen analyser (Leco Corporation, St. Joseph, MI, USA). Leaf [P] was analysed using ICPAES (Varian Vista MPX ICP-AES; Varian, Mulgrave, Australia) after dry-ashing pulverised plant material at 480°C for 8 h and dissolving in 16% HCl (Kalra 1998).

### *Collection and analysis of organic acids exuded by roots*

Between 300 to 600 mg FM of excised fine roots were kept on ice in a sterile 20-mL pill vial during harvesting. A volume of 15 mL of sH<sub>2</sub>O, containing 200 µM CaCl<sub>2</sub> for electrolytic conductivity and maintaining cellular integrity (Pearse et al. 2006a) was then added to each vial, submerging the roots. Organic acids were collected by agitating vials on an orbital shaker (160 rpm; Laboratory Marketing Services, Roodepoort, South Africa) for 1 h at 25°C. The solution was filtered through filter paper and the root material was recovered, dried at 60°C for 3 d and DM recorded. The filtrate was filtered through a 20-µm Acrodisc syringe filter (GVS Filter Technology, Indianapolis, IN, USA) and stored at -20°C until freeze dried and resuspended in 1 mL of sH<sub>2</sub>O. Analysis of citrate, malate, succinate, lactate and acetate concentration was performed at the Central Analytical Facility, Stellenbosch University, with enzymatic test kits in an Arena 20XT Enzyme Robot (Thermo Electron Oy, Vantaa, Finland). Concentration of malate, lactate and acetate in the sample was determined photometrically by measuring the increase in absorbance at 340 nm (Enzytec™ Fluid enzyme kits, Thermo Fisher Scientific Oy, Vantaa, Finland). The decrease in absorbance associated with the oxidation of NADH was used to determine the concentration of citrate and succinate (Yellow line enzyme kits, Roche, R-Biopharm AG, Darmstadt, Germany).

### *Analysis of root morphology*

At least 10% of the total root FM, the minimum amount recommended for accurate analysis of total root system morphology (Costa et al. 2000), was excised and stored in 30% isopropyl alcohol at 4°C. The seedlings of all four species had a modular fibrous root system which facilitated the selection of a

representative root sample, excised from the crown of the root to the root tips, in each plant. For staining, roots were decanted from the alcohol solution, rinsed with distilled water and then soaked in warmed 1% gentian violet solution for 15 min. The mixture was poured over a tea strainer and the roots were returned to the 30% isopropyl solution and stored at 4°C until ready for scanning. Roots were spread in water with minimal overlap in a perspex tray and scanned in grey level at 400 dpi resolution using an Epson Perfection V700 photo scanner (Epson, Long Beach, CA, USA). The image of the root sample was analysed for total root length, average diameter, length of roots in each specified diameter class, and root volume using WinRHIZO 2013 software (Regents Instruments, Quebec, Canada). The three diameter (dm) classes in mm, that accounted for 99% of the total root length, were  $0.0 < dm \leq 0.5$ ,  $0.5 < dm \leq 1.0$ , and  $1.0 < dm \leq 1.5$ , and are presented as % root length and denoted, respectively, as 0.25, 0.75 and 1.25 mm in diameter. After scanning, roots were dried at 60°C for 3 d and DM recorded.

#### *Field study of four Podalyria species*

Based on the location of representative specimens examined and listed in Schutte-Vlok and van Wyk (2011) or housed in the Bolus Herbarium at UCT, field collections were conducted in the CCR during November 2012. For each of the four *Podalyria* species, three sites (Table 2) were visited and at each site four plants were sampled for shoots and the associated soil. Soil was sampled below the canopy of the plant from the upper 15 cm of the soil profile using a soil corer or garden trowel after first removing leaf litter and debris from the surface of the ground. Altitude, temperature and precipitation for each of the 12 sites were obtained from the WorldClim ([www.worldclim.org](http://www.worldclim.org)) climate database (Hijmans et al. 2005).

#### *Analysis of leaf material and soil samples from the field*

Leaves were removed from stems, dried at 60°C for 3 d and milled in the Wiley Mill using a 0.5 mm mesh. Soil samples were air-dried and sieved through a 1 mm mesh before analysis. Leaf and soil [N] and C concentration ([C]) were determined using mass spectrometry. For leaf analysis, between 1.9 and 2.1 mg of milled leaf sample was weighed into 8 mm × 5 mm tin foil capsules (Elemental Microanalysis Ltd, Okehampton, UK) on a microbalance (Sartorius AG, Göttingen, Germany). For analysis of soil, between 20 and 40 mg soil per sample was used. The capsules were folded to enclose the samples, which were combusted in a Flash EA 1112 series elemental analyser (Thermo Electron, Milan, Italy). The resulting gases were fed into a Delta Plus XP IRMS (Thermo Finnigan, Bremen, Germany), via a Conflo III gas control unit (Thermo Finnigan). The in-house standards used were Merck Gel, a proteinaceous gel produced by Merck (Darmstadt, Germany), and dried leaves of *T. majus* collected from Woodbine Lane on UCT campus. The in-house standards have been calibrated

against IAEA (International Atomic Energy Agency) standards. Leaf [P] was analysed using ICPAES as described above. For the soil, available P was determined by extracting 2 g of soil in Bray II solution (Bray and Kurtz 1945), whereas total P in the soil was determined by acid digestion using a 1:1 mixture of 1 N nitric acid and hydrochloric acid at 80°C for 30 min (Sommers and Nelson 1972); the extracted solutions were then analysed using ICPAES. Soil pH was determined by shaking 2 g of soil in 20 mL 1 M KCl at 180 rpm for 60 min, passing the solution through filter paper and measuring the pH of the filtrate.

### *Statistical analysis*

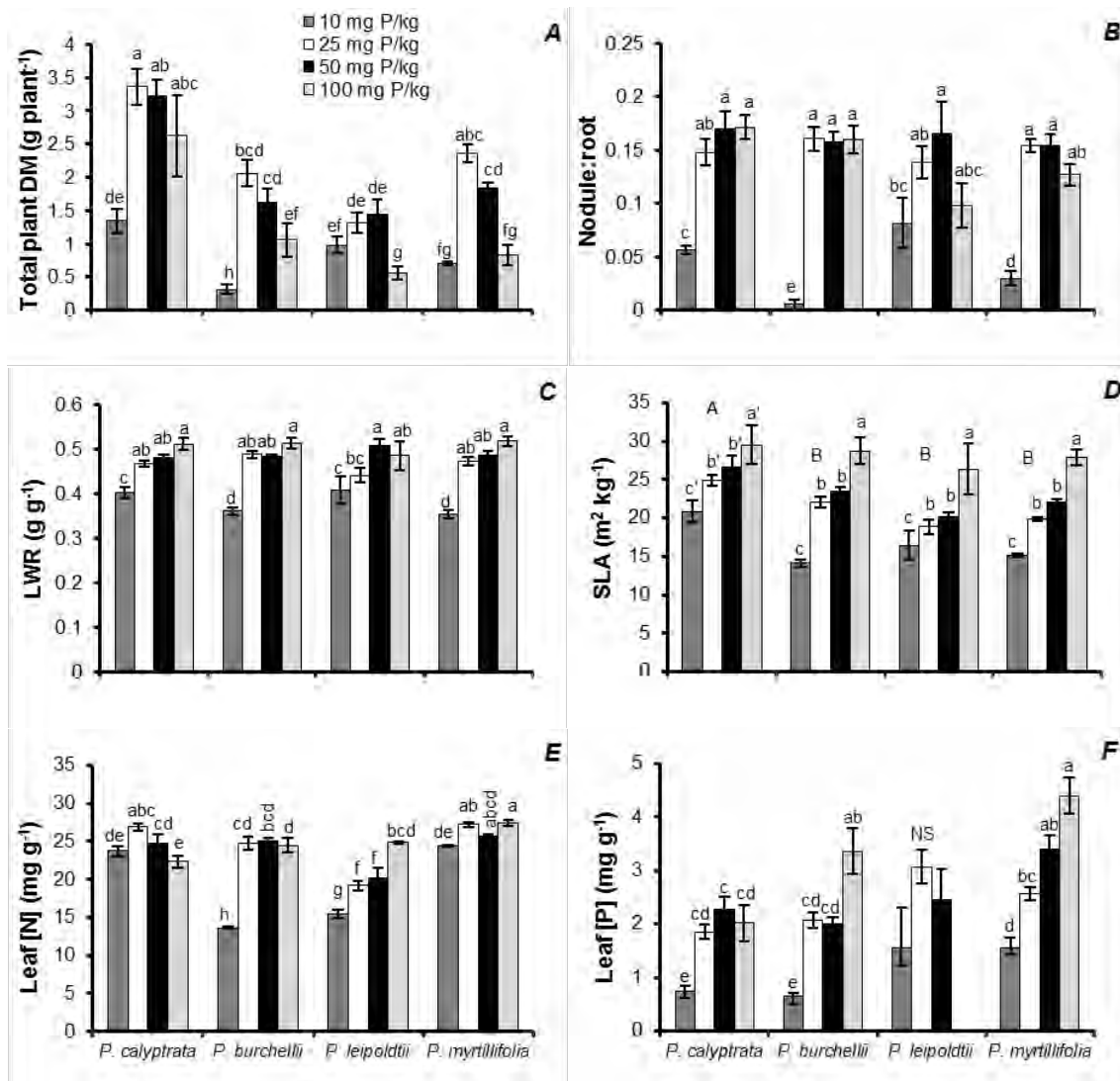
To reduce heteroscedasticity, all variables were  $\log_e$  transformed prior to statistical analysis except for percentage data which was arcsine square root transformed. A factorial ANOVA (STATISTICA version 11, StatSoft, Tulsa, OK, USA) was performed to test the species  $\times$  P-level interaction, except for leaf [P] in *P. leipoldtii*, assessed by one-way ANOVA. The species  $\times$  Fe-level interaction was assessed with a factorial ANOVA. Climate variables were analysed by one-way (Table 3) or factorial ANOVA (Table 4). For leaf or soil data in the field study, a nested ANOVA was conducted with site as a random effect nested in species. Means that were significantly different at  $P < 0.05$  were separated by Duncan's multiple range test.

## **4.3 Results**

### *Sand culture*

#### Accumulation of biomass and allocation to nodules and leaves, and leaf morphology

At low P supply of 10 mg P kg<sup>-1</sup>, the biomass of *P. calytrata* was similar to that of *P. leipoldtii*, but greater than the biomass of *P. myrtillifolia*, while *P. burchellii* was the smallest of the four species (Fig. 1A,  $F_{9,80}=5.63$ ,  $P < 0.001$ ). When P supply was increased to 25 mg P kg<sup>-1</sup>, *P. burchellii*, *P. myrtillifolia*, and *P. calytrata* increased accumulation of biomass by seven, 3.4, and 2.5-fold, respectively, but only *P. leipoldtii* did not respond significantly ( $P < 0.001$ ) to 25 mg P kg<sup>-1</sup> supply. With P supply increasing to 50 mg P kg<sup>-1</sup> none of the *Podalyria* species accumulated more biomass, whereas addition of 100 mg P kg<sup>-1</sup> decreased plant biomass in *P. burchellii*, *P. leipoldtii* and *P. myrtillifolia*, but not in *P. calytrata*. The ratio of nodule:root at 10 mg P kg<sup>-1</sup> was greatest in *P. leipoldtii* and *P. calytrata* and was threefold more than *P. myrtillifolia* and 10-fold greater than *P. burchellii* (Fig. 1B,  $F_{9,80}=12.50$ ,  $P < 0.001$ ). When P supply increased to 25 mg P kg<sup>-1</sup> or greater, the mass of nodule DM increased to approximately 17% of root DM in all four species. Leaf weight ratio was similar between *P. calytrata* and *P. leipoldtii* at 10 mg P kg<sup>-1</sup> and greater than the LWR of *P.*



**Fig. 1.** Effect of *Podalyria* species, *P. calytrata*, *P. burchellii*, *P. leipoldtii* and *P. myrtillofolia* and phosphorus (P) supply on (A) total plant dry matter (DM), (B) nodule:root ratio, (C) leaf weight ratio (LWR), (D) specific leaf area (SLA), (E) leaf nitrogen concentration ([N]) and (F) leaf P concentration ([P]). Means  $\pm$  s.e. ( $n=6$ ). For leaf [P], effect of species and P levels was assessed by factorial ANOVA for the three species except *P. leipoldtii* where the effect of P levels was assessed by one-way ANOVA (NS=not significant). Different lowercase letters indicate significantly different means between species and P levels at  $P<0.05$  from a factorial ANOVA. Different primed lowercase letters indicate significantly different means where no species  $\times$  P interaction occurred; different uppercase letters indicate significantly different means between species.

