Phosphorus uptake and utilization efficiency in cluster root and non-cluster root forming species of the Core Cape Subregion, South Africa

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Podalyria calyptrata, seedlings grown in sand in the glasshouse (Photo: D Basic)

Science: the creation of dilemmas by the solution of mysteries

Brian Herbert and Kevin J. Anderson

Declaration

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own. The thesis is submitted for the degree of Master of Science in the Department of Biological Sciences, University of Cape Town. It has not been submitted for any degree or examination at any other university.

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Dunja Basic

Abstract

The Core Cape Subregion (CCR) is made up of a mosaic of highly weathered and nutrient leached soil substrates in the Western Cape. Plant available phosphorus (P) in these soils is very low, generally ranging from 0.4-3.7 μg P g^{-1} soil and as a result plants have evolved a number of traits to enhance P-acquisition, such as increased root surface area (SA) and specific root length (SRL), cluster root and root hair proliferation and exudation of organic acids and acid phosphatases (APase) from the roots. Crop yield is limited worldwide due to the unavailability of P and P-fertilization is showing limited success due to soil retention. Sustainable management of this would include exploiting plants with natural adaptations for enhanced P acquisition and utilization. The aim of this study was to discover whether cluster root forming species are more efficient at P acquisition than non-cluster root species. This was achieved by focusing on two objectives: (1) to characterize root traits for increased P acquisition in different soils of the CCR and (2) comparing P-uptake and utilization efficiencies of cluster root species to non-cluster root species under glasshouse and natural conditions. Plants from Fabaceae, Polygalaceae, Proteaceae, Cyperaceae, and Juncaceae were grown in two different glasshouse experiments and observed in a field study. In chapter one, a potted glasshouse experiment was conducted where selected species were grown in three different soil types for nine months before being analysed for root adaptations. In addition, a field study experiment was conducted where root samples were collected from two sites in the CCR with different soil types and analysed for organic acid exudation and phosphatase activity, and leaf samples were analyzed for N and P concentrations. The second chapter was another glasshouse experiment where plants were grown in a potted sand experiment under two P concentrations (10 and 25 $mg.kg^{-1}$) for seven months before they were analysed for root adaptations. While cluster roots are a major adaptation for plants growing in P deficient conditions, they did not seem to give plants that produced them, an added advantage in P uptake or utilization. *Polygala myrtifolia* and *Ficina trispicata* were the most efficient species with regards to P uptake associated with their root morphology, while *A. linearis* and *Pod. calyptrata* were the most efficient species in P utilization most likely due to the addition of extra nitrogen they received through N_2-fixation. It was shown that morphological traits such as total root length, total root SA, SRL, root diameter and root:shoot biomass ratios, along with carboxylate exudation are prime components in P acquisition in fynbos species. There is however, substantial genetic variation in all these traits and therefore selective breeding could be used to select for specific traits.

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Chapter 1

General Introduction

The Greater Cape Floristic Region (GCFR) is constituted of two main sub-regions, the Core Cape Subregion (CCR) and the Extra Cape Subregion (ECR). The ECR is situated outside the CCR and incorporates the southern Namib, the western Richtersveld, Namaqualand, the Western Mountain Karoo and the Tanqua-southern Great Karoo, all of which covers an area of 98,869 km^2 (Snijman 2013). The CCR, known previously as the Cape Floristic Region or the Cape Floristic Kingdom, is located at the south-western tip of Africa and constitutes the most floristically diverse component within the GCFR (Born *et al.* 2006). It is a biodiversity hotspot home to an estimated 9,383 vascular plant species in an area covering 90,760 km^2, less than 4% of the South African subcontinent, of which just over 68% are endemic to the area (Manning and Goldblatt 2012). The CCR has a Mediterranean type climate, with rainfall mainly in the winter months in the west and rainfall throughout the year in the east (Manning and Goldblatt 2012). The CCR is primarily a mosaic made up of shale and sandstone derived substrates which give rise to different types of soils (Manning and Goldblatt 2012). This is further enhanced by local areas of limestone and granite which contribute significantly to edaphic diversity (Manning and Goldblatt 2012). The CCR has four biomes, Fynbos, Succulent Karoo, Albany Thicket and Afrotemperate Forest, with several unique vegetation types interspersed (Manning and Goldblatt 2012). Fynbos heathland is the most common vegetation type, covering more than 50% of the CCR and occurring typically on sandstone soils (Manning and Goldblatt 2012), while Renosterveld, another distinctive shrubland, is mainly restricted to richer, fine grained soils and shares little species with Fynbos even though they grow adjacently (Manning and Goldblatt 2012). The largest families in the CCR are Asteraceae (1,077 species) and Fabaceae (764 species), comprising almost 20% of total species together, followed by Iridaceae (758 species), Ericaceae (680 species), Aizoceae (624 species), Restionaceae (342) and Proteaceae (333 species). Due to the mosaic nature of the soils, phosphorus availability varies with each soil type (Kruger 1979; Stock and Lewis 1986; Witkowski and Mitchell 1987). A number of proteoid (Proteaceae), restioid (Restionaceae), ericoid (Ericaceae), sedge (Cyperaceae), and legume (Fabaceae) species have been identified to possess specialized root adaptations for enhanced P acquisition such as mycorrhizae and

root clusters which could be important for their long term persistence in the low P soils of the CCR (Power *et al.* 2010). Since the plants in the CCR have adapted to low P conditions through natural evolutionary processes, they provide an opportunity to explore wild indigenous species for potentially superior P acquisition root mechanisms (Power *et al.* 2010). Studying these root trait variations and their physiological functions for enhanced acquisition of P in the field is fundamental to identifying CCR genotypes with suitable traits as a possible germplasm source for the selection and breeding of these traits for increasing P acquisition to improve economically important species production in areas with low P (Lambers and Shane 2007).

1.1 Soils of the CCR

The CCR is mainly covered by soils derived from rocks of the pre-Carboniferous age (more than 400 mya; Manning and Goldblatt 2012). Most of these rocks are part of the Cape System which is comprised of an alternating series of quartzitic sandstones and fine grained shales. The Cape System experiences disparities in the weathering of its components and therefore has yielded two fundamentally different soil types; nutrient poor sandy soils and relatively nutrient rich clay soils. These soils differ greatly in their structure and water retention capabilities and as a result in their erosional patterns (Manning and Goldblatt 2012).

1.2 Phosphorus Availability

Phosphorus is one of the most essential elements for plant growth and development (Vance *et al.* 2003) due to its role in genetic, metabolic, structural and regulatory molecules (Raghothama and Karthikeyan 2005; White and Hammond 2008). However, soil P availability to the plant may be as low as 0.1-10 μM P (Hinsinger 2001) due to it adsorbing to calcium (Ca^{2+}), aluminium (Al^{3+}) or iron (Fe^{3+}) cations or its occurrence in organic form (Gahoonia and Nielson 2004). Thus P is regarded as one of the most unavailable and inaccessible macronutrients in the soil (Vance *et al.* 2003) and is often limiting to plant growth. Phosphorus deficiencies are more critical in highly weathered soils like in the tropics and subtropics as well as in soils of the Mediterranean basin (Hinsinger 2001). The use of phosphorus fertilizing in attempt to improve this situation is becoming increasingly uneconomical as well as ecologically flawed due to the efficiency of the added P fertilizer being as low as 10% (Russell 1973; Werft and Dekkers 1996) and due to the run-off from these P-loaded agricultural soils leading to environmental pollution (Runge-Metzger 1995; Bumb and Baanante 1996; Turner *et al.* 2002). This, included with the fact that phosphate

rock reserves are being depleted at a rate where in 30 to 200 years there will be none left, (Koppelaar and Weikard 2013; Ulrich *et al.* 2013) means that conserving rock phosphate reserves is vital. A viable alternative strategy, compared to addition of P to the soils via fertilizers, would be to enhance the total surface area and distribution of plant roots in the soil, as well as to enhance the physiological functions of plants to take up P (Lambers and Shane 2007).

The South African Cape Region has been shown to have the lowest total P (87 $mg.kg^{-1}$) out of five Mediterranean climate regions (Stock and Verboom 2012). The fynbos biome occurs predominantly on sandstone derived soils which are ancient, course-grained, highly leached and extremely poor in exchangeable bases, total N and available P (Kruger 1979; Witkowski and Mitchell 1987; Rebelo *et al*. 2006). The availability of P is particularly low in these soils due to their low pH, P adsorption to cations and its occurrence in organic form (Witkowski and Mitchell 1987; Gahoonia and Nielsen 2004). Most soils contain between 20-80% organic P (Raghothama 1999), however, Fynbos soils have been found to contain about 58-77% of organic P (Straker 1996) most of which is not available to plants due to immobilisation via microbial action (Vance *et al.* 2003). Inorganic P is bound to cations in the soils, with about 70% bound to iron, 5% to calcium and 5% is bound to aluminium (Mitchell *et al.* 1984). As a result, plant available P usually ranges from 0.4-3.7 µg P g^{-1} soil in fynbos soils (Witkowski and Mitchell 1987; Cramer 2010). In contrast to this, limestone, granite and shale derived soils are fine-grained and have higher nutrient status with higher concentrations of N and P (Kruger 1979; Witkowski and Mitchell 1987; Rebelo *et al.* 2006).

1.3 Adaptations to a low P lifestyle

Below (Table 1.1) are plant adaptations known to either enhance nutrient uptake; increased root surface area, enhanced expression of nitrate, phosphate or ammonium transporters, enhanced expression of phosphatases, increased organic acid synthesis and exudation and mycorrhizal symbioses; or conserve the nutrients by internal remobilization, decreased growth rate, higher phosphorus use efficiency and modified carbon and nitrogen metabolism (Vance 2001).

Table 1.1: Plant adaptations to low P reproduced from Vance (2001).

Strategy	Adaptation
Enhanced acquisition or uptake	• Expanded root surface area (more roots and root hairs, cluster roots, longer roots) • Enhanced expression of NO_3^-, PO_4^-, NH_4^+ transporters, phosphatases • Increased organic acid synthesis and exudation • Mycorrhizal symbioses
Conservation of use	• Internal remobilization • Decreased growth rate • More growth per unit P (phosphorus use efficiency) • Modified carbon and nitrogen metabolism

1.4 Mechanisms for phosphorus acquisition

1.4.1 Cluster Roots

The formation of proteoid roots or 'cluster' roots is considered to be one of the major adaptations to nutrient acquisition for plants adapted to severely impoverished soils (Skene 1998; Vance *et al.* 2003, Lambers *et al.* 2006). They were first noticed by Engler (1894) and later defined by Purnell (1960) in *Protea* species and hence termed 'proteoid' roots. The morphology of cluster roots differs between species but they can be separated into simple: single cluster of rootlets arising from the parent root, complex: a second cluster root emerging from within the first one; or compound: a cluster of simple cluster roots (Purnell 1960; Skene 1996). Cluster roots are concentrated in the first 100 mm of soil and their numbers as well as weight drop exponentially with increasing depth (Lamont 1983). They are also more likely to occur on older plants as seedlings are still dependent on the nutrient stores from the seed (Lamont 1983). They are known for having short life spans and forming on lateral roots as closely packed tertiary roots with dense coverings of root hairs (Richardson *et al.* 2009). In families where root clusters are prevalent, such as in Proteaceae, mycorrhizal symbioses are uncommon, however, exceptions have been found (e.g. *Hakea verrucosa*, Boulet and Lambers 2005). In contrast to this, many species of Fabaceae have mycorrhizal symbionts (Allsopp and Stock 1993) and most lack cluster roots (Cramer 2010; Power *et al.*

2010) but there have been reports of cluster roots and mycorrhizal symbioses in genera such as *Cyclopia* (Spriggs and Dakora 2009) and *Aspalathus* (Allsopp and Stock 1992, 1993; Sprent *et al.* 2010). The ability of these species to employ both cluster roots and mycorrhizal symbioses may seem beneficial in terms of maximising P-acquisition, however, it may be compromised by the high metabolic costs of cluster roots (Shane and Lambers 2005) and mycorrhizae (Lambers *et al*. 2002).

Certain Cyperaceae species have been observed to form 'swollen lateral roots' (Davies *et al*. 1973) that were later termed 'dauciform' roots by Lamont (1974) due to their carrot-like shape. It was found that factors controlling their formation are similar to those of proteoid roots but their origin and structure are different (Lamont 1974). Dauciform root development is restricted to species from certain genera belonging to the Cyperaceae (Shane *et al*. 2005) in low P soils, consistent with field observations (Davies *et al*. 1973; Lamont 1974) while there has been one report where dauciform root development occurred in two species of Juncaceae (Powell 1973). There has long been speculation that dauciform roots are similar in function to cluster roots (Davies *et al.* 1973; Lamont 1974, 1982, 1993; Muthukumar *et al.* 2004) and only recently have studies found this to be true, in that they also release carboxylates in large quantities during an exudative burst (Playsted *et al*. 2006; Shane *et al.* 2006) and their development is also suppressed when there are higher levels of P in the environment (Lambers *et al.* 2006).

Along with increasing the surface area for nutrient absorption, cluster roots have been found to exude organic acids, acid phosphatases, phenolics, mucilages and water which aid in mobilising nutrients from the soil (Lamont 1974; Watt and Evans 1999). One of the most interesting aspects of cluster roots is their ability to produce vast amounts of organic acids under P-deficiency and acidify the rhizosphere as was observed in a study by Dinkelaker *et al.* (1989) where cluster roots of white lupin acidified the rhizosphere from pH 7.5 to 4.8 in a calcareous soil (20% $CaCO_3$). In some cases, the pH of the rhizosphere can be decreased to as low as pH 3.6 by cluster roots (Li *et al.* 1997). It is well established that this acidification of the rhizosphere by cluster roots is due to the huge amount of organic acids being released from the roots, predominantly as citric and malic acids (Gardner *et al.* 1983; Dinkelaker *et al.* 1989; Li *et al*. 1997; Keerthisinghe *et al*. 1998; Neumann *et al.* 1999). The rate of export of organic acids from cluster roots is among the highest found in plants (Watt and Evans 1999). Highest exudation activity is found in mature cluster roots, while young and old cluster roots

release only a limited amount of organic acids (Keerthisinghe *et al.* 1998; Neumann *et al.* 1999; Watt and Evans 1999) and the fact that the rootlets are so closely situated leads to the acids accumulating and leading to high concentrations in the cluster root rhizosphere (Yan *et al.* 2002).

1.4.2 Root hairs

A large amount of plant available P is found within minute pores within the soil (Fawcett and Quick 1962). With the growth and proliferation of root hairs, which act as an extension of the root, plants are able to access these pores and obtain P readily (Gahoonia and Nielsen 1997; Lamont 2003). Root hairs are able to more effectively exploit the soil closest to the root surface by creating a uniform P-depletion zone, thus allowing for better P acquisition (Nye 1966; Misra *et al.* 1988). Root hairs are tube-like growths that form from the epidermal cells of roots and are vital in anchorage and increasing the surface area of roots for nutrient uptake (Gilroy and Jones 2000). They have been found to be the primary site of nutrient uptake in plants that lack mycorrhizal associations (Jungk 2001) and can form more than 70% of the root surface area in certain crop plants (Parker *et al.* 2000). In addition to genotypic variation (Hofer 1996), the formation and growth of root hairs is regulated primarily by the supply of P and nitrate (Hofer 1996; Gilroy and Jones 2000; Jungk 2001) and many plant species, including legume species, respond swiftly to P-deficiencies by increasing the length and abundance of their root hairs (Reid 1981; Jungk *et al.* 1990; Bates and Lynch 1996; Gahoonia and Nielsen 1997; Jungk 2001).

1.4.3 Acid Phosphatase

Phosphorus occurs in soils, not only as a mineral phosphate, but also as an organic compound form. Organic P can account for 30-80% of total P in agricultural soils (Tarafdar and Claassen 1988) however for these organic P sources to be usable by the plants, they must first be hydrolysed by phosphatases (Gilbert *et al.* 1999). There are two types of phosphatases that plants are associated with; alkaline phosphatase (ALPase) which functions optimally at a pH above 7 and is secreted by soil microorganisms including bacteria, fungi and earthworms in the soil (Herbien and Neal 1990). The other form, acid phosphatase (APase), functions optimally at a pH below 7 and originates from the plant itself (Tarafdar *et al.* 2001). Under P-deficient conditions, plants have been found to secrete more APase from their roots to enable hydrolysis of organic P (Dracup *et al.* 1984; Tadano and Sakai 1991; Li *et al.* 1997; Yun and Karppler 2001; White and Hammond 2008). A study by Tadano and Sakai (1991) found that

lupin secreted 20 times more APase from its roots when P was limiting compared to the P-sufficient controls. Similarly, it was found that there was a 1.5 times increase in APase activity of *Trifolium subterraneum* in P-deficient conditions (Dracup *et al.* 1984). In a separate study conducted by Adams and Pate (1992), it was determined that the cluster roots of white lupin, expressed a higher activity of APase than the non-cluster roots. The response of plants to secrete more APase in P-deficient conditions is considered evidence that APase is involved primarily in the hydrolysis of soil organic P for plant uptake (Hayes *et al.* 1999). Acid phosphatase differs from alkaline phosphatase by exhibiting a lower substrate specificity, therefore allowing it to act on a broad range of different physiologically relevant substrates, as well as having an optimal activity below pH 7.0 (Duff *et al.* 1994; Gilbert *et al.* 1999). However, Konietzny *et al.* (1995) have found that APase may show little or no activity against particular substrates such as phytate. It was also noted that acid phosphatase activity is seen to be regulated by developmental as well as environmental factors, including P-deficiency (Goldstein *et al*. 1988; Helal and Sauerbeck 1991; Tadano and Sakai 1991; Adams and Pate 1992; Tadano *et al*. 1993; Ascencio 1996, 1997).

1.4.4 Organic Acids

Under normal growth conditions plant roots exude a variety of organic compounds, one of which are organic acids (Tate 1984; Dinkelaker *et al.* 1989; Marschner 2012). Roots typically contain many organic acids with acetate, citrate, lactate, malate and succinate being a few of the primary anion components and their production and exudation rates are enhanced during P-deficiency (Tate 1984; Dinkelaker *et al.* 1989; Jones 1998; Ryan *et al.* 2001). Organic acids, when released, allow for chelation of Al^{3+}, Ca^{2+} and Fe^{3+} therefore replacing inorganic P on these bound forms and thus making it available to the plant (Jones 1998; Hinsinger 2001; Ryan *et al.* 2001). It has been found that the localised enhanced excretion of organic acids increased the effectiveness of exudates for nutrient mobilization, not only for P but also for micronutrients like zinc, iron and manganese (Marschener 1998).