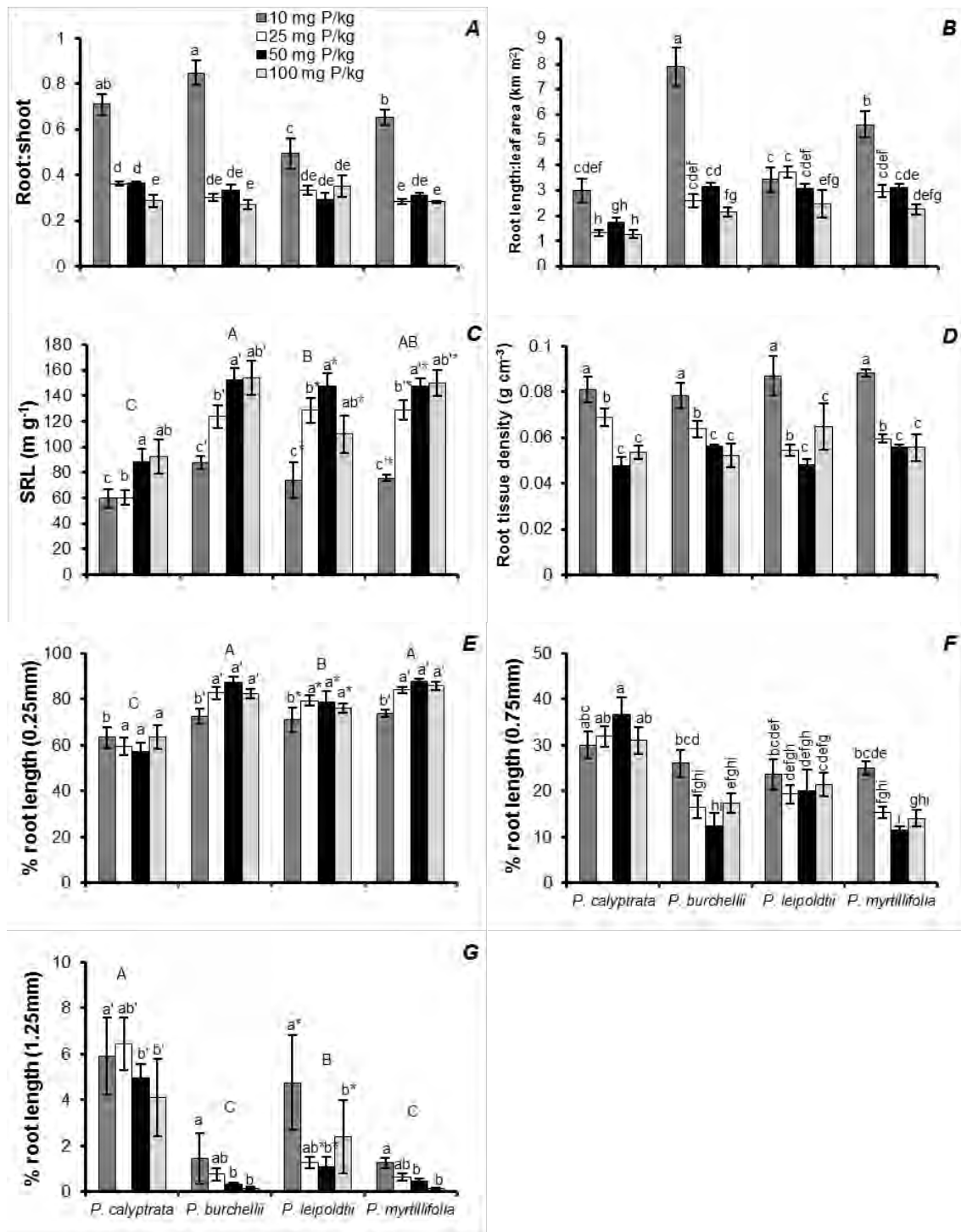
*burchellii* and *P. myrtillofolia* (Fig. 1C,  $F_{9,80}=5.63$ ,  $P<0.05$ ). With increased supply of P, however, the LWR increased, and was similar among the species. Specific leaf area (SLA), the ratio of leaf area to its mass, was greatest in *P. calytrata* but similar amongst *P. burchellii*, *P. leipoldtii* and *P. myrtillofolia* at all levels of P supply (Fig. 1D,  $F_{3,80}=11.54$ ,  $P<0.001$ ), and increased ( $F_{3,80}=52.99$ ,  $P<0.001$ ) with increasing P supply in all species.

## Leaf [N] and [P]

At low P supply of 10 mg P kg<sup>-1</sup>, leaf [N] was greatest in *P. calyptrata* and *P. myrtillifolia* with 24 mg N g<sup>-1</sup>, lower in *P. leipoldtii*, and least in *P. burchellii* (Fig. 1E,  $F_{9,80}=5.63$ ,  $P<0.001$ ). With greater P supply of 25 mg P kg<sup>-1</sup>, leaf [N] increased in all four species, and almost twofold in *P. burchellii* from 13 to 25 mg N g<sup>-1</sup> and was greater than that of *P. leipoldtii*, which had the lowest foliar [N] of 19 mg N g<sup>-1</sup>. With increasing supply of 100 mg P kg<sup>-1</sup>, leaf [N] decreased only in *P. calyptrata*. At 10 mg P kg<sup>-1</sup>, leaf [P] was similar between *P. calyptrata* and *P. burchellii*, possibly due to a dilution effect in the former species, and lower than *P. myrtillifolia* (Fig. 1F,  $F_{6,60}=3.04$ ,  $P<0.05$ ). Leaf [P] increased in all four species when supplied with 25 mg P kg<sup>-1</sup>, although the twofold increase in leaf [P] for *P. leipoldtii* was not statistically significant. With greater supply of 100 mg P kg<sup>-1</sup>, leaf [P] increased only in *P. burchellii* and *P. myrtillifolia*.

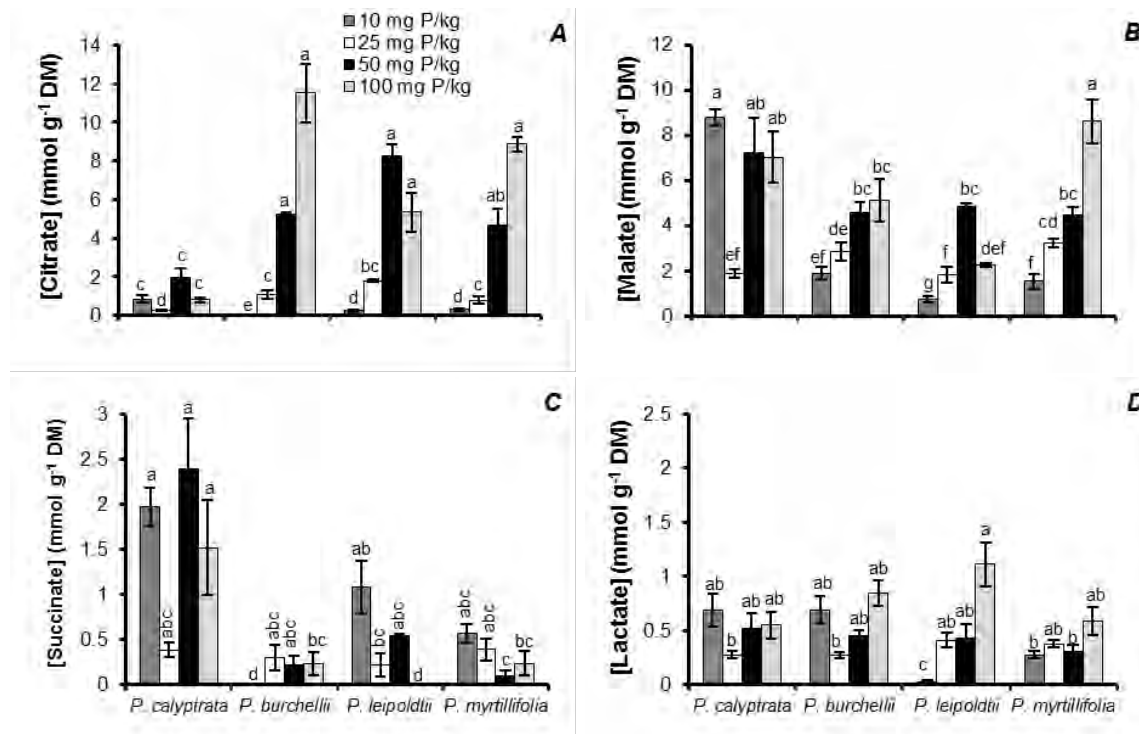
## Allocation of biomass to roots and morphology of roots

At low P supply, root:shoot ratio in *P. burchellii* was the same as that of *P. calyptrata*, but greater than the root:shoot ratio of *P. myrtillifolia* and *P. leipoldtii* which had the lowest ratio (Fig. 2A,  $F_{9,80}=4.50$ ,  $P<0.001$ ). With P supply increasing from 10 to 25 mg P kg<sup>-1</sup> the root:shoot ratio decreased by threefold in *P. burchellii*, by twofold in *P. myrtillifolia* and *P. calyptrata*, and by 1.5-fold in *P. leipoldtii*. The ratio of total root length:total leaf area (root length:leaf area) at 10 mg P kg<sup>-1</sup> was greatest in *P. burchellii*, followed by *P. myrtillifolia*, and lowest in *P. leipoldtii* and *P. calyptrata* (Fig. 2B,  $F_{9,80}=3.72$ ,  $P<0.001$ ). The root length:leaf area ratio decreased with the addition of 25 mg P kg<sup>-1</sup> in all species except *P. leipoldtii*. At all levels of P supply, *P. burchellii* had a greater root length:leaf area ratio than *P. calyptrata*. In all four species, SRL was lowest at 10 mg P kg<sup>-1</sup> and increased with greater P supply (Fig. 2C,  $F_{3,80}=27.32$ ,  $P<0.001$ ). The SRL of *P. calyptrata* roots was less than the SRL of the other three species at all levels of P supply, and the SRL of *P. leipoldtii* was also lower than that of *P. burchellii* ( $F_{3,80}=26.05$ ,  $P<0.001$ ). Root tissue density was similar amongst all four *Podalyria* species at each level of P and was highest at 10 mg P kg<sup>-1</sup> and decreased with increasing P supply (Fig. 2D,  $F_{3,80}=34.05$ ,  $P<0.001$ ). For the root diameter analysis, the Species  $\times$  P-level interaction was analysed for each of the three root diameters due to a significant Species  $\times$  P-level  $\times$  Root diameter interaction ( $F_{36,400}=2.10$ ,  $P<0.001$ ). *Podalyria burchellii* and *P. myrtillifolia* had the greatest proportion of roots that were 0.25 mm in diameter, followed by *P. leipoldtii*, and then *P. calyptrata*, which had the lowest proportion of the thinnest roots (Fig. 2E,  $F_{3,80}=35.19$ ,  $P<0.001$ ). When P supply increased from 10 to 50 mg P kg<sup>-1</sup> the proportion of thin roots increased in *P. burchellii*, *P. leipoldtii* and *P. myrtillifolia* by ca. 8–14% ( $F_{3,80}=5.19$ ,  $P<0.001$ ). In all four species, about 25% of their root length was 0.75 mm in diameter at low P supply (Fig. 2F). With increasing P supply this proportion remained unchanged in *P. calyptrata* and *P. leipoldtii* but decreased in *P.*



**Fig. 2.** Effect of *Podalyria* species, *P. calytrata*, *P. burchellii*, *P. leipoldtii* and *P. myrtilifolia* and phosphorus (P) supply on (A) root:shoot ratio, (B) root length:leaf area ratio, (C) specific root length (SRL), (D) root tissue density, and percent of root length that is (E) 0.25 mm, (F) 0.75 mm and (G) 1.25 mm in diameter. Means  $\pm$  s.e. ( $n=6$ ). Different lowercase letters indicate significantly different means between species and P levels at  $P<0.05$  from a factorial ANOVA. Different lowercase letters with the superscripts (') or (\*) or (\*') indicate significantly different means where no species  $\times$  P interaction occurred; different uppercase letters indicate significantly different means between species.

*burchellii* and *P. myrtillifolia* ( $F_{9,80}=2.45$ ,  $P<0.05$ ). All four species decreased the proportion of 1.25 mm thick roots with increasing P supply (Fig. 2G,  $F_{3,80}=4.02$ ,  $P<0.05$ ). However, *P. calyprata* had the greatest proportion of thick roots, followed by *P. leipoldtii*, whilst *P. burchellii* and *P. myrtillifolia* had the smallest fraction of thick roots ( $F_{3,80}=30.42$ ,  $P<0.001$ ).



**Fig. 3.** Effect of *Podalyria* species, *P. calyprata*, *P. burchellii*, *P. leipoldtii* and *P. myrtillifolia* and phosphorus (P) supply on the concentration of (A) citrate, (B) malate, (C) succinate and (D) lactate released by fine roots. Means  $\pm$  s.e. ( $n=3$ ). Different lowercase letters indicate significantly different means between species and P levels at  $P<0.05$  from a factorial ANOVA.

### Organic acids exuded by fine roots

At low supply of 10 mg P kg<sup>-1</sup>, *P. calyprata* roots released the greatest amounts of citrate when compared with the other species; exudation by *P. leipoldtii* and *P. myrtillifolia* roots led to similar concentrations of citrate (Fig. 3A,  $F_{9,32}=19.97$ ,  $P<0.001$ ). From the four species, *P. calyprata* roots also released the greatest quantities of malate at low P supply, with *P. burchellii* and *P. myrtillifolia* roots exuding greater amounts of malate than *P. leipoldtii* (Fig. 3B,  $F_{9,32}=15.05$ ,  $P<0.001$ ). Except for *P. calyprata*, all species increased exudation of citrate and malate by roots with increasing P supply, and only *P. leipoldtii* decreased malate exudation at 100 mg P kg<sup>-1</sup> supply. Similar amounts of succinate were released by *P. calyprata*, *P. leipoldtii* and *P. myrtillifolia* roots at low P supply, and the release of succinate decreased only in *P. leipoldtii* roots with increasing P supply (Fig. 3C,

$F_{9,32}=5.75$ ,  $P<0.001$ ). *Podalyria calytrata*, *P. burchellii* and *P. myrtillifolia* roots exuded similar amounts of lactate at 10 mg P kg<sup>-1</sup> supply, whereas *P. leipoldtii* roots increased production of lactate with increasing supply of P (Fig. 3D,  $F_{9,32}=8.51$ ,  $P<0.001$ ). Acetate was not detected in the analysis.