1.5 Problem Statement

This study investigated morphological and physiological mechanisms, as well as phosphorus use efficiency, in cluster and non-cluster root species. Phosphorus use efficiency in CCR Proteaceae, Fabaceae and Cyperaceae, and their sister families, Polygalaceae and Juncaceae was also investigated. The formation of proteoid roots or cluster roots is considered to be one of the major adaptations to nutrient acquisition, along with mycorrhizal symbioses and

nitrogen fixing nodules (Skene 1998; Vance *et al.* 2003, Lambers *et al.* 2006). Cluster roots were considered to be a normal component of Proteaceae (Lamont 1982, 1983) but further studies have shown that they have been found in members of Betulaceae, Casuarinaceae, Cucurbitaceae, Cyperaceae, Eleagnaceae, Fabaceae, Moraceae, Myricaceae, Restionaceae, all of which are adapted to low fertility soils (Dinkelaker *et al.* 1995; Skene 1998, 2000; Adams and Pate 2002). Among the legume species with cluster roots, the most extensively studied and used in agriculture is *Lupinus albus* (white lupin). This may be due to its ability to use a series of mechanisms to increase its P acquisition from soils (Kihara *et al.* 2003). It has the ability to produce cluster roots, induce the high-affinity P transport system (Neumann *et al.* 1999, Liu *et al.* 2001) and excrete compounds to solubilize P from soils like organic acids (Johnson *et al.* 1996a, b) and acid phosphatases (Wasaki *et al.* 1997). A study by Braum and Helmke (1995) showed that *L. albus* can access scarcely soluble inorganic P in soils better than any other crop plant and is able to secrete vast quantities of APase from its roots (Tadano and Sakai 1991). There are a great number of studies conducted on individual plants on cluster roots and their uses (Skene 1998, 2000; Lamont 1974, 1983; Watt and Evans 1999; Richardson *et al.* 2009), however, there is a general lack of studies performed at the whole plant level where P acquisition efficiency of cluster root plants are compared to non-cluster root plants with other P acquisition mechanisms including root length, root hairs and organic acid exudation and enzyme activity from "normal" roots. There are also no studies conducted on rooting mechanisms of Polygalaceae from the literature.

1.6 Hypothesis and thesis outline

Often, species with different mechanisms for enhanced P acquisition co-occur in an area and therefore it is not clear whether the cluster root mechanism is superior in P acquisition relative to other mechanisms. Since the plants in the CCR have evolved adaptations to low P conditions, they provide an opportunity to explore fynbos species for potentially superior root P acquisition mechanisms. The focal question in this study is whether cluster root forming species are more efficient at P acquisition than non-cluster root species. This will be achieved by focusing on two objectives: (1) to characterise root traits for increased P acquisition in different soils of the CCR and (2) to compare P-uptake efficiency of cluster root plants to non-cluster root species under glasshouse conditions.

This study focused on root adaptations in Proteaceae, Fabaceae, Cyperaceae as well as Polygalaceae and Juncaceae which are sister families to Fabaceae and Cyperaceae, respectively. The study was separated into two experimental chapters; the first was a potted

glasshouse experiment where selected plant ecotypes were grown in three different soil types for nine months before being analysed for root adaptations and a field study experiment where plant leaves and roots were sampled from two sites with different soil types and analysed for organic acid exudation, phosphatase activity and leaf N and P concentrations. The second chapter was another glasshouse experiment where plants were grown in a potted sand experiment under two P concentrations (10 and 25 $mg.kg^{-1}$) for seven months before their root adaptations were analysed.

Chapter 2

Morphological and physiological mechanisms for phosphorus acquisition of Fynbos species in different soils of the Western Cape

2.1 Introduction

The Core Cape Subregion (CCR) is made up of a mosaic of sandstone and shale substrates and pockets of granite and limestone that give rise to a wide range of different soil types, greatly contributing to the edaphic diversity of the area (Manning and Goldblatt 2012). The sandstone system yields sandy soils which are course grained and nutrient poor, while the shale substrates are clay soils with richer nutrient content and both the granite and the limestone derived soils have intermediate status (Groves *et al*. 1983; Manning and Goldblatt 2012). The CCR covers four biomes and several distinctive vegetation types, the most distinctive and common being Fynbos (Manning and Goldblatt 2012). Vegetation in the Fynbos biome is characterized by high levels of endemism where distribution of species is defined and restricted, among other factors, by soil type (Cowling and Holmes 1992). A study by Stock and Verboom (2012) showed that the South African Cape Region is the least fertile out of five regions with Mediterranean climates, with the lowest pH (about 4.8), total P (87 $mg.kg^{-1}$) and second lowest total N (973 $mg.kg^{-1}$). Due to the CCR soils being highly weathered and nutrient-leached, mineral nutrients in the soil are largely unavailable to plants possibly due to low content in the parent substrate and being fixed to various cations (Witkowski and Mitchell 1987). Microorganisms also immobilise some of the organic P in the soil during decomposition, rendering the P unavailable to plants (Vance *et al.* 2003). As a result, plant available P has been estimated to generally range from 0.4 – 3.7 $\mu g\ P\ g^{-1}$ soil in Fynbos soils (Witkowski and Mitchell 1987; Cramer 2010). To survive in these P limited soils, species in the Fynbos biome evolved various mechanisms to enhance their acquisition of P (Vance 2001; Neumann and Martinoia 2002; Lambers *et al.* 2006).

In a review by Lambers *et al.* (2008) it was shown that plants have several contrasting and complementary mechanisms for increasing their acquisition of both N and P and that these mechanisms do not occur randomly across biomes but are dependent on the age of the

landscape and its soil nutrient status. In ancient soils, where most P is adsorbed to soil particles and where there is little P in solution, the strategy to mine the soils is dominant, which is achieved by the exudation of large amounts of carboxylates to mobilise the sorbed P, whereas in younger soils where soil P is higher, scavenging mechanisms are dominant which include increases in root proliferation, development of root hairs and symbioses with mycorrhizae (Lambers *et al.* 2008). The diversity of nutrient-acquisition mechanisms across soils helps explain the observed species richness across biomes (Lamont 2003; Orians and Milewski 2007; Lambers *et al*. 2008).

Root morphological parameters are closely associated with a plant's capacity for nutrient acquisition and uptake (Denton *et al.* 2006; Lynch 2007) and changes in root morphology are essential for P-acquisition efficiency (Lambers *et al*. 2006). Responses in root morphology include adaptations to increase the total surface area by producing more and longer roots, finer roots, more branched systems of roots, an increase in specific root length (SRL), root hair proliferation, production of cluster roots and microbial association with mycorrhizal fungi, all of which enable the plant to explore a greater volume of soil and acquire nutrients more efficiently (Lamont 1982; Allsopp and Stock 1993; Schachtman *et al*. 1998; Lynch and Brown 2001; Vance 2001; Pang *et al*. 2010b). Physiologically, plants can enhance the expression of phosphatases, which solubilise organically bound P (Marschner *et al*. 1986; Vance 2001), and increase the synthesis and exudation of organic acids, which mobilise inorganic P in the soil (Pang *et al*. 2010b).

2.1.1 Root Adaptations

The growth and configuration of a plant's root system is called 'root architecture' (morphology) and varies in response to nutrient availability in the soil (Lynch and Brown 2001; Vance *et al.* 2003). Root morphology is therefore vital with regards to P acquisition due to the low mobility of P within the soil (Lynch and Brown 2001). Phosphorus in soils moves mainly via diffusion at a slow rate (10^{-12} to 10^{-15} $m^2.s^{-1}$) and, as such, a zone of depletion is usually present around plant roots (Schachtman *et al.* 1998; Lynch and Brown 2001). Longer and finer roots are better distributed than shorter and thicker roots in low nutrient soils to allow plants to cover a larger surface area for nutrient absorption (Schachtman *et al*. 1998; Vance *et al.* 2003). Definitive studies conducted by Drew (1975) and Jackson *et al*. (1990) demonstrated the effects of localised supplies of soil P on the proliferation of roots in grass species. A noteworthy finding was that there was also

genotypic variation in the changes in root morphology to facilitate P acquisition in response to P deficiency (Ge *et al.* 2000; Liao *et al.* 2001; Lynch and Brown 2001).

The majority of root biomass is usually comprised of lateral roots (i.e. roots forming from the main root axes of the tap, basal and adventitious roots) which are an important component in root morphology and their formation is greatly enhanced to improve foraging for P (Lynch and Brown 2001). A study by Williamson *et al.* (2001) has shown that, grown under P-deficient conditions, species of *Arabidopsis* had a reduction in primary root elongation and an increase in lateral root density and elongation. It was also reported that P-deficiency induced alterations in root morphology, particularly the development of lateral roots, is usually accompanied by an increase in root hair density and length (Vance *et al.* 2003). Root hairs are protrusions of root epidermal cells and their role in P uptake as well as plant survival have been shown in many studies with various plant species and root hair mutants (Yan *et al.* 1995; Bates and Lynch 2000; Jungk 2001; Gahoonia and Nielsen 2003). The increase in root surface area through the proliferation of root hairs in low P soils is a carbon saving (Hetrick 1991) and efficient strategy for P acquisition (Bates and Lynch 2000). In addition to increasing the surface area for P uptake for plants, recent studies have also found that they assist in the release of organic acids (Narang *et al.* 2000; Hinsinger 2001; Ryan *et al.* 2001) and acid phosphatase (Gahoonia *et al.* 2001) into the rhizosphere. Gahoonia and Nielsen (2003) looked at the effects of root hairs on growth and P-uptake on a barley root-hairless mutant, *Hordeum vulgare*, and root hair producing genotype, Pallas, under high and low P conditions. It was found that under low-P conditions, the hairless mutant did not survive longer than 30 days while Pallas continued to grow and had a 2-times higher P-uptake than did the hairless mutant. Under high-P conditions, both genotypes survived and continued to grow, however the root hair length of Pallas was shown to be shorter under high-P conditions compared to low-P conditions. This confirmed the importance of root hairs in the growth of plants under low-P conditions but at high-P, root hairs became unnecessary.

Plants species growing in the most heavily leached soils in the world, such as Western Australia and Western Cape, South Africa, have evolved specialised structures, cluster roots, to enhance acquisition of P (Pate *et al.* 2001; Vance *et al.* 2003). The growth of cluster roots is seasonal and starts when the winter rains begin, to allow efficient uptake of nutrients from the leaf litter produced during winter and spring (Lamont 1982; Pate and Watt 2001). When the soil surface dries out in summer, the cluster roots senesce (Vance *et al.* 2003). Shrubs and

trees that form cluster roots are generally slow-growing, sclerophyllous and grow on highly leached soils with very low P (Dinkelaker *et al.* 1995; Pate *et al.* 2001; Lambers *et al.* 2002). Cluster roots produce large quantities of root hairs compared to normal lateral plant roots which produce highly regulated root hairs from a discrete number of epidermal cells (Ridge 1995; Malamy and Benfey 1997; Dolan 2001; Vance *et al*. 2003). This increase in root hair density comes with an increase in surface area of about 100 times that of normal roots (Vance *et al.* 2003). These dense areas of cluster roots are able to mobilise sparingly soluble P by exuding organic acids and phosphatases in localised patches in the soil (Gardner *et al.* 1983; Dinkelaker *et al*. 1995; Lambers *et al.* 2002). The formation of cluster roots is generally stimulated by a deficiency in P (Walker and Pate 1986; Skene *et al.* 1996) and as P availability increases, the formation of cluster roots is suppressed (Dinkelaker *et al.* 1995; Keerthisinghe *et al.* 1998; Watt and Evans 1999) which can be seen in Proteaceae (Dell *et al.* 1980; Skene *et al.* 1996) and other groups of plants (Trinick 1977; Walker and Pate 1986; Louis *et al.* 1990; Racette *et al.* 1990; Crocker and Schwintzer 1993; Hurd and Schwintzer 1996, 1997).

Nutrient acquisition in low-nutrient soils is also supported by plants that live symbiotically with organisms in the soils (Smith *et al.* 2010). The most common symbiotic relationship is that with mycorrhizal fungi of which there are six types; arbuscular, arbutoid, ecto, ericoid, monotropoid and orchid mycorrhizae, which are involved with almost 90% of land plants (Smith *et al.* 2000, 2010; He *et al.* 2003). Arbuscular mycorrhizal (AM) symbioses are the most frequent occurrence, involving up to 80% of vascular plant species and approximately 150 fungal species (Smith *et al.* 2010). These AM fungi take up inorganic P as well as Zn and N via their external hyphae and transfer these to the plant, which in turn exchanges this for organic carbon (Smith *et al.* 2010). Where there are large amounts of organic matter accumulation in soils, ectomycorrhizal (ECM) and ericoid mycorrhizal (ERM) symbioses dominate as they both have a robust capacity to mobilise both P and N from available and recalcitrant organic forms (Smith *et al.* 2010). The three remaining mycorrhizal fungi types; arbutoid, monotropoid and orchid mycorrhizae are associated with Ericales, Monotropaceae and Orchidaceae respectively and, while not as referred to as the rest, are as important to plants as the other types (He *et al.* 2003).

Under normal growth conditions plant roots exude a wide variety of organic compounds (Marschner 2012) which can alter the chemistry of the rhizosphere, soil microbial

populations and plant growth (Vance *et al.* 2003). Organic acids are one of these exudates and their production and exudation rates are enhanced when there is P or Fe deficiency, Al toxicity and/or exposure to heavy metals (Tate 1984; Dinkelaker *et al.* 1989; Jones 1998; Neumann *et al*. 2000; Ryan *et al.* 2001). Among the variety of organic acids produced and exuded, a study by Jones (1998) indicated that citric, malic and oxalic acid are the most efficient at solubilizing unavailable P due to their rapid complexation with metals (Zhang *et al.* 1997; Hinsinger *et al.* 2006). In a study by Power *et al.* (2010) it was found that Proteaceae species produced and exuded more carboxylates from their roots than Fabaceae species, *Aspalathus nivea* and *A. subtingens*. Another compound plants utilize are phosphatases which are enzymes that originate either from the plant itself or are secreted by microorganisms within the soil (Tarafdar and Jungk 1987; Herbien and Neal 1990). Since a large proportion of soil P occurs in an organic form (Straker 1996; Raghothama 1999) phosphatase enzymes, APases and ALPases, catalyse the cleavage of P from the organic phosphate esters in both alkaline and acidic low P soils (Asmar *et al.* 1995) thereby making P available for uptake by plant roots. There are both intra- and extracellular APases evident in plants and while intracellular APases function primarily in releasing P from senescent tissue for remobilization and utilization, extracellular APases are involved in P acquisition from the soil (Marschner *et al.* 1986; Tarafdar and Claassen 1988; Duff *et al*. 1989; Duff *et al.* 1994).

2.1.2 Legume VS non-legume and soil types

Members of Fabaceae are best known for their ability to form symbiotic relationships with soil bacteria, known as rhizobia, which colonise the roots and form special organs called nodules (Lamont 1982; Sprent *et al.* 2013). Within these nodules the bacteria are able to reduce N from the atmosphere to ammonia which is then passed onto the host plant to be assimilated into organic compounds such as amino acids and nucleotides (Sprent *et al.* 2013). A number of studies have found that legumes are superior in extracellular APase activity (Nuruzzaman *et al.* 2006; Houlton *et al.* 2008), organic acid exudation (Ae *et al.* 1990; Jones 1998; Keerthisinghe *et al.* 1998; Veneklaas *et al.* 2003; Nuruzzaman *et al.* 2006) and P-uptake in low P soils when compared to other non-legume plant species. Acid phosphatases are present in the rhizosphere of most plants and its activity increases with P-deficiency (Tadano and Sakai 1991) and other work, with an extensive variety of crop species, have shown similar results (Goldstein *et al*. 1988; Helal and Sauerbeck 1991; Adams and Pate 1992; Tadano *et al*. 1993; Ascencio 1996, 1997). Studies have also found that where N_2-fixing plants were present, extracellular APase activity was almost three times higher in the

soils compared to soils where there were no N_2-fixers (Zou *et al.* 1995; Allison *et al.* 2006; Nuruzzaman *et al.* 2006; Houlton *et al.* 2008). For example, in the study by Nuruzzaman *et al.* (2006) three legume crop species, *Lupinus albus* (White Lupin), *Pisum sativum* (Field Pea) and *Vicia faba* (Faba Bean) were compared with a cereal species, *Triticum aestivum* (Spring Wheat), and it was found that the three legume species had consistently higher APase activity compared to the cereal species especially in low-P soils and that *L. albus* produced significant amounts of citrate while there were no organic acids detected in the rhizosphere of *P. sativum*, *V. faba* and *T. aestivum*. However, *V. faba*, which neither produced cluster roots nor exuded large amounts of organic acids, had the greatest P content in its tissues probably due to its greater root dry mass accumulation compared to the other species. The three legumes also had better growth than that of *T. aestivum* suggesting that they have a special adaptation to acquiring P from low P soils. Furthermore, N_2-fixers produce nitrogen-rich litter which adds more nitrogen to the soils stimulating the production of phosphatases (Olander and Vitousek 2000; Treseder and Vitousek 2001) thereby increasing the availability of P. This is indicative of a nitrogen-rich strategy of P acquisition that is specifically suited to N_2-fixing species (Houlton *et al.* 2008). Another study by Fageria *et al.* (2014) looked at the growth and P-uptake of five legumes species in three levels of P; 0, 100 and 200 mg P kg^{-1} soil. They found that all the plants' root dry weight and their root:shoot ratios increased until 100 mg P kg^{-1} soil and decreased at higher P, while their SRL's increased with decreasing P. All five species varied in their P-uptake which increased with increasing P and overall P-uptake efficiency was higher at low P and decreased with increasing P. Legumes with cluster roots have been found to be remarkably plastic whereby they increase their root mass over 100% in P enriched pockets in the soil and the subsequent increase in root P concentrations suggests that the significant increases in P uptake are a result of targeted plastic root growth (Adams *et al.* 2002). A study on two legumes, *Cicer arietinum* (Chickpea) and *L. albus*, in different soils found that there were large differences in the root exudates of the two species between the different soils and it was suggested that pH may be the soil factor responsible in that case (Veneklaas *et al.* 2003).

2.1.3 Problem statement and objective

There are three main problem statements to this study, firstly that cluster root formation has long been associated with superior P acquisition. It is thought that plants that produce cluster roots are better adapted to accessing sparingly soluble forms of P that are predominant in ancient, highly weathered and leached soils (Lambers *et al.* 2008) compared to other forms of

P acquisition mechanisms plants exhibit such as enhanced root:shoot ratios, root morphological changes, root exudation of organic acids and APase as well as symbioses with mycorrhizae (Lamont 1982; Allsopp and Stock 1993; Schachtman *et al*. 1998; Lynch and Brown 2001; Vance 2001; Pang *et al*. 2010b; Lambers *et al.* 2006, 2008). Secondly, it is of the general opinion that legumes are not as well adapted as some other families like Cyperaceae, Proteaceae and Restionaceae to P acquisition which leads to their inability to persist into late succession in the fynbos (Power *et al.* 2010). Lastly, P acquisition mechanisms have been found to be influenced by soil types (Ae *et al.* 1990; Cowling *et al.* 1994; Lambers *et al.* 2002; Yan *et al.* 2002; Gahoonia and Nielsen 2003; Lamont 2003; Linder 2003; Veneklaas *et al.* 2003; Orians and Milewski 2007; Lambers *et al.* 2008).