Accumulation of biomass and exudation of organic acids by fine roots in response to increasing Fe supply

Both *P. calytrata* and *P. myrtillifolia* accumulated greatest biomass when receiving 47 mg Fe kg<sup>-1</sup> and the accumulation of biomass decreased dramatically with increased supply of up to 190 mg Fe kg<sup>-1</sup>, possibly due to Fe toxicity (Table 1,  $F_{2,24}=10.25$ ,  $P<0.001$ ). The root:shoot ratio increased with increasing Fe supply in both species ( $F_{2,24}=12.11$ ,  $P<0.001$ ). In addition, the proportion of nodule DM which was ca. 14–16% of root DM at 47 mg Fe kg<sup>-1</sup>, decreased with increasing Fe supply ( $F_{2,24}=12.06$ ,  $P<0.001$ ). Similar to plant biomass and nodulation DM, release of citrate ( $F_{2,12}=77.36$ ,  $P<0.001$ ), succinate ( $F_{2,12}=14.84$ ,  $P<0.001$ ) and malate (data not shown,  $F_{2,12}=7.62$ ,  $P<0.01$ ) by *P. calytrata* and *P. myrtillifolia* roots decreased when Fe supply increased from 47 to 190 mg Fe kg<sup>-1</sup>.

### Field Study

#### Soil nutrients

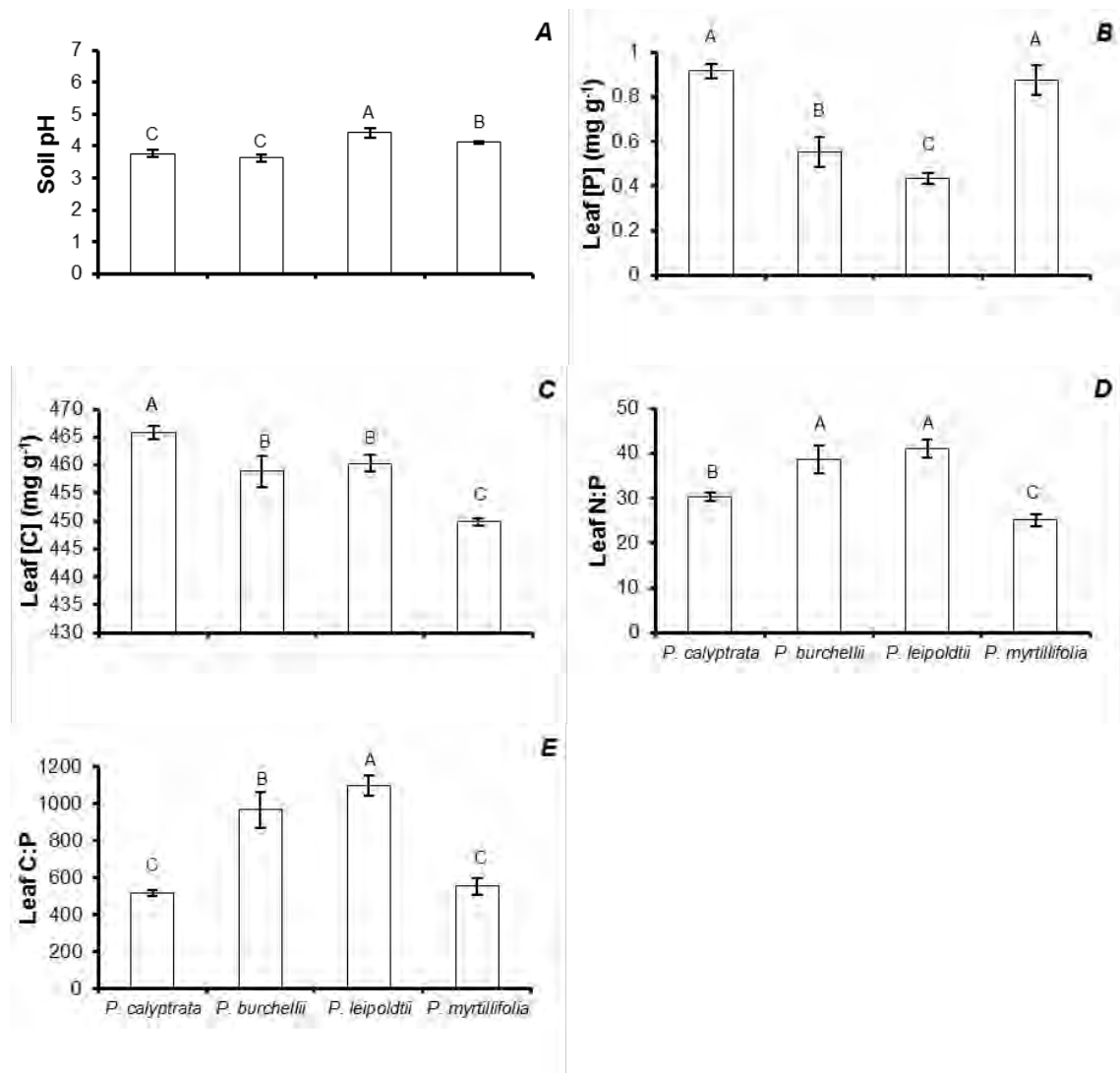
All species occurred in acidic soil with a pH range of 3.7–4.4 (Fig. 4A,  $F_{3,8}=4.89$ ,  $P<0.05$ ). The nested ANOVA showed that there was no significant difference ( $P<0.05$ ) in total soil [P] with a mean of ca. 73 mg P kg<sup>-1</sup>, Bray II extractable soil [P] of 6–12 mg P kg<sup>-1</sup>, total soil [N] of 1–1.5 g N kg<sup>-1</sup>, soil [C], and soil N:P ratio of ca. 22 amongst the sites of the four species (data not shown).

#### Leaf nutrients

Leaf [N] with a mean of ca. 21 mg N g<sup>-1</sup> was similar amongst all four *Podalyria* species (data not shown). In contrast, leaf [P] in *P. calytrata* and *P. myrtillifolia* of about 0.9 mg P g<sup>-1</sup> was greater than leaf [P] in *P. burchellii*, with leaf [P] of 0.4 mg P g<sup>-1</sup> in *P. leipoldtii* the lowest of the four species (Fig. 4B,  $F_{3,8}=5.87$ ,  $P<0.05$ ). *Podalyria calytrata* also recorded the highest leaf [C] whilst *P. myrtillifolia* had the lowest, with leaf [C] in *P. burchellii* and *P. leipoldtii* intermediate to these species (Fig. 4C,  $F_{3,8}=5.0$ ,  $P<0.05$ ). Plants of *P. leipoldtii* had both the highest leaf N:P ratio (Fig. 4D,  $F_{3,8}=5.07$ ,  $P<0.05$ ) and leaf C:P ratio (Fig. 4E,  $F_{3,8}=5.63$ ,  $P<0.05$ ) followed by *P. burchellii*. The leaf N:P and C:P ratios in *P. calytrata* were lower than that of *P. burchellii*; *P. myrtillifolia* had both the lowest leaf N:P ratio and leaf C:P ratio.

Table 1. Effect of *Podalyria* species and Fe supply on total plant dry matter (DM), root:shoot and nodule:root ratio, and concentration of citrate and succinate released by fine roots in nodulated *P. calytrata* and *P. myrtilifolia*, grown in sand culture with 25 mg P kg<sup>-1</sup>. Means ± s.e. (n=5) for biomass parameters and (n=3) for organic acids. Different lowercase letters in each column indicate significantly different means between species and Fe levels at P<0.05 from a factorial ANOVA; different lowercase letters with a prime symbol (') indicate significantly different means where no species × Fe interaction occurred.

Species	Fe-level (mg kg <sup>-1</sup> )	Total plant DM (mg plant <sup>-1</sup> )	Root:shoot	Nodule:root	[Citrate] (mmol g <sup>-1</sup> DM)	[Succinate] (mmol g <sup>-1</sup> DM)
<i>P. calytrata</i>	47	6.29±0.60 <sup>a</sup>	0.44±0.02 <sup>c</sup>	0.143±0.007 <sup>a</sup>	0.70±0.09 <sup>a</sup>	0.65±0.08 <sup>a</sup>
	95	2.88±0.28 <sup>b</sup>	0.66±0.03 <sup>b</sup>	0.052±0.007 <sup>bc</sup>	0.14±0.02 <sup>c</sup>	0.15±0.03 <sup>c</sup>
	190	0.43±0.03 <sup>c</sup>	0.98±0.04 <sup>a</sup>	0.043±0.005 <sup>c</sup>	0.41±0.06 <sup>b'</sup>	0.53±0.01 <sup>ab</sup>
<i>P. myrtilifolia</i>	47	8.41±0.46 <sup>a</sup>	0.21±0.01 <sup>d</sup>	0.164±0.004 <sup>a</sup>	0.78±0.20 <sup>a</sup>	0.38±0.01 <sup>b</sup>
	95	2.58±0.60 <sup>b</sup>	0.36±0.05 <sup>c</sup>	0.073±0.016 <sup>b</sup>	0.09±0.01 <sup>c</sup>	0.12±0.02 <sup>cd</sup>
	190	0.18±0.02 <sup>d</sup>	0.97±0.07 <sup>a</sup>	0 <sup>d</sup>	0.23±0.04 <sup>b</sup>	0.09±0.00 <sup>d</sup>



**Fig. 4.** (A) Soil pH, leaf (B) phosphorus concentration ([P]), (C) carbon concentration ([C]), (D) N:P ratio and (E) C:P ratio for *Podalyria* species, *P. calytrata*, *P. burchellii*, *P. leipoldtii* and *P. myrtilifolia* in the Core Cape Subregion. Means  $\pm$  s.e. ( $n=3$ ). Different uppercase letters indicate significantly different means between species at  $P < 0.05$  assessed from a nested ANOVA with site as a random effect nested in species.

## Climate

*Podalyria myrtilifolia* sites in the CCR (Table 2) were at a similar altitude above sea level as *P. calytrata* sites but lower than *P. burchellii* and *P. leipoldtii* sites in the mountains of the CCR (Table 3,  $F_{3,8}=4.76$ ,  $P < 0.05$ ). *Podalyria calytrata* received the greatest amount of annual precipitation, *P. burchellii* and *P. myrtilifolia* received intermediate amounts, whereas *P. leipoldtii* received the lowest precipitation in a year ( $F_{3,8}=13.64$ ,  $P < 0.01$ ). There was no difference amongst species in the maximum temperature of their hottest month. *Podalyria leipoldtii*, however, experienced a twofold lower minimum temperature than *P. calytrata* and *P. myrtilifolia* in their coldest month ( $F_{3,8}=4.86$ ,

$P < 0.05$ ), and the largest temperature range between its hottest and coldest temperatures ( $F_{3,8} = 4.54$ ,  $P < 0.05$ ). When the temperature range was averaged over the year as the mean diurnal range, *P. leipoldtii* also recorded a greater variation than *P. calytrata* and *P. myrtillifolia* ( $F_{3,8} = 6.04$ ,  $P < 0.05$ ). In terms of the combined effects of moisture and temperature, the driest and warmest three months experienced by *P. burchellii* were ca. 3–4°C cooler than the temperature experienced by the other species during their driest and warmest quarter (Table 4,  $F_{3,16} = 3.67$ ,  $P < 0.05$ ). During their driest and warmest parts of the year, *P. leipoldtii* sites received threefold less moisture than *P. burchellii*, *P. myrtillifolia* and *P. calytrata* sites ( $F_{3,16} = 19.92$ ,  $P < 0.001$ ); and also had the coldest temperature during their wettest and coolest quarters ( $F_{3,16} = 6.73$ ,  $P < 0.01$ ). During these winter periods, *P. calytrata* received the most moisture, and *P. myrtillifolia* received 50% more precipitation than *P. burchellii*, and *P. leipoldtii* ( $F_{3,16} = 24.06$ ,  $P < 0.001$ ).

Table 2. The four *Podalyria* species investigated in this study, with their respective sites and coordinates in the Core Cape Subregion.

Species	Site	Site coordinates
<i>P. calytrata</i>	Silvermine Reserve	-34.08 S; 18.40 E
	Tafelberg Road	-33.95 S; 18.42 E
	Newlands Forest	-33.97 S; 18.44 E
<i>P. burchellii</i>	Bergplaas	-33.91 S; 22.67 E
	Outeniqua Pass	-33.89 S; 22.40 E
	Robinson Pass	-33.88 S; 22.02 E
<i>P. leipoldtii</i>	Algeria Reserve 1	-32.37 S; 19.05 E
	Kleinklip Huis	-32.14 S; 18.96 E
	Algeria Reserve 2	-32.37 S; 19.06 E
<i>P. myrtillifolia</i>	Swartvlei	-34.00 S; 22.73 E
	Salmondsdam	-34.45 S; 19.58 E
	Tulbagh	-33.40 S; 19.24 E

Table 3. Metres above sea level (MASL), mean annual precipitation (MAP), maximum (Max) and minimum (Min) temperature (T) of the hottest and coldest month, the range between this Max and Min, and the mean diurnal range (the difference between the mean of the monthly Max T and Min T) for each *Podalyria* species in the Core Cape Subregion. Means  $\pm$  s.e. ( $n=3$ ). Different lowercase in each column indicate significantly different means between species at  $P<0.05$  from a one-way ANOVA.

Species	MASL (m)	MAP (mm)	Max T of hottest month (°C)	Min T of coldest month (°C)	T annual range (°C)	Mean T diurnal range (°C)
<i>P. calyptrata</i>	312 $\pm$ 71 <sup>bc</sup>	1026 $\pm$ 34 <sup>a</sup>	24.9 $\pm$ 0.6	7.6 $\pm$ 0.1 <sup>a</sup>	17.4 $\pm$ 0.7 <sup>b</sup>	9.5 $\pm$ 0.4 <sup>b</sup>
<i>P. burchellii</i>	585 $\pm$ 102 <sup>ab</sup>	638 $\pm$ 84 <sup>b</sup>	24.9 $\pm$ 0.5	5.2 $\pm$ 0.9 <sup>ab</sup>	19.7 $\pm$ 1.2 <sup>b</sup>	11.6 $\pm$ 0.7 <sup>ab</sup>
<i>P. leipoldtii</i>	802 $\pm$ 170 <sup>a</sup>	383 $\pm$ 39 <sup>c</sup>	29.3 $\pm$ 1.2	3.2 $\pm$ 0.6 <sup>b</sup>	26.1 $\pm$ 0.6 <sup>a</sup>	14.8 $\pm$ 0.4 <sup>a</sup>
<i>P. myrtilifolia</i>	234 $\pm$ 90 <sup>c</sup>	722 $\pm$ 91 <sup>b</sup>	26.4 $\pm$ 1.8	6.4 $\pm$ 1.3 <sup>a</sup>	20.0 $\pm$ 3.1 <sup>b</sup>	10.9 $\pm$ 1.5 <sup>b</sup>

Table 4. Mean temperature (T) or precipitation during the driest and warmest three months of the year (quarter), and during the wettest and coolest quarters for each *Podalyria* species in the Core Cape Subregion. A separate factorial ANOVA assessed the following species and climate interactions: Species  $\times$  Mean T of the driest or warmest quarter, Species  $\times$  Precipitation in the driest or warmest quarter, Species  $\times$  Mean T of the wettest or coolest quarter, and Species  $\times$  Precipitation in the wettest or coolest quarter. Means  $\pm$  s.e. ( $n=3$ ). Different lowercase letters in each column indicate significantly different means between the main effect of species at  $P<0.05$  as no interaction occurred.

Species	Mean T (°C) of driest and warmest quarters	Precipitation (mm) in driest and warmest quarters	Mean T (°C) of wettest and coolest quarters	Precipitation (mm) in wettest and coolest quarters
<i>P. calyptrata</i>	19.4 $\pm$ 0.2 <sup>a</sup>	100 $\pm$ 3 <sup>a</sup>	12.7 $\pm$ 0.2 <sup>a</sup>	458 $\pm$ 9 <sup>a</sup>
<i>P. burchellii</i>	16.7 $\pm$ 1.3 <sup>b</sup>	153 $\pm$ 14 <sup>a</sup>	13.7 $\pm$ 1.0 <sup>a</sup>	159 $\pm$ 14 <sup>c</sup>
<i>P. leipoldtii</i>	20.5 $\pm$ 0.7 <sup>a</sup>	34 $\pm$ 4 <sup>b</sup>	10.4 $\pm$ 0.6 <sup>b</sup>	182 $\pm$ 10 <sup>c</sup>
<i>P. myrtilifolia</i>	19.4 $\pm$ 1.0 <sup>a</sup>	108 $\pm$ 25 <sup>a</sup>	12.5 $\pm$ 0.7 <sup>a</sup>	268 $\pm$ 42 <sup>b</sup>

#### 4.4 Discussion

In this study the hypothesis of trait and niche difference between closely related *Podalyria* species was supported because the responses to P supply in the accumulation and allocation of biomass, SRL and organic acids released by fine roots, and leaf [P] and the climate of habitats, differed between the closely related *Podalyria* species. Notably also, and contrary to expectation, SRL and exudation of organic acids by roots increased with greater P supply in the N<sub>2</sub>-fixing *Podalyria* species. This

observation of increased expression of traits for P acquisition with increasing P supply is, however, consistent with plant growth strategies for fast growth at high P supply but stress tolerance and survival at low P supply (Grime 1977; Chapin 1980).

In controlled glasshouse conditions all four nodulated *Podalyria* species exhibited traits that would enable growth in the Cape soils with low P availability of 6–12 mg P kg<sup>-1</sup>. By increasing allocation to root biomass over shoot biomass with low P supply, each *Podalyria* species showed an ability for enhanced acquisition of P (Nielsen et al. 2001; Lambers et al. 2006; Power et al. 2010; Poorter et al. 2012). The species, however, were differentiated by their combined root:shoot ratio and LWR allocation responses, and there was clearly an absence of phylogenetic signal between the closely related species; *P. burchellii* and *P. myrtilifolia* showed greater limitation by P at low P supply, as suggested by Bloom et al. (1985), through allocating relatively more to root biomass for P acquisition and less to leaf biomass for C acquisition, compared with *P. calytrata* and *P. leipoldtii*. Moreover, greater responsiveness to increased P supply in root:shoot ratio, LWR, accumulation of biomass and nodulation, also reflect greater limitation by low P supply in *P. burchellii* and *P. myrtilifolia*. With the lowest root:shoot ratio at low P supply, and with biomass accumulation, and a root:shoot ratio and LWR that were least responsive to P supply, growth of *P. leipoldtii* may be adapted to low availability of P (Fig. 4B). This species also attained its maximum biomass with a lower leaf [N] than the other species, showing low N demand atypical of legumes (McKey 1994) but characteristic of species from infertile landscapes (Lambers et al. 2010). In contrast, *P. calytrata* showed the ability to accumulate N at low P supply, with large plant size (Fig. 1A) and high leaf [N]. Thus, consistent with an earlier report of superior nodulation in the species (Maistry et al. 2013), greater N acquisition in *P. calytrata* indicates tolerance of nodule growth and possibly N<sub>2</sub>-fixation to low availability of P.

Variation in SRL is a function of two components, namely RTD and root diameter (Eissenstat 1991; Comas et al. 2002). Although increasing RTD in all species contributed to the decreasing SRL observed in all four species as P supply decreased, the differences in the proportion of thin and thick roots amongst species (Fig. 2E–G) may have led to the species differences in SRL. Roots with high tissue density have a longer lifespan and lower turnover rate than those with lower RTD (Ryser 1996; Eissenstat et al. 2000). Hence the plants that grew more slowly at low P supply constructed roots with low SRL (Fig. 2C, Roumet et al. 2006), analogous to the observed low SLA of long lived sclerophyllous leaves (Lambers et al. 2010). This would be to reduce the rate of nutrient loss associated with short lifespan and a high turnover rate of tissue. This trade-off in growth strategies (Grime 1977; Chapin 1980) was equally evident in the root morphology adjustments in all four *Podalyria* species, with high RTD and low SRL for efficient use of resources possibly through root persistence at low P supply, but low RTD and high SRL for maximising root exploration and P-uptake (Eissenstat 1991; Comas et al. 2002) at high P supply. Root diameter morphology, however,

contrasted between close relatives with *P. calytrata* and *P. leipoldtii* having a lower proportion of the thinnest roots but a larger fraction of the thickest roots compared with *P. burchellii* and *P. myrtillifolia*. For *P. burchellii* and *P. myrtillifolia*, the cost of a faster turnover rate in thin roots would be feasible in their conducive easterly habitats with less climate seasonality, allowing for more rapid rates of proliferation into new soil not yet depleted of its phosphate by roots and thereby increasing rates of P-uptake. Furthermore, *P. burchellii*, with a similar SRL and SLA but greater root:shoot ratio and root length:leaf area ratio than *P. myrtillifolia* at low P supply, allocates and invests more biomass to root growth for enhanced P acquisition relative to *P. myrtillifolia*. In the broad-leaved (high SLA) and bigger *P. calytrata* plants, large diameter roots would function for greater structural support; for *P. leipoldtii* which experiences the greatest temperature extremes and the driest summers compared to the other species, thicker roots would confer persistence and fortification against cold and desiccation (Eissenstat et al. 2000).

Although *P. calytrata* had the lowest SRL, its roots produced the highest concentration of organic acids amongst the four species at low P supply. Increased exudation of organic acids by roots at low P supply is a mechanism for enhanced acquisition of P (Hinsinger 2001; Lambers et al. 2006), with the tricarboxylated citrate reported to be the most effective at solubilising P, followed by dicarboxylated malate and succinate (Ryan et al. 2001). Thicker roots of *P. calytrata* that can only proliferate at a slower rate towards the relatively immobile phosphate ion, may instead rely on chemical modification of the rhizosphere for solubilising P and increasing the concentration available at the root surface. With a SRL that gives the lowest turnover of roots, and a higher SLA and leaf [N] permitting greater C acquisition in habitats that are not limited by moisture, *P. calytrata* can allocate more resources to the production of C-rich citrate, malate and succinate. Consistent with higher respiration rates of roots with greater SRL (Comas et al. 2002), the *Podalyria* species increased exudation of organic acids, especially of citrate and malate, with increasing P supply. The plant roots did not release a greater concentration of organic acids to avert Fe toxicity because exudation of organic acids decreased with increasing Fe supply (Table 1) unlike the greater exudation of organic acids in response to increased Al supply (Ryan et al. 1995; Yang et al. 2000). However, as in *L. angustifolius* and *Phaseolus vulgaris* L., increasing organic acid anion release by roots with increasing P supply may function as a counter ion for charge balance associated with greater proton release from enhanced N<sub>2</sub>-fixation (Fig. 1B, Tang et al. 2004; Wang et al. 2008). In addition, to prevent pH increases in the rhizosphere, the N<sub>2</sub>-fixing *Podalyria* legumes found in acid soils may favour releasing organic anions instead of hydroxide ions to balance charge. Furthermore, in *L. angustifolius* that did not form cluster roots at low P supply, high nitrate supply stimulated cluster root formation and citrate exudation at adequate P supply (Hocking and Jeffery 2004). It is known that increased amounts of P in the solution or in the plant tissue may increase the demand and capacity for uptake of N and vice versa (Cole et al. 1963; Hills et al. 1970; Chapin 1980; Fageria 2001). Hence, when P supply

increased to 25 mg P kg<sup>-1</sup> or greater, all four *Podalyria* species increased nodulation so that approximately 17% of root DM was invested into nodule DM. Both the leaf [N] (Fig. 1E) and leaf [P] (Fig. 1F) increased and there was a positive interaction between P supply and N accumulation because both biomass and leaf [N] increased simultaneously. Increasing LWR in all species showed increased investment in leaf DM for acquisition of C with increasing P supply, indicating greater demand for C. Therefore, with greater P supply, increased nodulation and release of organic acids from roots and a higher SRL were plant growth responses for enhanced acquisition of resources.

In the field, the allopatric distribution ranges of the four species were indeed apparent, so that with the existing climatic diversity (Tables 3, 4; Cowling et al. 2009), each species would occupy a different niche in their CCR habitats. *Podalyria calytrata* was found in the south-west along the lower slopes of Table Mountain National Park, *P. burchellii* in the east on the slopes of the Outeniqua Mountains, *P. leipoldtii* occurred in the north-west in the Cederberg Mountain ranges, and *P. myrtillifolia* was more widespread in the lowlands of the south-east CCR (Tables 2, 3; Schutte-Vlok and van Wyk 2011). Although all species occurred in acidic soils low in Bray II P of 6–12 mg P kg<sup>-1</sup>, *P. leipoldtii* experiences severe drought, receiving threefold less moisture than *P. myrtillifolia* during their driest and warmest summer periods, and also had the coldest winter periods. In contrast to warmer and wetter soils that could stimulate decomposition, mineralisation and weathering of soil nutrients (Austin and Vitousek 1998; Lukac et al. 2010), these dry and cold conditions would reduce availability of nutrients from the soil. Therefore, with the lowest leaf [P] of 0.4 mg P g<sup>-1</sup>, whereas other legume species in the CCR and global vegetation have foliar [P] of 0.8–1.0 mg P g<sup>-1</sup> (Lambers et al. 2010; Power et al. 2011), and with a leaf N:P ratio of 41, whereas foliar N:P ratios of wild plants from other infertile soils are between 22–27 (Lambers et al. 2010; Stock and Verboom 2012), *P. leipoldtii* has evolved to grow with a low demand for P, as was clearly evidenced in the results from the glasshouse. On the other hand, for *P. myrtillifolia*, greater leaf [P] of 0.9 mg P g<sup>-1</sup> and the lowest leaf N:P ratio of 25 reflects relatively more fertile growth conditions, so that the lowest leaf [C] in *P. myrtillifolia* may be due to construction of less sclerophyllous leaves for when nutrient and drought stress is not severe (Lambers et al. 2010). *Podalyria burchellii*, meanwhile, receives a more reliable year-round rainfall of ca. 150 mm, and with ca. 4°C cooler driest and warmest periods than that of the other species, *P. burchellii* benefits from lower levels of drought and nutrient stress (Turner and Gilliam 1976) during summer as indicated by a greater leaf [P] than in *P. leipoldtii*. Furthermore, the greatest amount of annual precipitation received by *P. calytrata* may also enable its higher leaf [P] (Turner and Gilliam 1976) and relatively lower leaf N:P and C:P ratios than *P. burchellii*. Therefore, *P. leipoldtii* with extreme climatic conditions and the lowest leaf [P] in its fynbos habitat, occupied the most oligotrophic niche; *P. burchellii* had a more nutrient limited niche than *P. calytrata*, and *P. myrtillifolia* was found in the relatively more fertile niche. The similar soil fertility levels but different climate, physiology and morphology between closely related species is consistent with a

model of speciation in allopatry (Wiens 2004; Bentley et al. 2014) following lineage splitting during the middle to late Miocene and early Pliocene (Boatwright et al. 2008; Schnitzler et al. 2011) that coincides with geological uplift or climatic changes (Cowling et al. 2009). It is possible that habitat contraction associated with changing climate may have isolated the species, or that adaptation to climate and the associated nutrient availability was more recent and occurred after the vicariant geomorphic event.

#### **4.5 Conclusion**

The mechanisms for P acquisition, growth, and the ecological niche differed in closely related *Podalyria* species. The absence of phylogenetic signal (trait or niche conservatism) suggests that ecological factors override phylogenetic relatedness in determining the presence of P acquisition traits in these species; and of these factors, climate rather than soil nutrient levels *per se*, may drive distribution and speciation in the fynbos. *Podalyria calyptрата* nodulated well at low P supply with greater exudation of organic acids by its thick roots. *Podalyria burchellii* had a greater proportion of fine roots, similar to *P. myrtillifolia*, but with greater allocation of biomass to roots. In comparison, biomass accumulation in *P. leipoldtii* did not have a high demand for N and P so that the species invested less in P acquisition traits. Overall, the *Podalyria* species exhibited plasticity in response to changing resource availability, with strategies that augur well for their persistence given changing climate and increasing nutrient deposition in the nutrient limited fynbos.