The overall objective of this chapter was to determine whether cluster root species are more efficient at P acquisition compared to non-cluster root species. It was hypothesized that *A. linearis*, a cluster root forming legume species, adapted to infertile soil and drier conditions of the CCR, would be more efficient at P-acquisition than the other species tested, and that plants in Silvermine sandstone soils, which is considered to be most impoverished soil, will form superior P-acquisition mechanisms. Four fynbos species consisting of two legume and two non-legume species, with one cluster root species and a non-cluster root congeneric pair, were grown in potted soil from three sites around the CCR, with contrasting soil types and P-content, under glasshouse conditions for nine months. At harvest, plant biomass, root morphology, APase activity, organic acid exudation, as well as tissue N and P concentrations were measured. This allowed multiple assessments of P-acquisition mechanisms in a plant, a novelty of this study, in contrast to single mechanisms commonly reported in literature (Gahoonia and Nielsen 1997; Zhang *et al.* 1997; Gilbert *et al.* 1999; Neumann and Römheld 1999; Veneklaas *et al.* 2003; Gahoonia and Nielsen 2004; Vandamme *et al.* 2013). A field study was conducted alongside this glasshouse experiment to determine root exudation of organic acids and APase activity of the glasshouse species or close relatives under conditions in the field. Eight fynbos species, consisting of four legumes and four non-legume species, two of which are cluster root forming were sampled from two sites in the field.

2.2 Materials and Methods

2.2.1 Species and growth conditions

The root morphology and physiological responses in the four plant species namely; *Aspalathus linearis* (Burm. f.) R. Dahlgreen*, Podalyria calyptrata* (Retz.) Willd.*, Polygala myrtifolia* (L.) and *Leucadendron argenteum* (L.) R. Br., were investigated in a potted soil experiment in a well-ventilated glasshouse situated at the University of Cape Town (S 33° 57.353'; E 018° 27.742') with an average temperature of 14.5°C and a range of 6-25°C. This study compared species consisting of two legume (*A. linearis* and *Pod. calyptrata*) and two non-legume species, (*Pol. myrtifolia* and *L. argenteum*) with *A. linearis* and *L. argenteum* as cluster root forming species. *Aspalathus linearis* and *Pod. calyptrata* plants were grown from seed obtained from Silverhill Seeds and Books, Cape Town, South Africa. The *A. linearis* seeds were mechanically scarified by being rubbed with sand paper while the *Pod. calyptrata* seeds were soaked in boiled water overnight to break dormancy. Seeds were sown into trays of acid washed sand to germinate. *Leucadendron argenteum* and *Pol. myrtifolia* failed to germinate from seed and were therefore bought as seedlings from New Plant Nursery (George, South Africa). Each species was grown in soil collected from their natural locations in the CCR and in areas where they do not naturally occur for a soil type treatment (Table 2.1).

Soils were collected in South Africa from two sites with sandstone as the parent material; Silvermine (S 34° 05.33'; E 018° 25.17') and Niewoudtville (S 31° 22.33'; E 019° 06.33'), and one site with shale derived soils; Rhodes Memorial (S 33° 57.08'; E 018° 27.32') and were passed through a 5mm sieve to remove any large debris. These sites represented the natural habitats for *Pod. calyptrata*, *A. linearis* and *L. argenteum*, respectively whereas *Pol. myrtifolia* is a wide spread species. Four germinated seedlings of *A. linearis* and *Pod. calyptrata* were planted into six replicated pots filled with 4 kg of soil and this was repeated for each soil type. One seedling of *Pol. myrtifolia* and *L. argenteum,* that were 5 and 20 cm high respectively, measured from the base of the stem to the furthest point of the leaves, were also planted into potted soils as described above to give a total of 72 pots. After one month of growth, seedlings of *A. linearis* and *Pod. calyptrata* were thinned down to one plant per pot. Plants were watered as needed but no extra nutrients were supplied. Pots were arranged at random on trolleys in the glasshouse and were re-arranged every two weeks. Plants were left to grow for nine months, from March to November 2013, and then harvested (Figure 2.1).

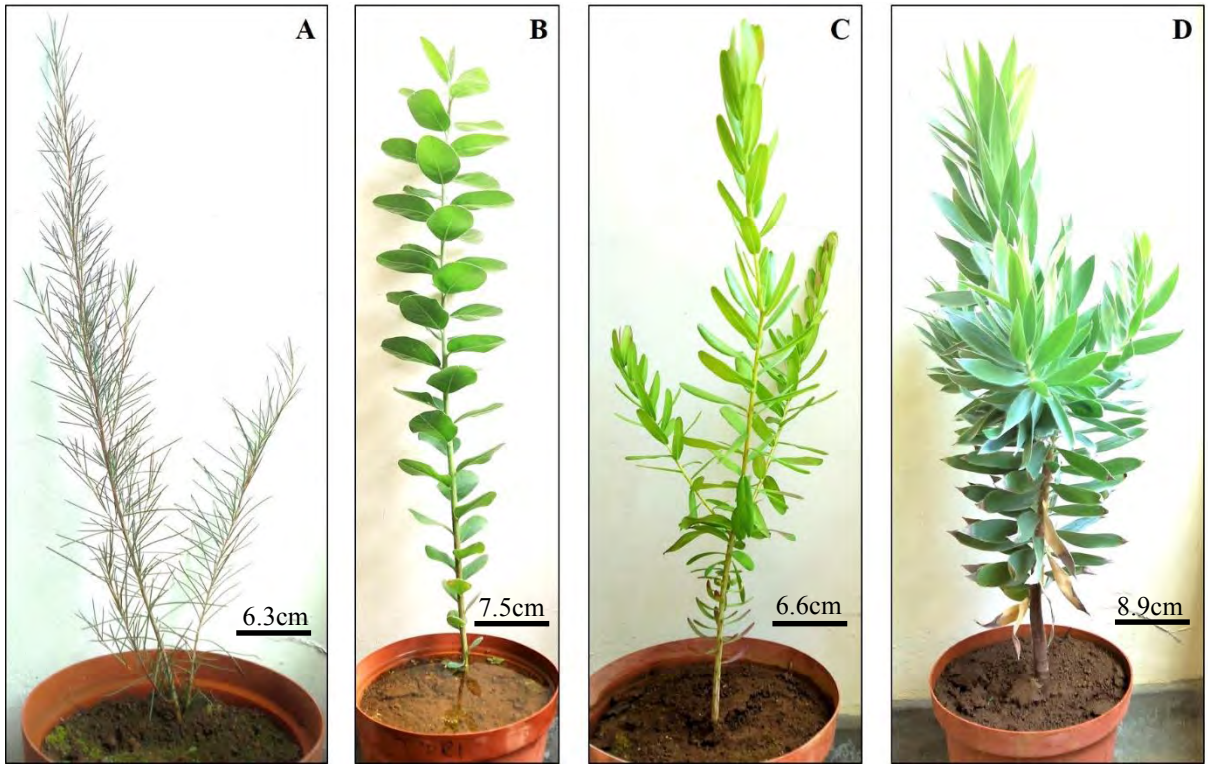

Figure 2.1: The four species, *Aspalathus linearis* (A), *Podalyria calyptrata* (B), *Polygala myrtifolia* (C) and *Leucadendron argenteum* (D), grown in Rhodes Memorial soils, at time of harvest.

2.2.2 Fieldwork

Two sites in the CCR were sampled, Silvermine (S 34° 05.433'; E 018° 25.282') and Rhodes Memorial (S 33° 57.7'; E 018° 27.25') during September and October 2012. Species of Fabaceae, Polygalaceae and Proteaceae, which were used in the glasshouse study, were sampled from both sites. The species sampled in Rhodes Memorial were *Aspalathus macrantha* Harv., *Leucadendron argenteum, Podalyria calyptrata* and *Polygala myrtifolia* and the species sampled in Silvermine were *Psoralea pinnata* L., *Leucadendron laureolum* (Lam.) Fourc., *Podalyria sericea* (Andrews) R.Br. ex Aiton f. and *Muraltia heisteria* (L.) DC. At each site, five replicates were taken per species. For each plant, roots were traced from the first 10cm of soil and samples of root and cluster root, if present, were taken, for APase activity and organic acid exudation analyses. Loose soil on the roots was carefully removed and the roots were then rinsed with distilled water to remove any remaining rhizosphere soil. Samples of leaves were also taken for N and P concentrations. At each site four soil samples were also collected at random. The soil samples were taken from the upper

15 cm of soil at each site using a soil corer or a trowel and labelled. Methods for the various analyses are as described below.

2.2.3 Nutritional analysis of soil

In the laboratory the soil samples were air dried and sieved through a 1mm mesh to remove debris prior to analysis. Determination of pH was performed by shaking 2 g of soil material in 20 ml 1M KCl at 180 rpm for 60 min and then centrifuging at 4,000 g for 20 min in an Eppendorf 5810 R Centrifuge (Hamburg, Germany). The supernatant was used to measure the pH using a pH 320 meter (WTW GmbH, Weilheim, Germany). Concentration of Total N in the soil was measured at the Archaeology Department at the University of Cape Town using mass spectrometry. Samples were combusted in a Flash EA 1112 series elemental analyser (Thermo Electron, Milan, Italy) and the gases were passed to a Delta Plus XP isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany), via a Conflo III gas control unit (Thermo Finnigan, Bremen, Germany), for the determination of concentration of Total N. Total P and available P (Bray II P) were analysed at the analytical laboratory Bemlab (Pty) Ltd. (Somerset West, South Africa). Total P in soil was determined by a method adapted from Sommers and Nelson (1972) where the P was extracted from the soil through an acid digestion using a 1:1 mixture of 1N nitric acid and hydrochloric acid at 80°C for 30 minutes. The P concentration in the extract was then determined with an optical emission spectrometer (ICP-OES, Varian, United States). Available P was determined using the Bray II method where the P was extracted from the soil using Bray II solution as the extractant (Bray and Kurtz 1945). The extractant was then measured colourimetrically based on the reaction with ammonium molybdate and the development of the 'Molybdenum Blue' colour. Soil concentrations of carbon (C), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg) and sodium (Na) were analysed at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenberg (Stellenbosch, South Africa), according to the Handbook of Standard Soil Testing Methods for Advisory Purposes (1990). Carbon was measured using the Walkley-Black method where organic C was oxidised with acidic dichromate followed by a titration of excess dichromate with ferrous sulphate. The C was calculated from the difference between the total dichromate added and the amount of dichromate left unreacted after C oxidation. The Ca, Fe, K, Mg and Na concentrations were determined by ICP-OES (Thermo Fisher Scientific Inc., NYSE: TMO) in 1% citric acid extractions.