Comprising about 805 species, legumes (Fabaceae) form the second largest plant family to Asteraceae in the CCR (Goldblatt and Manning 2012). Given their role as a biogenic source of N and hence as potential regulators of biogeochemical processes in ecosystems, legumes are an important functional group in the oligotrophic fynbos. By increasing N availability and soil fertility in natural ecosystems, N<sub>2</sub>-fixation can lead to increased primary productivity. It is thus disconcerting that very little is known about the nutritional physiology and ecology of Cape legumes (Lambers et al. 2006; Lynch and Brown 2006). Although cluster roots, for instance, have been observed in *A. linearis* (Lambers et al. 2006; Hawkins et al. 2011; Maistry et al. 2013), their functioning has not been explored. Therefore, understanding the physiology of the unique and largely understudied legume biodiversity in the nutrient poor CCR was compelling motivation for the study, and reporting on the physiological mechanisms enabling tolerance of low availability of P in *A. linearis* and *P. calytrata*, as model legume species of the CCR (Maistry et al. 2013), was the primary objective of this thesis. For this purpose it was anticipated that low P supply would limit plant growth and increase expression of traits for P acquisition (Chapters 2–4).

Studies designed to report on the physiological mechanisms enabling tolerance of low P availability, typically observe plant responses to decreasing P supply. N and P colimitation of N<sub>2</sub>-fixing and combined-N supplied legumes and non-legumes (Power et al. 2010; Maistry et al. 2013), however, indicate that consideration of single resource P limitation is misleading, requiring instead to consider the potential interactive effect of N and P on legume growth responses to nutrient supply (Bloom et al. 1985; Craine 2009; Cramer 2010). Because the responses to increased P supply were dependant on the level of N supplied (Maistry et al. 2013), ecologically relevant levels of increasing N supply (Glass et al. 2002; Britto and Kronzucker 2005) were used to investigate in a more mechanistic way N and P limitation of plant growth. Furthermore, single resource P limitation experiments that merely demonstrate the physiological differences between species (Tadano and Sakai 1991; Pearse et al. 2006a; Pang et al. 2010), lack the potential to provide deeper insights into how these differences influence or shape ecosystems. Cramer et al. (2014) note that the question of how ecophysiological specialisations vary across environmental gradients and contribute to species richness in the Cape flora, has received little attention. Hence, closely related species were compared in the glasshouse and in the field to make inferences about P acquisition traits and the distribution of species in the fynbos.

The scarcity of nutrients in the soil has long been considered as the causal antecedent of distribution patterns and speciation in the CCR and the heathland vegetation of Australia (Beadle 1954, 1962; Ozanne and Specht 1981; Lamont 1982; Cowling and Holmes 1992; Richards et al. 1997; Lambers et

al. 2008; Shane et al. 2008). Cramer et al. (2014), however, identify two main sources of niche diversity in the CCR, namely the west-east climate gradient and nutrient poor soil, and conclude that ecophysiological specialisation (of species) to the mosaic of niches created by the interaction of these two factors, is a key reason for plant species richness in the Cape (Linder 2005). The observation from our study on *Podalyria* (Chapter 4) shows that speciation may indeed be the result of the interaction between climate and nutrient availability. The conclusion that climate may drive the allopatric distribution of the closely related *Podalyria* species is consistent with a framework proposed by Pearson and Dawson (2003) showing the spatial scales across which different factors can act to affect the distribution of species. Thus climate is the main driver of species distribution over the regional scale (200–2000 km), whereas soil type (and associated nutrient availability) would drive speciation at a local (1–10 km) or site scale (10–1000 m). A study that compares closely related sympatric species would therefore be pertinent in the case of soil type, and should show that the closely related species occur on different soils. Although *P. calytrata* (exudation of organic acids by roots), *P. burchellii* (an extensive length of fine roots) and *P. myrtillifolia* (fine roots) exhibited traits that would enhance P acquisition, and showed plasticity in responses to increasing P supply, the accumulation and allocation of biomass in *P. leipoldtii* was relatively unresponsive to P supply. The low degree of plasticity in *P. leipoldtii* can be correlated with a high degree of ecophysiological specialisation to a specific niche. Indeed *P. leipoldtii* was observed to have a very restricted distribution (Table 2, Chapter 4; Schutte-Vlok and van Wyk 2011). This species should be investigated, as a model leguminous plant, for the molecular mechanisms and genes involved in N<sub>2</sub>-fixation and drought tolerance, to help in understanding the functioning of other legumes from arid environments, with potential applications in agriculture, agroforestry and land rehabilitation.

In addition to climatic and edaphic variation, the prevalence of fire in the fynbos (Cocks and Stock 2001) adds another layer of complexity into our attempts to understand legume nutrition and ecosystem processes. In soils that are generally oligotrophic, fire can result in pulses of N and P to the system (Brown and Mitchell 1986; Stock and Lewis 1986). Hence, examining the responses of P acquisition mechanisms and biomass to increased supply of combined-N at low P and high P supply in *A. linearis* and *P. calytrata*, is analogous to the temporal fluctuations in the relative availability of N and P experienced by legume species in the nutrient poor fynbos soils. Comparing nutrient concentration values across studies must be done with caution due to differences in growth stages of the plants and the dilution effect associated with biomass accumulation. However, the fact that tissue [P] for *P. calytrata* of 0.5 mg P g<sup>-1</sup> at 10 μM P supply, and 2 mg P g<sup>-1</sup> at 100 μM P supply are on either side of the value of 1 mg P g<sup>-1</sup> found in the field plants, does indicate that the conditions in the sand experiment closely approximated the conditions of low and high P supply that one would expect in the field. It was clear (in Chapter 2 and 3, and also in Chapter 4) that increasing the supply of N or P induced a greater demand for the opposite ion, because the mechanism for the acquisition of that ion

was stimulated. It was shown empirically that the ratio of supply of N and P is critical to determining the level of expression of P acquisition mechanisms, nodulation and growth (Chapter 2 and 3), and that *A. linearis* and *P. calyptрата* have the ability to compensate for an imbalanced supply N:P ratio by allocating resources to the acquisition of the more limiting resource. For instance, increased phosphatase activity by roots in *A. linearis* may have increased available P, because the demand N:P ratio was lower than the more N limiting supply N:P ratio, and enhanced plant growth; prolific nodulation in *P. calyptрата* increased the available N:P ratio and the accumulation of biomass to the levels of that in plants receiving high levels of nitrate. Balanced nutrition, therefore, is clearly important to plant growth, as has been demonstrated by the prevalence of synergistic growth responses to simultaneous addition of N and P (Elser et al. 2007; Bishop et al. 2010; Craine and Jackson 2010; Harpole et al. 2011; Maistry et al 2013).

Legumes are noted to be abundant after fire (Brown and Mitchell 1986; Manders 1990). This may be the result of their ability to cope with changes and imbalances in soil nutrients during this time. It has been argued, however, that the inability of legumes to persist late into the post-fire succession may be due to low allocation of biomass to roots relative to non-legumes (Power et al. 2010). Although the increased expression of P acquisition mechanisms in response to low P supply (in *A. linearis*, *P. calyptрата*, *P. burchellii* and *P. myrtillifolia*) are noteworthy, it is the substantial responses in cluster root formation, and phosphatase and organic acid exudation (*A. linearis*) and root organic acid exudation and PEPC and MDH activity (*P. calyptрата*) to severe P limitation induced by greater N supply, which suggests that legumes are actually well adapted for acquiring or recycling P. Further experiments, comparing *A. linearis*, *P. calyptрата* and model non-legume species in Proteaceae (Hawkins et al. 2007; Shane et al. 2008; Lambers et al. 2011), for instance, would be required. Other hypotheses, investigating ecological processes such as competitive exclusion of legumes by non-legumes based on differences in drought or shade tolerance or both, should also be considered.

Whereas balanced nutrition favours synergistic growth, colimitation by N and P poses a challenge when investigating the physiology of wild legumes and non-legumes, with no previous history on adequate nutrient requirements (Power et al. 2010; Maistry et al. 2013). Thus there is a need to establish the correct concentration of combined-N and P to supply to legumes and non-legume species for future studies, so that plants are supplied adequate levels of combined-N at low and high P supply, allowing for suitable comparisons to N<sub>2</sub>-fixing plants. For instance, as discussed in Chapter 2, choosing 100 µM P as high P supply may require 2.3 mM N supply to meet the N demand of the P supplied, but supply of 2.3 mM N and 10 µM P will not support plant growth. Our results suggest that an experimental design with N<sub>2</sub>-fixing and 500 µM N supply as the N treatments at a low P level of 10 µM P and a high P level of 25 µM P in sand culture, should be feasible. The results on colimitation of plant growth are also of applied physiological interest. In agricultural systems,

farmers are interested in knowing the maximum amount of combined-N that can be supplied before nodulation is inhibited and the benefits of the freely available fixed  $N_2$  are diminished. Findings on nodulated *P. calyptрата* show that the appropriate level of combined-N supply will vary depending on the level of P supplied. For instance, at low P supply of 10  $\mu\text{M}$  P, more than 100  $\mu\text{M}$  N supply may reduce  $N_2$ -fixation, whereas at high P supply, plants may receive up to 500  $\mu\text{M}$  N before nodulation is depressed. Therefore the level of P in the soil will have to be diagnosed and then the appropriate amount of N should be supplied, after first conducting N  $\times$  P studies on the crop. It needs to be recognised, however, that there is a minimum concentration of each element that can support growth, and below which the ratio of the different elements is not important. Above these thresholds of nutrient concentrations, the ratio of elements becomes meaningful, and it is above these thresholds where colimitation can occur.

$N_2$ -fixation is presumed to be demanding of P (Israel 1987; Hellsten and Huss-Danell 2002), a premise that forms the basis of most theories and hypotheses on legume physiology and ecology (Vitousek et al. 2002; Power et al. 2010; Cramer et al. 2014). This premise is based on the significant increase in biomass accumulation that legumes show when supplied more P (Jakobsen 1985; Israel 1987; Pereira and Bliss 1987; Drevon and Hartwig 1997). This result is juxtaposed against the unresponsiveness of non-legumes to P fertilisation (Hawkins et al. 2007; Power et al. 2010) which is interpreted to signify a lower demand for P by plants acquiring combined-N. It was clearly apparent, however, that limitation by N can prevent an increase in biomass accumulation to increased P supply (non-nodulated *A. linearis*), whereas in  $N_2$ -fixing plants, the ability to meet the N demand of greater P supply (*P. calyptрата*) results in enhanced biomass accumulation. Colimitation of nodulation by N and P showed that nodulation was a regulated response to N demand. Furthermore, the similar response to increased P supply (Robson 1983) and similar biomass and leaf N:P ratio between  $N_2$ -fixing and  $\text{NO}_3$ -supplied *P. calyptрата* plants receiving high P supply of 15  $\mu\text{M}$  P indicates that the  $N_2$ -fixing and  $\text{NO}_3$ -supplied plants have the same demand for P. Hence, embracing the concept of colimitation can lead to a more nuanced understanding of resource limitation of plant growth.

The five legume species in this study have each shown unique responses at the whole plant level, within the broad categories of adaptations to P stress (Vance et al. 2003). Although the current work has elucidated previously unknown functional adaptations within the fynbos species, this should form the basis for future studies to focus on how these mechanisms are controlled at the cellular level. In this regard, the use of modern genomic tools such as metabolome, transcriptome, or proteome profiles may reveal the underlying mechanisms that underpin these whole plant responses. In model legumes and non-legumes (Hammond et al. 2004; Hernández et al. 2007; Hernández et al. 2009), this approach towards cellular mechanisms has already revealed different levels of control over the response to P deficiency. Moreover, the “-omics” level analyses would enable the understanding of network

interactions among gene expression, protein synthesis and metabolites, in a P stress “interactome”, in order to validate the whole plant level responses. This would be of particular interest in legumes that have evolved on nutrient poor soils.

## References

- Almeida JPF, Hartwig UA, Frehner M, Nösberger J, Lüscher A (2000) Evidence that P deficiency induces N feedback regulation of symbiotic N<sub>2</sub> fixation in white clover (*Trifolium repens* L.). *Journal of Experimental Botany* 51: 1289–1297.
- Almeida JPF, Lüscher A, Frehner M, Oberson A, Nösberger J (1999) Partitioning of P and the activity of root acid phosphatase in white clover (*Trifolium repens* L.) are modified by increased atmospheric CO<sub>2</sub> and P fertilization. *Plant and Soil* 210: 159–166.
- Al-niemi TS, Kahn ML, McDermott TR (1998) Phosphorus uptake by bean nodules. *Plant and Soil* 198: 71–78.
- Appels MA, Haaker H (1988) Identification of cytoplasmic nodule associated forms of malate dehydrogenase involved in the symbiosis between *Rhizobium leguminosarum* and *Pisum sativum*. *European Journal of Biochemistry* 171: 515–522.
- Araujo AP, Plassard C, Drevon JJ (2008) Phosphatase and phytase activities in nodules of common bean genotypes at different levels of phosphorus supply. *Plant and Soil* 312: 129–138.
- Austin AT, Vitousek PM (1998) Nutrient dynamics on a precipitation gradient in Hawai'i. *Oecologia* 113: 519–529.
- Beadle NCW (1954) Soil phosphate and the delimitation of plant communities in Eastern Australia. *Ecology* 35: 370–375.
- Beadle NCW (1962) Soil phosphate and the delimitation of plant communities in Eastern Australia II. *Ecology* 43: 281–288.
- Bentley J, Verboom GA, Bergh NG (2014) Erosive processes after tectonic uplift stimulate vicariant and adaptive speciation: evolution in an Afrotemperate-endemic paper daisy genus. *BMC Evolutionary Biology* 14: 27.
- Bishop JG, O'Hara NB, Titus JH, Apple JL, Gill RA, Wynn L (2010) N-P colimitation of primary production and response of arthropods to N and P in early primary succession on Mount St. Helens volcano. *PLoS ONE* 5: 1–9.
- Bloom AJ, Chapin III FS, Mooney HA (1985) Resource limitation in plants—an economic analogy. *Annual Review of Ecology and Systematics* 16: 363–392.
- Boatwright JS, Savolainen V, van Wyk B-E, Schutte-Vlok AL, Forest F, van der Bank M (2008) Systematic position of the anomalous genus *Cadia* and the phylogeny of the tribe Podalyrieae (Fabaceae). *Systematic Botany* 33: 133–147.
- Bobbink R (1991) Effects of nutrient enrichment in Dutch chalk grassland. *Journal of Applied Ecology* 28: 28–41.
- Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* 134: 189–207.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

- Bray RH, Kurtz LT (1945) Determination of total, organic and available forms of phosphorus in soils. *Soil Science* 59: 39–45.
- Britto DT, Kronzucker HJ (2005) Plant nitrogen transport and its regulation in changing soil environments. *Journal of Crop Improvement* 15: 1–23.
- Brown G, Mitchell DT (1986) Influence of fire on soil phosphorous status in sand plain lowland fynbos, south-western Cape. *South African Journal of Botany* 52: 67–72.
- Brundett MC (2002) Coevolution of roots and mycorrhizas of land plants. *New Phytologist* 154: 275–304.
- Carroll JA, Caporn SJM, Johnson D, Morecroft MD, Lee JA (2003) The interactions between plant growth, vegetation structure and soil processes in semi-natural acidic and calcareous grasslands receiving long-term inputs of simulated pollutant nitrogen deposition. *Environmental Pollution* 121: 363–376.
- Casarin V, Plassard C, Hinsinger P, Arvieu JC (2004) Quantification of ectomycorrhizal effects on the bioavailability and mobilization of soil P in the rhizosphere of *Pinus pinaster*. *New Phytologist* 163: 177–195.
- Cavender-Bares J, Ackerly DD, Baum DA, Bazzaz FA (2004) Phylogenetic overdispersion in Floridian oak communities. *American Naturalist* 163: 823–843.
- Cernusak LA, Winter K, Turner BL (2011) Transpiration modulates phosphorus acquisition in tree seedlings. *Tree Physiology* 31: 878–885.
- Chapin FS III. (1980) The mineral nutrition of wild plants. *Annual Review of Ecology and Systematics* 11: 233–260.
- Cocks MP, Stock WD (2001) Field patterns of nodulation in fifteen *Aspalathus* species and their ecological role in the fynbos vegetation of South Africa. *Basic Applied Ecology* 2: 115–125.
- Coker GT, Schubert KR (1981) Carbon dioxide fixation in soybean roots and nodules. *Plant Physiology* 67: 691–696.
- Cole C, Grunes D, Porter L, Olsen S (1963) The effects of nitrogen on short term phosphorus absorption and translocation in corn (*Zea mays*). *Soil Science Society of America Proceedings* 27: 671–674.
- Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant Journal* 39: 487–512.
- Comas LH, Bouma TJ, Eissenstat DM. (2002) Linking root traits to potential growth rate in six temperate tree species. *Oecologia* 132: 34–43.
- Comas LH, Eissenstat DM (2004) Linking fine root traits to maximum potential growth rate among 11 mature temperate tree species. *Functional Ecology* 18: 388–397.
- Copeland L, Quinnell RG, Day DA (1989) Malic enzyme activity in bacteroids from soybean nodules. *Journal of General Microbiology* 135: 2005–2011.

- Costa C, Dwyer LM, Hamilton RI, Hamel C, Nantais L, Smith DL (2000) A sampling method for measurement of large root systems with scanner-based image analysis. *Agronomy Journal* 92: 621–627.
- Cowling RM, Holmes PM (1992) Flora and Vegetation. In: Cowling RM, ed. *The Ecology of Fynbos - Nutrients, Fire and Diversity*. Oxford University Press, Cape Town, pp 23–61.
- Cowling RM, Procheş Ş, Partridge TC (2009) Explaining the uniqueness of the Cape Flora: incorporating geomorphic evolution as a factor for explaining its diversification. *Molecular Phylogenetics and Evolution* 51: 64–74.
- Craine JM (2009) *Resource strategies of wild plants*. Princeton University Press, Princeton, NJ, USA.
- Craine JM, Jackson RD (2010) Plant nitrogen and phosphorus limitation in 98 North American soils. *Plant and Soil* 334: 73–84.
- Cramer MD (2010) Phosphate as a limiting resource: Introduction. *Plant and Soil* 334: 1–10.
- Cramer MD, West AG, Power SC, Skelton R, Stock WD (2014) Plant Ecophysiological Diversity. In: Allsopp N, Colville JF, Verboom GA, eds. *Fynbos: Ecology, Evolution, and Conservation of a Megadiverse Region*. Oxford University Press, Oxford, pp 248–272.
- Cramer MD, Lewis OAM, Lips SH (1993) Inorganic carbon fixation and metabolism in maize roots as affected by nitrate and ammonium nutrition. *Physiologia Plantarum* 94: 425–432.
- Crews TE (1993) Phosphorus regulation of nitrogen fixation in a traditional Mexican agroecosystem. *Biogeochemistry* 21: 141–166.
- Davidson EA, Howarth RW (2007) Nutrients in synergy. *Nature* 449: 1000–1001.
- Dinkelaker B, Hengeler C, Marschner H (1995) Distribution and function of proteoid roots and other root clusters. *Botanica Acta* 108: 183–200.
- Dinkelaker B, Römheld V, Marschner H (1989) Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell & Environment* 12: 285–292.
- Drevon J-J, Hartwig UE (1997) Phosphorus deficiency increases the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta* 201: 463–469.
- Duff SMG, Moorhead GBG, Lefebvre DD, Plaxton WC (1989) Phosphate starvation inducible ‘bypasses’ of adenylate and phosphate dependant glycolytic enzymes in *Brassica nigra* suspension cells. *Plant Physiology* 90: 1275–1278.
- Duff SMG, Sarath G, Plaxton WC (1994) The role of acid phosphatases in plant phosphorus metabolism. *Physiologia Plantarum* 90: 791–800.
- Eissenstat DM (1991) On the relationship between specific root length and the rate of root proliferation: a field study using citrus rootstocks. *New Phytologist* 118: 63–68.
- Eissenstat DM, Wells CE, Yanai RD, Whitbeck JL (2000) Building roots in a changing environment: implications for root longevity. *New Phytologist* 147:33–42.

- Elser JJ, Bracken MES, Cleland EE, Gruner DS, Harpole WS, Hillebrand H, Ngai JT, Seabloom EW, Shurin JB, Smith JE (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters* 10: 1135–1142.
- Elser JJ, Fagan WF, Kerkhoff AJ, Swenson NG, Enquist BJ (2010) Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. *New Phytologist* 186: 593–608.
- Elser JJ, Sterner RW, Gorokhova E, Fagan WF, Markow TA, Cotner JB, Harrison JF, Hobbie SE, Odell GM, Weider J (2000). Biological stoichiometry from genes to ecosystems. *Ecology Letters* 3: 540–550.
- Fageria VD (2001) Nutrient interactions in crop plants. *Journal of Plant Nutrition* 24: 1269–1290.
- Fine PVA, Daly DC, Villa Muñoz G, Mesones I, Cameron KM (2005) The contribution of edaphic heterogeneity to the evolution and diversity of Burseraceae trees in the western Amazon. *Evolution* 59: 1464–1478.
- Fischinger SA, Schulze J (2010) The importance of nodule CO<sub>2</sub> fixation for the efficiency of symbiotic nitrogen fixation in pea at vegetative growth and during pod formation. *Journal of Experimental Botany* 61: 2281–2291.
- Foehse D, Jungk A (1983) Influence of phosphate and nitrate supply on root hair formation of rape, spinach and tomato plants. *Plant and Soil* 74: 359–368.
- Freeden AL, Rao IM, Terry N (1989) Influence of phosphorous nutrition on growth and carbon partitioning in *Glycine max*. *Plant Physiology* 89: 225–230.
- Fujita Y, de Ruiter PC, Wassen MJ, Heil GW (2010) Time dependent, species-specific effects of N:P stoichiometry on grassland plant growth. *Plant and Soil* 334: 99–112.
- Gadd GM (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. *Advances in Microbiology Physiology* 41: 47–92.
- Gahoonia TS, Nielsen NE (2004) Root traits as tools for creating phosphorus efficient crop varieties. *Plant and Soil* 260: 47–57.
- Garrish V, Cernusak LA, Winter K, Turner BJ (2010) Nitrogen to phosphorus ratio of plant biomass versus soil solution in a tropical pioneer tree, *Ficus insipida*. *Journal of Experimental Botany* 61: 3735–3748.
- Garten CT (1976) Correlations between concentrations of elements in plants. *Nature* 261:686–688.
- Gentili F, Huss-Danell K (2003) Local and systemic effects of phosphorus and nitrogen on nodule function in *Alnus incana*. *Journal of Experimental Botany* 54: 2757–2767.
- Gilbert GA, Knight JD, Vance CP, Allan DL (1999) Acid phosphatase activity in phosphorus-deficient white lupin roots. *Plant Cell & Environment* 22: 801–810.
- Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Herbert JK, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles S, Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. *Journal of Experimental Botany* 53: 855–864.
- Gniazdowska A, Krawczak A, Mikulska M, Rychter AM (1999) Low phosphate nutrition alters bean plants' ability to assimilate and translocate nitrate. *Journal of Plant Nutrition* 22: 551–563.

- Gordon C, Wynn JM, Woodin SJ (2001) Impacts of increased nitrogen supply on high Arctic heath; the importance of bryophytes and phosphorus availability. *New Phytologist* 149: 461–471.
- Grime JP (1977) Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *American Naturalist* 111: 1169–1194.
- Güsewell S (2004) N:P ratios in terrestrial plants: variation and functional significance. *New Phytologist* 164: 243–266.
- Hammond JP, Broadley MR, White PJ (2004) Genetic responses to phosphorus deficiency. *Annals of Botany* 94: 323–332.
- Harpole WS, Ngai JT, Cleland EE, Seabloom EW, Borer ET, Bracken MES, Elser JJ, Gruner DS, Hillebrand H, Shurin JB, Smith JE (2011) Nutrient co-limitation of primary producer communities. *Ecology Letters* 14: 852–862.
- Hawkins H-J, Hettasch H, Cramer MD (2007) Putting back what we take out, but by how much? Phosphorus and nitrogen additions to farmed *Leucodendron* ‘Safari Sunset’ and *Leucospermum* ‘Succession’ (Proteaceae). *Scientia Horticulturae* 111: 378–388.
- Hawkins H-J, Malgas R, Bienabe E (2011) Ecotypes of wild rooibos (*Aspalathus linearis* (Burm. F) Dahlg., Fabaceae) are ecologically distinct. *South African Journal of Botany* 77: 360–370.
- Hawkins H-J, Wolf G, Stock WD (2005) Cluster roots of *Leucadendron laureolum* (Proteaceae) and *Lupinus albus* (Fabaceae) take up glycine intact: an adaptive strategy to low mineral N in soils? *Annals of Botany* 97: 1275–1282.
- Hellsten A, Huss-Danell K (2000) Interaction effects of nitrogen and phosphorous on nodulation in red clover (*Trifolium pratense* L.). *Acta Agriculturae Scandinavia* 50: 13–142.
- Hernández G, Ramírez M, Valdés-López O, Tesfaye M, Graham MA, Czechowski T, Schlereth A, Wandrey M, Erban A, Cheung F, Wu HC, Lara M, Town CD, Kopka J, Udvardi MK, Vance CP (2007) Phosphorus stress in common bean: root transcript and metabolic responses. *Plant Physiology* 144: 752–767.
- Hernández G, Valdés-López O, Ramírez M, Goffard N, Weiller G, Aparicio-Fabre R, Fuentes SI, Erban A, Kopka J, Udvardi MK, Vance CP (2009) Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. *Plant Physiology* 151: 1221–1238.
- Hijmans, RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25: 1965–1978.
- Hills EJ, Sailsberry RL, Ulrich A, Sipitanos KM (1970) Effect of phosphorus on nitrate in sugar beet (*Beta vulgaris* L.). *Agronomy Journal* 62: 91–92.
- Hinsinger P (2001) Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant and Soil* 237: 173–195.
- Hocking PJ, Jeffery S (2004) Cluster-root production and organic anion exudation in a group of old-world lupins and a new-world lupin. *Plant and Soil* 258: 135–150.
- Hopper SD (2009) OCBIL theory: towards an integrated understanding of the evolution, ecology and conservation of biodiversity on old, climatically buffered, infertile landscapes. *Plant and Soil* 322: 49–86.