2.2.4 Plant biomass

At harvest of the glasshouse-grown plants, the plants were separated into leaves, stems, roots, nodules and cluster roots (if present; Figure 2.1 and 2.2). Samples of roots were collected, as described below, for extracellular root phosphatase activity, organic acid exudation and root morphology and root hair analyses. The remaining biomass was weighed and oven dried at 60°C for three days. After drying, the organs were re-weighed and milled using a Hammer Mill (United Scientific Pty Ltd, South Africa) for nutrient analysis. Similarly, about 200 g of leaves were taken from species sampled in the field and oven dried at 60°C for three days after which the leaves were milled using a Hammer Mill (United Scientific Pty Ltd, South Africa) for nutrient analysis.

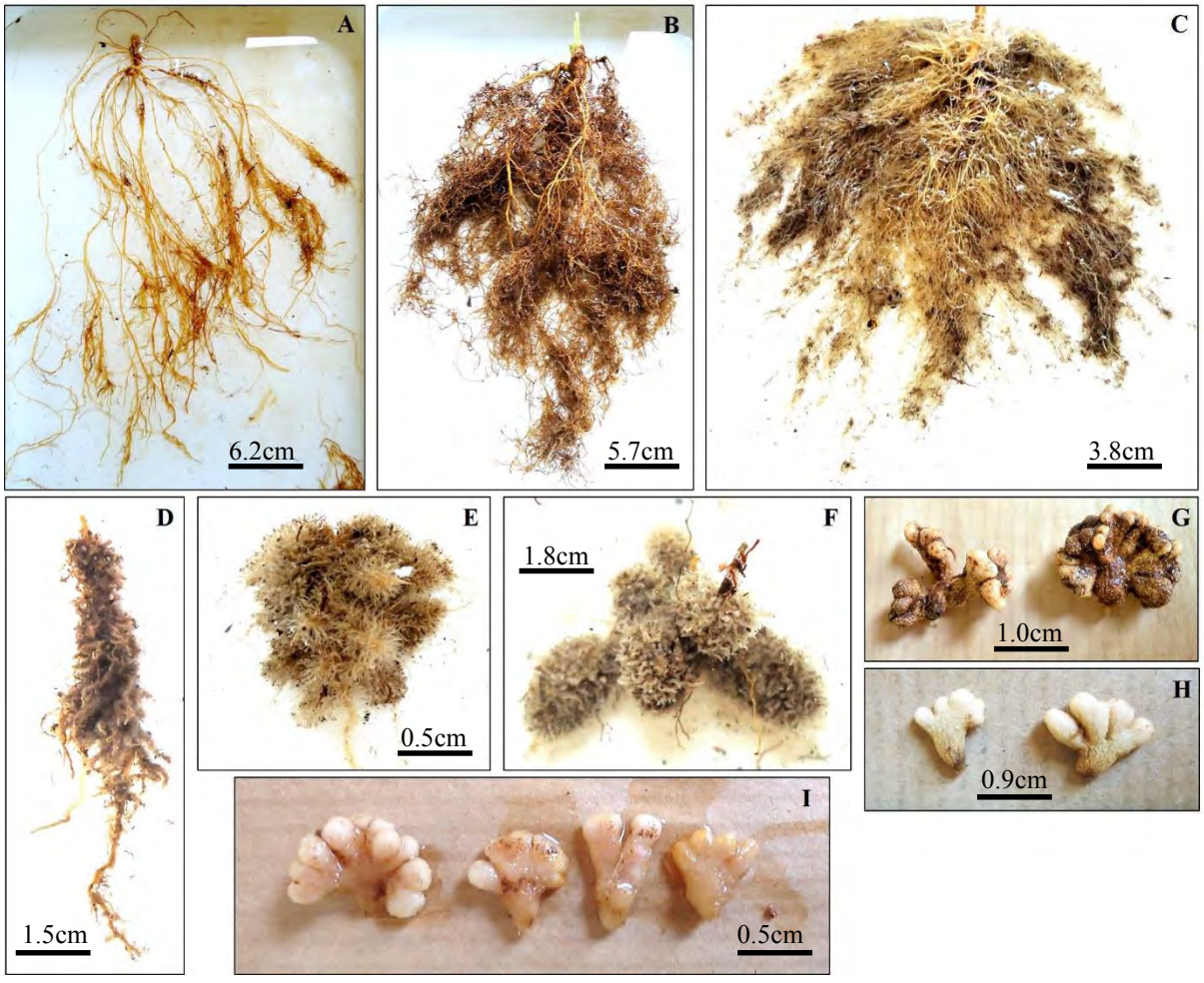

Figure 2.2: Whole root systems of the three species, *Aspalathus linearis* (A), *Podalyria calyptrata* (B) and *Polygala myrtifolia* (C), cluster roots from *Aspalathus linearis* (D) and *Leucadendron argenteum* (E-F) and root nodules from *Podalyria calyptrata* (G-H) and *Aspalathus linearis* (I) all grown in Rhodes Memorial soils.

2.2.5 Extracellular acid phosphatase activity

About 300-600 mg of root biomass was collected fresh at harvesting, weighed and kept on ice until ready to assay for APase activity. Roughly the same amount of root biomass was collected in the field and kept on ice until return to the lab where they were weighed for fresh weight and kept on ice until ready for assay. Method of assay is detailed in Tabatabai and Bremner (1969) and modified by Hedley *et al.* (1982). The analysis involved colourimetric estimation of the p-nitrophenol released by phosphatase activity after incubation of root material with 4 ml of MES 15 mM/ $CaCl_2$ 500 µM buffer (pH 5.5) at 28°C for 30 minutes. The reaction was stopped with 0.5 M NaOH and absorbance was measured spectrophotometrically at 412 nm. The detection limit for absorbance was < 0.001nm. One unit of acid phosphatase activity was defined as the activity per gram of root which produced 1 µmol p-nitrophenol per hour. The roots were recovered and then oven dried at 60°C for three days and re-weighed for dry weight.

2.2.6 Organic acid exudation

Between 300-600 mg of roots were collected from both glasshouse and field at harvest and transferred to vials containing 15 ml of 200 µM $CaCl_2$ to ensure cell integrity (Pearse *et al.* 2006) for the determination of root exudation of organic acids. Samples were gently shaken on an orbital shaker (Laboratory Marketing Services, Roodepoort, South Africa) at 160 rpm for one hour to collect organic acid exudates. The solution from each vial was then filtered through filter paper and stored at -20°C. The root material was recovered and oven dried at 60°C for three days and re-weighed for dry weight. The filtrates, that were stored at -20°C, were filtered through 20 µm syringe filters, freeze dried and then re-suspended in 1 ml of sterilized water. Samples were analysed for acetic, citric, lactic, malic and succinic acids by the Central Analytical Facility of Stellenbosch University (Stellenbosch, South Africa). Acetic, lactic and malic acid concentrations were determined photometrically by measuring the increase in absorbance at 340 nm (Enzytec™ Fluid enzyme kits, Thermo Fisher Scientific, Oy, Finland). Citric and succinic acid concentrations were determined by the decrease in absorbance associated with the oxidation of NADH (Yellow line enzyme kits, Roche, R-Biopharm AG, Darmstadt, Germany). Detection limits for the organic acids were as follows; < 0.003 $g.l^{-1}$ for acetic acid, <0.0005 $g.l^{-1}$ for citric acid, < 0.0021 $g.l^{-1}$ for lactic acid, < 0.0025 $g.l^{-1}$ for malic acid and < 0.00015 $g.l^{-1}$ for succinic acid.

2.2.7 Assessment of root morphology using WinRHIZO

A sub-sample of approximately 15% of the total root mass, as recommended by Vandamme *et al.* (2013), was collected for root morphological analyses and stored in a 10% ethanol solution in the fridge. Roots were rinsed with distilled water and stained by submersion in a warmed, 2% solution of gentian violet for five minutes. The excess dye was then rinsed off and samples were stored in ethanol until ready for scanning. Total root length (cm), average diameter (cm) and total surface area (cm^2) were measured using a STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Quebec, Canada). The root samples were recovered and oven dried for three days at 60°C and weighed for dry weight. Total root length (cm) and total surface area (cm^2) were converted to whole root results by multiplying by their relevant conversion factors to 100% of the root. Specific Root Length (SRL) was calculated by dividing the total root DW (g) by total root length (m).

2.2.8 Assessment of root hairs

For the assessment of root hairs, ten random root segments of 1 cm length were collected, randomly, from the root system, from each plant during harvesting. The samples were stored in a 200 µM $CaCl_2$ solution in the fridge to preserve cell integrity until use. The ten root sub-samples were mounted on microscope slides with Hoyers solution and were examined under a DM500 Leica compound microscope (Wetzler, Germany) where root hair density, root hair length and width were measured at 40 and 100X magnification.

2.2.9 Nitrogen and phosphorus analysis in plant tissue

Concentration of N in plant samples was measured at the Archaeology Department at the University of Cape Town using mass spectrometry. Samples were weighed to 2.5 – 2.7 µg on a Sartorius (AG, Göttingen, Germany) micro balance and measured as explained for soil N above.

Phosphorus analysis in the plant samples was conducted at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenberg (Stellenbosch, South Africa) using the dry ashing method (ALASA 1998).

Whole plant P for the glasshouse study, was determined using the following equation:

$$\text{Whole plant [P]}$$

$$= \frac{(\text{leaf [P]} \times \text{leaf mass}) + (\text{stem[P]} \times \text{stem mass}) + (\text{root [P]} \times \text{root mass})}{\text{total plant biomass}}$$

2.2.10 Phosphorus Uptake Efficiency (PUpE)

Phosphorus Uptake Efficiency (PUpE) was calculated using the following equation from Hammond *et al.* (2009):

$$\text{PUpE} = \frac{(P_{HIGH} \times Y_{HIGH}) - (P_{LOW} \times Y_{LOW})}{\Delta P_{APP}}$$

where P_{HIGH} was whole plant P ($mg.g^{-1}$) in Niewoudtville soil, Y_{HIGH} was total plant biomass (g) in Niewoudtville soil, P_{low} was whole plant P ($mg.g^{-1}$) in Silvermine soil, Y_{low} was total plant biomass (g) in Silvermine soil and ΔP_{APP} was the difference between total P in Niewoudtville and Silvermine soils. These two soils were chosen as they were both Sandstone derived soils with different concentrations of P.

2.2.11 Statistics

2.2.11.1 Glasshouse study

Variables were log transformed as necessary and all statistical analyses were conducted in Statistica 12 (StatSoft, Inc., Tulsa, U.S.A.). Data were analysed by analysis of variance (ANOVA) to evaluate species effects and means were compared by Tukey HSD tests at a 5% probability level. Data for *L. argenteum* plants were analysed and presented separately (Table 2.4) due to the seedlings being transplanted when they were already well established (>20 cm) plants. Thus, only a comparison among soil types was done for *L. argenteum*. A forward stepwise regression was used to determine which phosphorus acquisition mechanisms contributed significantly towards biomass accumulation of plants.

2.2.11.2 Fieldwork

Variables were log transformed where necessary and statistical analyses were conducted in Statistica 12 (StatSoft, Inc., Tulsa, U.S.A.). Data were analysed using a nested analysis of

variance (ANOVA) with species nested to site. Means were compared by Tukey HSD tests at a 5% probability level.

2.3 Results

2.3.1 Soil chemical characteristics

The soils collected from all the sites were highly acidic, with Silvermine soil being the most acidic with a pH of 4.1, followed by Niewoudtville with a pH of 4.8 and Rhodes Memorial soil was the least acidic with a pH of 5.3 (Table 2.1). Rhodes Memorial soils had the greatest concentration of carbon and all other nutrients measured except available P which was lower than in Niewoudtville soils. Conversely, Silvermine soils had the lowest concentration of all the nutrients except carbon which was higher than that of Niewoudtville soils. Rhodes Memorial soils had 3 and 12 times more Total P than Niewoudtville and Silvermine soils, respectively.

Table 2.1: Soil data from Niewoudtville, Rhodes Memorial and Silvermine. Means and standard errors followed by different letters in the rows are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001.

Variable	Niewoudtville	Rhodes Mem.	Silvermine	*F* ratio
pH	4.82 ± 0.04 b	5.34 ± 0.04 c	4.14 ± 0.03 a	291.52***
Total P ($mg.kg^{-1}$)	41.18 ± 2.25 b	118.73 ± 2.62 c	9.79 ± 1.53 a	192.84***
Available P Bray II ($mg.kg^{-1}$)	11.41 ± 1.10 c	7.39 ± 0.14 b	2.11 ± 0.20 a	121.28***
Nitrogen ($mg.g^{-1}$)	0.25 ± 0.02 a	2.67 ± 0.02 b	0.29 ± 0.04 a	338.25***
Carbon ($mg.g^{-1}$)	4.25 ± 0.19 a	39.58 ± 1.54 c	5.53 ± 0.40 b	557.47***
Calcium ($cmol(+).kg^{-1}$)	0.71 ± 0.06 b	6.07 ± 0.34 c	0.31 ± 0.02 a	539.95***
Iron ($mg.kg^{-1}$)	35.50 ± 1.74 b	48.95 ± 1.19 c	15.59 ± 1.11 a	136.41***
Potassium ($mg.kg^{-1}$)	34.00 ± 0.91 b	286.25 ± 3.90 c	10.25 ± 0.48 a	2670.62***
Magnesium ($cmol(+).kg^{-1}$)	0.25 ± 0.02 b	2.30 ± 0.09 c	0.18 ± 0.00 a	911.21***
Sodium ($mg.kg^{-1}$)	14.50 ± 0.65 a	57.00 ± 0.82 b	15.50 ± 0.50 a	540.50***

2.3.2 Biomass

Among the three species, *Pod. calyptrata* generally accumulated greater biomass followed by *Pol. myrtifolia* and *A. linearis*. However, there were significant interactions ($p < 0.001$) between species and soil types on the accumulation of biomass. *Polygala myrtifolia* accumulated greater total plant biomass in Rhodes Memorial soils compared to plants in Niewoudtville and Silvermine soils while *Pod. calyptrata* had higher total plant biomass in Niewoudtville soils compared to both Rhodes Memorial and Silvermine soils ($p < 0.001$). *Aspalathus linearis*, however, showed no significant differences between soils (Figure 2.3.I). Similar to the biomass accumulation data, biomass allocation to root and shoot varied with species and soil type. For instance, *A. linearis* exhibited higher root:shoot ratios in Silvermine soils compared to plants from both Rhodes Memorial and Niewoudtville soils ($p < 0.001$; Figure 2.3.II), while the root:shoot ratios in *Pod. calyptrata* and *Pol. myrtifolia* were not significantly different between soils. Nodulation occurred for both *A. linearis* and *Pod. calyptrata* with no differences between the two species, the soils, nor any interaction between species and soils ($p > 0.05$; Figure 2.4). *Aspalathus linearis*, which is a cluster root forming species, produced cluster roots only in Silvermine soils.

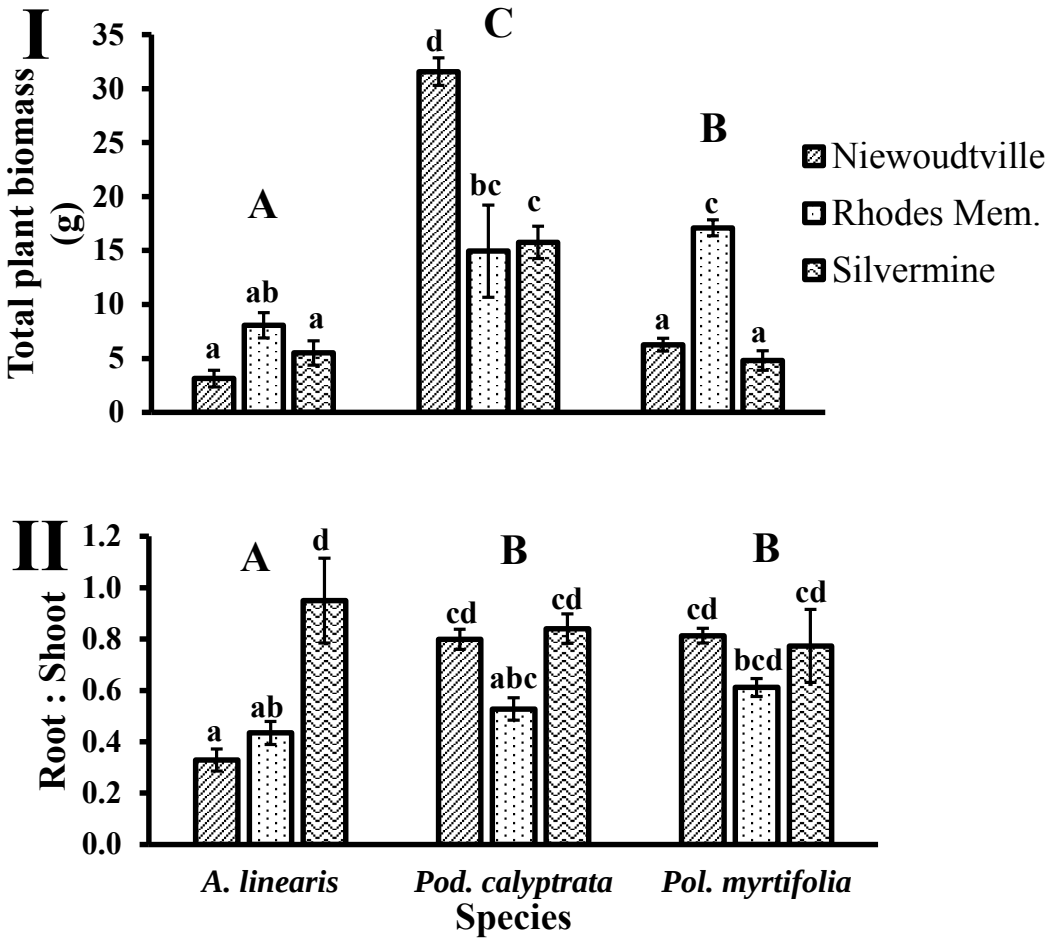

Figure 2.3: Total plant biomass (I) and root:shoot ratio (II) of *A. linearis, Pod. calyptrata* and *Pol. myrtifolia* grown in Niewoudtville, Rhodes Memorial and Silvermine soils. Upper case letters indicate significant differences between species and lower case letters indicate significant differences between soils by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard error.