- Houlton BZ, Wang Y-P, Vitousek PM, Field CB (2008) A unifying framework for dinitrogen fixation in the terrestrial biosphere. *Nature* 454: 327–330.
- Huppe HC, Turpin DH (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. *Annual Review of Plant Physiology and Plant Molecular Biology* 45: 577–607.
- Israel DW (1987) Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* 84: 835–840.
- Imсандe J, Touraine B (1994) N demand and the regulation of nitrate uptake. *Plant Physiology* 105: 3–7.
- Jakobsen I (1985) The role of phosphorus in nitrogen fixation by young pea plants (*Pisum sativum*). *Physiologia Plantarum* 64: 190–196.
- Jeschke WD, Kirkby EA, Peuke AD, Pate JS, Hartung W (1997) Effects of P deficiency on assimilation and transport of nitrate and phosphate in intact plants of castor bean (*Ricinus communis* L.). *Journal of Experimental Botany* 48: 75–91.
- Jones DL, Darrah PR (1994) Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant and Soil* 166: 247–257.
- Jones HE, Dighton J (1993) The use of nutrient bioassays to assess the response of *Eucalyptus grandis* to fertilizer application. 2. A field experiment. *Canadian Journal of Forest Research* 23: 7–13.
- Johnson JF, Allan DL, Vance CP, Weiblen G (1996) Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*. Contribution to organic acid exudation by proteoid roots. *Plant Physiology* 112: 19–30.
- Johnson JF, Vance CP, Allan DL (1994) Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiology* 104: 657–66.
- Johnson D, Leake JR, Lee JA (1999) The effects of quantity and duration of simulated pollutant nitrogen deposition on root-surface phosphatase activities in calcareous and acid grasslands: a bioassay approach. *New Phytologist* 141: 433–442.
- Jungk A (2001) Root hairs and the acquisition of plant nutrients from soil. *Journal of Plant Nutrition and Soil Science* 164: 121–129.
- Kalra YP (1998) *Handbook of standard methods of plant analysis*. CRC Press, Boca Raton, FL, USA.
- Keerthisinghe G, Hocking PJ, Ryan PR, Delhaize E (1998) Effect of phosphorus supply on the formation and function of proteoid roots of white lupin (*Lupinus albus*). *Plant Cell & Environment* 21: 467–478.
- King BJ, Layzell DB, Canvin DT (1986) The role of dark carbon dioxide fixation in root nodules of soybean. *Plant Physiology* 81: 200–205.
- Laing WA, Christeller JT, Sutton WD (1979) Carbon dioxide fixation by lupin root nodules. II. Studies with <sup>14</sup>C-labeled glucose, the pathway of glucose catabolism and the effects of some treatments that inhibit nitrogen fixation. *Plant Physiology* 63: 450–454.
- Lajtha K, Klein M (1988) The effect of varying nitrogen and phosphorus availability on nutrient use by *Larrea tridentata*, a desert evergreen shrub. *Oecologia* 75: 348–353.

- Lajtha K, Harrison AF (1995) Strategies of phosphorus acquisition and conservation by plant species and communities. In: Tiessen H, ed. Phosphorus in the global environment. John Wiley & Sons, Chichester, pp 139–147.
- Lambers H, Brundrett MC, Raven JA, Hopper SD (2010) Plant mineral nutrition in ancient landscapes: high species diversity on fertile soils is linked to functional diversity for nutritional strategies. *Plant and Soil* 334: 11–31.
- Lambers H, Finnegan PM, Laliberte E, Pearse SJ, Ryan MH, Shane MW, Veneklaas EJ (2011) Phosphorus nutrition of Proteaceae in severely phosphorus-impooverished soils: are there lessons to be learned for future crops? *Plant Physiology* 156: 1058–1066.
- Lambers H, Raven JA, Shaver GR, Smith SE (2008) Plant nutrient-acquisition strategies change with soil age. *Trends in Ecology and Evolution* 23: 95–103.
- Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ (2006) Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. *Annals of Botany* 98: 693–713.
- Lamont B (1972) The effect of soil nutrients on the production of proteoid roots by *Hakea* species. *Australian Journal of Botany* 20: 27–40.
- Lamont B (1982) Mechanisms for enhancing nutrient uptake in plants, with particular reference to Mediterranean South Africa and Western Australia. *The Botanical Review* 48: 597–689.
- Lamont B, Pérez-Fernández M, Rodríguez-Sánchez J (2015). Soil bacteria hold the key to cluster root formation. *New Phytologist* 206: 1156–1162.
- Le Roux MR, Khan S, Valentine AJ (2008) Organic acid accumulation may inhibit N<sub>2</sub> fixation in phosphorus-stressed lupin nodules. *New Phytologist* 177: 956–964.
- Leidi EO, Rodriguez-Navarro DN (2000) Nitrogen and phosphorus availability limit N<sub>2</sub> fixation in bean. *New Phytologist* 147: 337–346.
- Li H, Shen J, Zhang F, Tang C, Lambers H (2008) Is there a critical level of shoot phosphorus concentration for cluster-root formation in *Lupinus albus*? *Functional Plant Biology* 35: 328–336.
- Linder PH (2005) Evolution of diversity: the Cape flora. *Trends in Plant Science* 10: 536–541.
- Losos JB (2008) Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity between species. *Ecology Letters* 11: 995–1007.
- Lukac M, Calfapietra C, Lagomarsino A, Loreto F (2010) Global climate change and tree nutrition: effects of elevated CO<sub>2</sub> and temperature. *Tree Physiology* 30: 1209–1220.
- Lynch JP, Brown KM (2001) Topsoil foraging – an architectural adaptation of plants to low phosphorus availability. *Plant and Soil* 237: 225–237.
- Lynch JP, Brown KM (2006) Whole plant adaptations to low phosphorous availability. In: Huang B, ed. *Plant-environment interactions*, 3rd edn. CRC Press, Boca Raton, FL, pp 209–242.

- Maistry PM (2010) Phosphorus requirement of indigenous N<sub>2</sub>-fixing legumes and rhizobial diversity in the low P soils of the Cape Floristic Region, South Africa. MSc Thesis, University of Cape Town, South Africa.
- Maistry PM, Cramer MD, Chimphango SBM (2013) N and P colimitation of N<sub>2</sub>-fixing and N-supplied fynbos legumes from the Cape Floristic Region. *Plant and Soil* 373: 217–228.
- Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2014) Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis*. *Functional Plant Biology* doi: org/10.1071/FP14100.
- Manders PT (1990) Fire and other variables as determinants of forest/ fynbos boundaries in the Cape Province. *Journal of Vegetation Science* 1: 483–490.
- Manning J, Goldblatt P (2012) Plants of the Greater Cape Floristic Region 1: the Core Cape Flora, *Strelitzia* 29. South African National Biodiversity Institute, Pretoria.
- Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, London, UK.
- Matzek V, Vitousek PM (2009) N:P stoichiometry and protein:RNA ratios in vascular plants: an evaluation of the growth rate hypothesis. *Ecology Letters* 12: 765–771.
- McKey D (1994) Legumes and nitrogen: the evolutionary ecology of nitrogen-demanding lifestyle. In: Sprent JI, McKey D, eds. *Advances in Legume Systematics: The Nitrogen Factor*. Royal Botanic Gardens, Kew, pp 211–228.
- Miller SS, Liu J, Allan DL, Menzhuber CJ, Fedorova M, Vance CP (2001) Molecular control of acid phosphatase secretion into the rhizosphere of proteoid roots from phosphorus-stressed white lupin. *Plant Physiology* 127: 594–606.
- Mitchell DT, Brown G, Jongens-Roberts SM (1984) Variations of forms of phosphorus in the sandy soils of coastal fynbos, south–western Cape. *Journal of Ecology* 72: 575–584.
- Morton JF (1983) Rooibos tea, *Aspalathus linearis*, a caffeineless, low-tannin beverage. *Economic Botany* 37: 164–173.
- Mucina L, Rutherford MC, eds. (2006) *The vegetation of South Africa, Lesotho and Swaziland*. South African National Biodiversity Institute, Pretoria.
- Muofhe ML, Dakora FD (1999) Nitrogen nutrition in nodulated field plants of the shrub tea legume *Aspalathus linearis* assessed using <sup>15</sup>N natural abundance. *Plant and Soil* 209: 181–186.
- Neumann G, Massonneau A, Martinoia E, Römheld V (1999) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* 208: 373–382.
- Neumann G, Römheld V (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil* 211: 121–130.
- Nielsen KL, Eshel A, Lynch JP (2001) The effect of phosphorus availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. *Journal of Experimental Botany* 52: 329–339.
- Niinemets Ü, Kull K (2005) Co-limitation of plant primary productivity by nitrogen and phosphorus in a species-rich wooded meadow on calcareous soil. *Acta Oecologica* 28: 345–356.

- Ocaña A, del Pilar Cordovilla M, Ligeró F, Lluch C (1996) Phosphoenolpyruvate carboxylase in root nodules of *Vicia faba*: partial purification and properties. *Physiologia Plantarum* 97: 724–730.
- Olander LP, Vitousek PM (2000) Regulation of soil phosphate and chitinase activity by soil N and P availability. *Biogeochemistry* 49: 175–190.
- Olivera M, Tejera N, Iribarne C, Ocaña A, Lluch C (2004) Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris*): effect of phosphorus. *Physiologia Plantarum* 121: 498–505.
- Ostertag R (2010) Foliar nitrogen and phosphorus accumulation responses after fertilization: an example from nutrient-limited Hawaiian forests. *Plant and Soil* 334: 85–98.
- Ozanne PG, Specht RL (1981) Mineral nutrition of Heathlands: Phosphorus toxicity. In: Specht RL, ed. *Ecosystems of the world. Heathlands and related shrublands*. Elsevier Scientific, Amsterdam, pp 277–289.
- Pang J, Ryan MH, Tibbett M, Cawthray GR, Siddique KHM, Bolland MDA, Denton MD, Lambers H (2010) Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus supply. *Plant and Soil* 331: 241–255.
- Parsons R, Stanforth A, Raven JA, Sprent JI (1993) Nodule growth and activity may be regulated by a feedback mechanism involving phloem nitrogen. *Plant Cell & Environment* 16: 125–136.
- Pate JS, Layzell DB, Atkins CA (1979) Economy of carbon and nitrogen in a nodulated and nonnodulated (NO<sub>3</sub>-grown) legume. *Plant Physiology* 64: 1083–1088.
- Paungfoo-Lonhienne C, Schenk PM, Lonhienne TGA, Brackin R, Meier S, Rentsch D, Schmidt S (2009) Nitrogen affects cluster root formation and expression of putative peptide transporters. *Journal of Experimental Botany* 60: 2665–2676.
- Pearse SJ, Veneklaas EJ, Cawthray GR, Bolland MDA, Lambers H (2006a) Carboxylate release of wheat, canola and 11 grain legume species as affected by phosphorus status. *Plant and Soil* 288: 127–139.
- Pearse SJ, Veneklaas EJ, Cawthray GR, Bolland MDA, Lambers H (2006b) *Triticum aestivum* shows greater biomass response to a supply of aluminium phosphate than *Lupinus albus*, despite releasing fewer carboxylates into the rhizosphere. *New Phytologist* 169: 515–524.
- Pearse SJ, Veneklaas EJ, Cawthray GR, Bolland MDA, Lambers H (2007) Carboxylate composition of root exudates does not relate consistently to a crop species' ability to use phosphorus from aluminium, iron or calcium phosphate source. *New Phytologist* 173: 181–190.
- Pearson RG, Dawson TP (2003). Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Global Ecology & Biogeography* 12: 361–371.
- Pereira PAA, Bliss FA (1987) Nitrogen fixation and plant growth of common bean (*Phaseolus vulgaris* L.) at different levels of phosphorus availability. *Plant and Soil* 104: 79–84.
- Phoenix GK, Booth RE, Leake JR, Read DJ, Grime JP, Lee JA (2003) Simulated pollutant nitrogen deposition increases P demand and enhances root-surface phosphatase activities of three plant functional types in a calcareous grassland. *New Phytologist* 161: 279–289.
- Pieters AJ, Paul MJ, Lawlor DW (2005) Low sink demand limits photosynthesis under P<sub>i</sub> deficiency. *Journal of Experimental Botany* 52: 1083–1091.

- Poorter H, Niklas KJ, Reich PB, Oleksyn J, Poot P, Mommer L (2012) Biomass allocation to leaves, stems and roots: meta analyses of interspecific variation and environmental control. *New Phytologist* 193: 30–50.
- Power SC, Cramer MD, Verboom GA, Chimphango SBM (2010) Does phosphate acquisition constrain legume persistence in fynbos of the Cape Floristic Region? *Plant and Soil* 334: 33–46.
- Power SC, Cramer MD, Verboom GA, Chimphango SBM (2011) Legume seeders of the Cape Floristic Region inhabit more fertile soils than congeneric resprouters—sometimes. *Plant Ecology* 212: 1979–1989.
- Prinzing A, Durka W, Klotz S, Brandl R (2001) The niche of higher plants: evidence for phylogenetic conservatism. *Proceedings of the Royal Society of London* 268: 2383–2389.
- Radin JW, Eidenbock MP (1984) Hydraulic conductance as a factor limiting leaf expansion of phosphorus-deficient cotton plants. *Plant Physiology* 75: 372–377.
- Raghothama KG, Karthikeyan AS (2005) Phosphate acquisition. *Plant and Soil* 274: 37–49.
- Richards MB, Stock WD, Cowling RM (1997) Soil nutrient dynamics and community boundaries in the fynbos vegetation of South Africa. *Plant Ecology* 130: 143–153.
- Roumet C, Urcelay C, Diaz S (2006) Suites of root traits differ between annual and perennial species growing in the field. *New Phytologist* 170: 357–368.
- Rath M, Salas J, Parhy B, Norton R, Menakuru H, Sommerhalter M, Hatlstad G, Kwon J, Allan DL, Vance CP, Uhde-Stone C (2010) Identification of genes induced in proteoid roots of white lupin under nitrogen and phosphorus deprivation, with functional characterization of a formidase. *Plant and Soil* 334: 137–150.
- Reddell P, Yun Y, Shipton WA (1997) Cluster roots and mycorrhizae in *Casuarina cunninghamiana*: their occurrence and formation in relation to phosphorus supply. *Australian Journal of Botany* 45: 41–51.
- Robson AD (1983) Mineral Nutrition. In: WJ Broughton, ed. *Nitrogen fixation*. Clarendon Press, Oxford, pp 36–55.
- Roelofs RFR, Rengel Z, Cawthray GR, Dixon KW, Lambers H (2001) Exudation of carboxylates in Australian Proteaceae: chemical composition. *Plant Cell & Environment* 24: 891–903.
- Rosendahl L, Vance CP, Pedersen WB (1990) Products of dark CO<sub>2</sub> fixation in pea root nodules support bacteroid metabolism. *Plant Physiology* 93: 12–19.
- Rufty TW, MacKown CT, Israel DW (1990) Phosphorus effects on assimilation of nitrate. *Plant Physiology* 94: 328–333.
- Ryan MH, Ehrenberg S, Bennett RG, Tibbett M (2009) Putting the P in *Ptilotus*: a phosphorus accumulating herb native to Australia. *Annals of Botany* 103: 901–911.
- Ryan P, Delhaize E, Jones D (2001) Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* 52: 527–560.
- Ryser P (1996) The importance of tissue density for growth and life span of leaves and roots: a comparison of five ecologically contrasting grasses. *Functional Ecology* 10: 717–723.

- Sa T, Israel DW (1991) Energy status and functioning of phosphorus-deficient soybean nodules. *Plant Physiology* 97: 928–935.
- Sas L, Rengel Z, Tang C (2002) The effect of nitrogen nutrition on cluster root formation and proton extrusion by *Lupinus albus*. *Annals of Botany* 89: 435–442.
- Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. *Plant Physiology* 116: 447–453.
- Schnitzler J, Barraclough TG, Boatwright JS, Goldblatt P, Manning JC, Powell MP, Rebelo T, Savolainen V (2011) Causes of plant diversification in the Cape Biodiversity Hotspot of South Africa. *Systematic Biology* doi: 10.1093/sysbio/syr006.
- Schulze J, Temple G, Temple SJ, Beschow H, Vance CP (2006) Nitrogen fixation by white lupin under phosphorus deficiency. *Annals of Botany* 98: 731–740.
- Schutte-Vlok AL, van Wyk B-E (2011) A taxonomic revision of *Podalyria* (Fabaceae). *Systematic Botany* 36: 631–660.
- Shane MW, Cramer MD, Funayama-Noguchi S, Cawthray G, Millar HA, Day DA, Lambers H (2004) Developmental physiology of cluster root carboxylate synthesis and exudation in Harsh Hakea. Expression of phosphoenolpyruvate carboxylase and the alternative oxidase. *Plant Physiology* 135: 549–560.
- Shane MW, Cramer MD, Lambers H (2008) Root of edaphically controlled Proteaceae turnover on the Agulhas Plain, South Africa: phosphate uptake regulation and growth. *Plant Cell & Environment* 31: 1825–1833.
- Shane MW, Lambers H (2005) Cluster roots: a curiosity in context. *Plant and Soil* 274: 101–125.
- Shaver GR, Melillo JM (1984) Nutrient budgets of marsh plants: efficiency concepts and relation to availability. *Ecology* 65: 1491–1510.
- Siddiqi MY, Glass ADM, Ruth TJ, Rufty TW (1990) Studies on the uptake of nitrate in barley. 1. Kinetics and  $^{13}\text{NO}_3^-$  influx. *Plant Physiology* 93: 1426–1432.
- Sinclair TR, Park WI (1993) Inadequacy of the Liebig limiting factor paradigm for explaining varying crop yields. *Agronomy Journal* 85: 742–746.
- Smith AM (1985) Capacity for fermentation in roots and *Rhizobium* nodules of *Pisum sativum* L. *Planta* 166: 264–270.
- Smith CR, Knowles VL, Plaxton WC (2000) Purification and characterization of cytosolic pyruvate kinase from *Brassica napus* (rapeseed) suspension cell cultures. Implications for the integration of glycolysis with nitrogen assimilation. *European Journal of Biochemistry* 267: 4477–4485.
- Smith FW, Jackson WA (1987) Nitrogen enhancement of phosphate transport in roots of *Zea mays* L. 1. Effects of ammonium and nitrate pretreatment. *Plant Physiology* 84: 1314–1318.
- Smith SE, Smith FAS, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology* 133: 16–20.
- Smith VH (1992) Effects of nitrogen: phosphorus supply ratios on nitrogen fixation in agricultural and pastoral ecosystems. *Biogeochemistry* 18: 19–35.

- Smith WH (1976) Character and significance of forest tree exudates. *Ecology* 57: 324–331.
- Sommers LE, Nelson DW (1972) Determination of total phosphorus in soils: a rapid perchloric acid digestion procedure. *Proceedings of Soil Science Society America* 36: 902–904.
- Sprent JI (1994) Nitrogen acquisition systems in the Leguminosae. In: Sprent JI, McKey D, eds. *Advances in Legume Systematics 5: The Nitrogen Factor*. Royal Botanical Gardens, Kew, pp 1–16.
- Sprent JI, Ardley JK, James EK (2013) From north to south: a latitudinal look at legume nodulation processes. *South African Journal of Botany* 89: 31–41.
- Spriggs AC (2004) Symbiotic N<sub>2</sub>-fixation in cultivated *Cyclopia* Vent. Spp. (honeybush): Towards sustainable cultivation in the Western Cape of South Africa. PhD Thesis, University of Cape Town, South Africa.
- Stock WD, Lewis OAM (1986) Soil nitrogen and the role of fire as a mineralizing agent in a South African coastal fynbos ecosystem. *Journal of Ecology* 74: 317–328.
- Stock WD, Verboom GA (2012) Phylogenetic ecology of foliar N and P concentrations and N:P ratios across Mediterranean-type ecosystems. *Global Ecology and Biogeography* 21: 1147–1156.
- Stock WD, Wienand KT, Baker AC. 1995. Impacts of invading N<sub>2</sub>-fixing *Acacia* species on patterns of nutrient cycling in two Cape ecosystems: evidence from soil incubation studies and <sup>15</sup>N natural abundance values. *Oecologia* 101: 375–382.
- Stöcklin J, Schweizer K, Körner C (1998) Effects of elevated CO<sub>2</sub> and phosphorus addition on productivity and community composition of intact monoliths from calcareous grassland. *Oecologia* 116: 50–56.
- Streeter J (1988) Inhibition of legume nodule formation and N<sub>2</sub> fixation by nitrate. *Critical Reviews in Plant Sciences* 7: 1–23.
- Suliman S, Van Ha C, Schulze J, Phan Tran L-S (2013) Growth and nodulation of symbiotic *Medicago truncatula* at different levels of phosphorus availability. *Journal of Experimental Botany* 64: 2701–2712.
- Tabatabai MA, Bremner JM (1969) Use of p-Nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* 1: 301–307.
- Tadano T, Sakai H (1991) Secretion of acid phosphatase by the roots of several crop species under phosphorus-deficient conditions. *Soil Science and Plant Nutrition* 37: 129–140.
- Tang C, Drevon JJ, Jaillard B, Souche G, Hinsinger P (2004) Proton release of two genotypes of bean (*Phaseolus vulgaris* L.) as affected by N nutrition and P deficiency. *Plant and Soil* 260: 59–68.
- Tarafdar JC, Claassen N (1988) Organic phosphorus compounds as a phosphorus source for higher plants through the activity of phosphatases produced by plant roots and microorganisms. *Biology and Fertility of Soils* 5: 308–312.
- Thien SJ, McFee WW (1972) Effect of nitrogen on phosphorus transport systems in *Zea mays* L. *Soil Science Society of America Proceedings* 36: 617–620.
- Theodorou ME, Plaxton WC (1993) Metabolic adaptations of plant respiration to nutritional phosphate deprivation. *Plant Physiology* 101: 339–344.

- Treeder KK, Vitousek PM (2001) Effects of soil nutrient availability on investment in acquisition of N and P in Hawaiian rain forests. *Ecology* 82: 946–954.
- Turner FT, Gilliam JW (1976) Increased P diffusion as an explanation of increased P availability in flooded rice soils. *Plant and Soil* 45: 365–377.
- Uhde-Stone C, Gilbert G, Johnson JF, Litjens R, Zinn KE, Temple SJ, Vance CP, Allan DL (2003) Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. *Plant and Soil* 248: 99–116.
- Vance CP, Boylan KLM, Maxwell CA, Heichel GH, Hardman LL (1985) Transport and partitioning of CO<sub>2</sub> fixed by root nodules of ureide and amide producing legumes. *Plant Physiology* 78: 774–778.
- Vance CP, Heichel GH (1991) Carbon in N<sub>2</sub> fixation: limitation or exquisite adaptation. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 373–392.
- Vance CP, Stade S, Maxwell CA (1983) Alfalfa root nodule carbon dioxide fixation. *Plant Physiology* 72: 469–473.
- Vance CP, Udhe-Stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a non-renewable resource. *New Phytologist* 157: 423–447.
- Vincent JM (1970) A manual for the study of root-nodule Bacteria. International Biological Program. Handbook no 15. Blackwell Scientific Publications, Oxford.
- Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm N, Howarth RW, Marino R, Martinelli L, Rasetter EB, Sprent JI (2002) Towards and ecological understanding of biological nitrogen fixation. *Biogeochemistry* 57(58): 1–45.
- Vitousek PM, Porder S, Houlton BZ, Chadwick A (2010) Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecology Applications* 20: 5–15.
- Wall LG, Hellsten A, Huss-Danell K (2000) Nitrogen, phosphorus, and the ratio between them affect nodulation in *Alnus incana* and *Trifolium pratense*. *Symbiosis* 29: 91–105.
- Wang B, Shen J, Tang C, Rengel Z (2008) Root morphology, proton release, and carboxylate exudation in lupin in response to phosphorus deficiency. *Journal of Plant Nutrition* 31: 557–570.
- Wang YH, Garvin DF, Kochian LV (2001) Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology* 127: 345–359.
- Wasaki J, Yamamura T, Shinano T, Osaki M (2003) Secreted acid phosphatase is expressed in cluster roots of lupin in response to phosphorus deficiency. *Plant and Soil* 248: 129–136.
- Watt M, Evans JR (1999) Linking development and determinacy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO<sub>2</sub> concentration. *Plant Physiology* 120: 705–716.
- Webb CO (2000) Exploring the phylogenetic structure of ecological communities: an example for rain forest trees. *American Naturalist* 156: 145–155.

- Wiens JJ (2004) Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. *Evolution* 58: 193–197.
- Wiens JJ, Graham CH (2005) Niche conservatism: integrating evolution, ecology and conservation biology. *Annual Review of Ecology Evolution and Systematics* 36: 519–539.
- Willems JH, Peet RK, Bik L (1993) Changes in chalk-grassland structure and species richness resulting from selective nutrient additions. *Journal of Vegetation Science* 4: 203–212.
- White PJ, Hammond JP (2008) Phosphorous nutrition of terrestrial plants. In: White PJ, Hammond JP, eds. *The ecophysiology of plant-phosphorus interactions*. Springer, Dordrecht, Netherlands, pp 51–81.
- Witkowski ETF, Mitchell DT (1987) Variations in soil phosphorus in the fynbos biome, South Africa. *Journal of Ecology* 75: 1159–1171.
- Yang MZ, Sivaguru M, Horst WJ, Matsumoto H (2000) Aluminium tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). *Physiologia Plantarum* 110: 72–77.

## **APPENDIX A**

---



Biological  
Sciences

Department of Biological Sciences  
University of Cape Town

Private Bag X3, Rhodes Gift, 7701 South Africa  
Tel: +27 (0) 21 650 3604 Fax: +27 (0) 21 650 3301  
<http://www.biologicalsciences.uct.ac.za/>



---

APPENDIX A1

5<sup>th</sup> December 2014

To Whom It May Concern

I hereby testify that Pravin Mark Maistry, the first author of the following published papers:

- a) Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2014) Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis*. *Functional Plant Biology* doi.org/10.1071/FP14100, and
- b) Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2015) Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume *Podalyria calyptrata*. *Journal of Plant Physiology* 174: 16–25,

was the lead researcher in the work done towards completing the papers.

Mr Maistry conceptualized and designed the research, and independently conducted the experiments, data collection, and statistical analysis. He independently wrote the manuscripts supported by comments and suggestions from the coauthors and dealt with referees comments in collaboration with the coauthors.

My contribution to the project was through providing specialist expertise, facilities, supervision and comments and suggestions on the manuscript.

Yours sincerely,

Dr Samson Chimphango



Biological  
Sciences

Department of Biological Sciences  
University of Cape Town

Private Bag X3, Rhodes Gift, 7701 South Africa  
Tel: +27 (0) 21 650 3604 Fax: +27 (0) 21 650 3301  
<http://www.biologicalsciences.uct.ac.za/>



---

APPENDIX A2

5<sup>th</sup> December 2014

To Whom It May Concern

I hereby testify that Pravin Mark Maistry, the first author of the following published papers:

- a) Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2014) Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis*. *Functional Plant Biology* doi.org/10.1071/FP14100, and
- b) Maistry PM, Muasya AM, Valentine AJ, Chimphango SBM (2015) Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume *Podalyria calyptrata*. *Journal of Plant Physiology* 174: 16–25,

was the lead researcher in the work done towards completing the papers.

Mr Maistry conceptualized and designed the research, and independently conducted the experiments, data collection, and statistical analysis. He independently wrote the manuscripts supported by comments and suggestions from the coauthors and dealt with referees comments in collaboration with the coauthors.

My contribution to the project was through providing specialist expertise, facilities, supervision and comments and suggestions on the manuscript.

Yours sincerely,

A/ Prof Muthama Muasya



UNIVERSITEIT STELLENBOSCH  
UNIVERSITY OF STELLENBOSCH

APPENDIX A3

5th December 2014

To Whom It May Concern

I hereby testify that Pravin Mark Maistry, the first author of the following published papers:

- a) Maistry PM, Muasya AM, Valentine AJ, Chiphango SBM (2014) Increasing nitrogen supply stimulates phosphorus acquisition mechanisms in the fynbos species *Aspalathus linearis*. *Functional Plant Biology* doi.org/10.1071/FP14100, and
  - b) Maistry PM, Muasya AM, Valentine AJ, Chiphango SBM (2015) Balanced allocation of organic acids and biomass for phosphorus and nitrogen demand in the fynbos legume *Podalyria calyptata*. *Journal of Plant Physiology* 174: 16–25,
- was the lead researcher in the work done towards completing the papers.

Mr Maistry conceptualized and designed the research, and independently conducted the experiments, data collection, and statistical analysis. He independently wrote the manuscripts supported by comments and suggestions from the coauthors and dealt with referees comments in collaboration with the coauthors.

My contribution to the project was through providing specialist expertise, facilities, supervision and comments and suggestions on the manuscript.

Yours sincerely,

Prof Alexander J. Valentine  
Legume Physiology Group,  
University of Stellenbosch