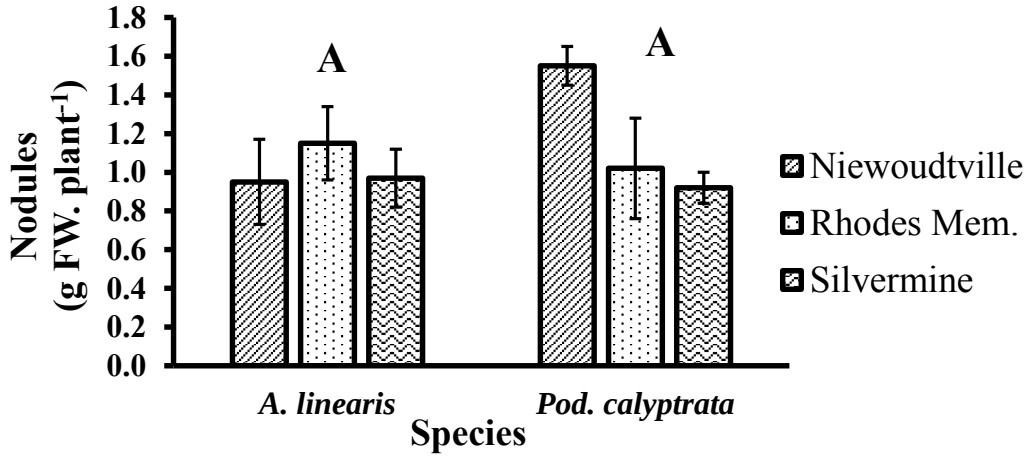

Figure 2.4: Nodules per plant in *A. linearis* and *Pod. calyptrata* grown in soil from Niewoudtville, Rhodes Memorial and Silvermine. Letters indicate significant differences between species by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard error.

2.3.3 Root exudation of organic acids and phosphatase activity (glasshouse)

Root exudation of citric acid was similar between *A. linearis* and *Pod. calyptrata* but was significantly greater in *Pol. myrtifolia* ($p < 0.05$; Figure 2.5.I). Succinic acid exudation, however, was greatest in *A. linearis* and lower and similar among *Pod. calyptrata* and *Pol. myrtifolia* species ($p < 0.05$; Figure 2.5.II). All three species produced and exuded more citric acid than succinic acid ($p < 0.05$). There were no differences in root exudation for both citric and succinic acids associated with soil types ($p > 0.05$).

The activity of APase also differed between species ($p < 0.001$; Figure 2.6) where *A. linearis* had two times and four times more activity than *Pod. calyptrata* and *Pol. myrtifolia* respectively. However, similar to the organic acids, the soil types did not affect the activity of root APase.

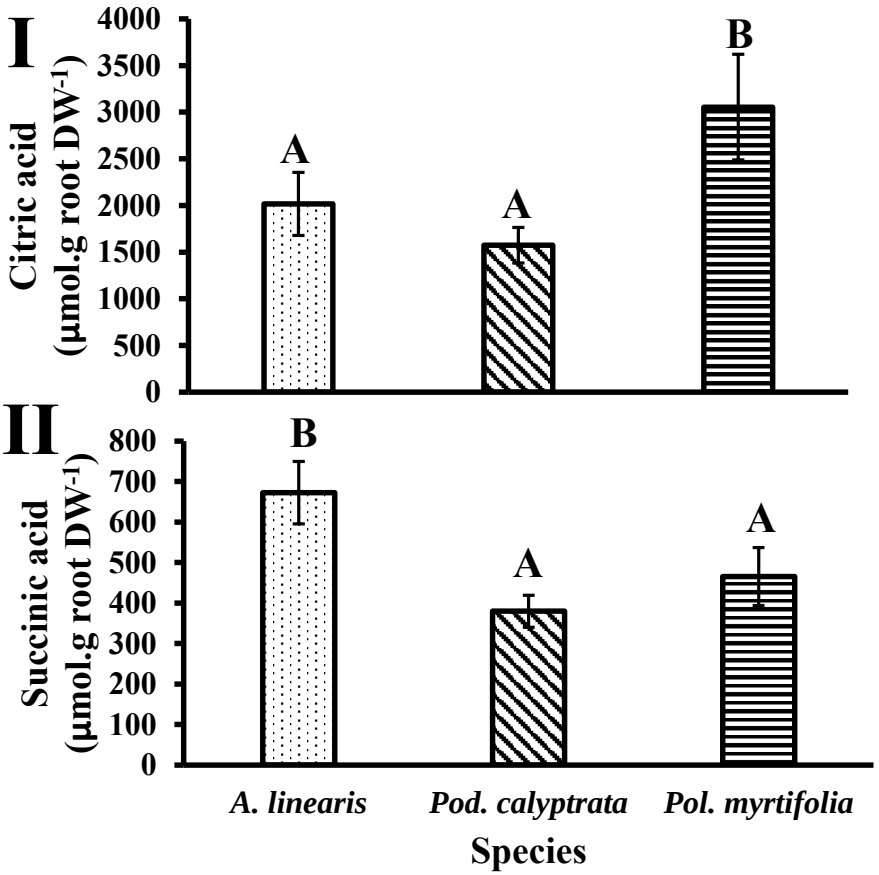

Figure 2.5: Root exudation of citric acid (I) and succinic acid (II) of *A. linearis, Pod. calyptrata* and *Pol. myrtifolia*. There were no differences between soils therefore only species and exudation rates were analyzed. Letters indicate significant differences between species by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

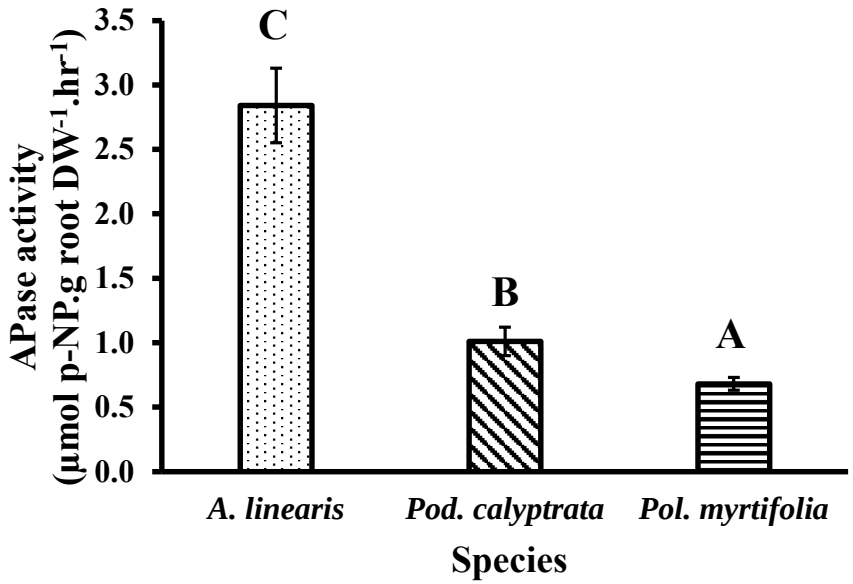

Figure 2.6: Acid phosphatase (APase) activity in the roots of *A. linearis, Pod. calyptrata* and *Pol. myrtifolia.* There were no differences between soils therefore only species and APase activity were analyzed Letters indicate significant differences between species by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

2.3.4 Root exudation of organic acids and phosphatase activity (fieldwork)

Root exudation of citric acid was highest from *A. macrantha* from the Rhodes Memorial site, ranging from 2-100 times more citric acid exuded than the other species ($p = 0.006$), while there were no differences in succinic acid exudation between species ($p > 0.05$; Table 2.2). Overall, six times more citric acid was exuded from the plants on the Rhodes Memorial site compared to plants from the Silvermine site ($p = 0.003$).

The activity of APase did not differ between species and soils except for activity of the cluster roots of *L. argenteum* from the Rhodes Memorial soils, which were significantly lower in their APase activity than the rest of the roots from the other species ($p = 0.014$; Table 2.2). Overall APase activity was higher in Rhodes Memorial soils than in Silvermine soils.

Table 2.2: Phosphatase activity, organic acid exudations and tissue concentrations of several fynbos species observed at Rhodes Memorial and Silvermine in the fieldwork study. Means and standard errors followed by different letters in the columns are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001. – denotes data not available.

Site	Species	APase activity (µmol p-NP. g root DW^{-1}. hr^{-1})	Citric acid exudation (µmol. g root DW^{-1})	Succinic acid exudation (µmol. g root DW^{-1})	Leaf P (mg. g DW^{-1})	Leaf N (mg. g DW^{-1})	Leaf N:P
Rhodes Memorial	*A. macrantha*	1.83 ± 0.29 b	13446 ± 0.0 b	5674 ± 3049.4 a	0.62 ± 0.06 b	49.98 ± 2.14 d	82.23 ± 4.50 c
	Pod. calyptrata	1.44 ± 0.40 b	938 ± 426.2 a	410 ± 262.7 a	0.72 ± 0.07 bcd	26.65 ± 2.10 b	37.88 ± 2.89 b
	Pol. myrtifolia	2.93 ± 0.81 b	5835 ± 2400.0 ab	2720 ± 837.2 a	3.50 ± 0.25 f	16.88 ± 1.37 a	4.93 ± 0.60 a
	L. argenteum	0.98 ± 0.17 ab	873 ± 0.0 a	200 ± 80.8 a	0.68 ± 0.04 bc	-	-
	L. argenteum cluster	1.88 ± 0.69 b	279 ± 222.7 a	67 ± 33.5 a	-	-	-
Silvermine	*Ps. pinnata*	1.17 ± 0.22 b	602 ± 238.8 a	592 ± 333.3 a	1.44 ± 0.05 e	73.27 ± 1.82 e	51.00 ± 1.20 bc
	Pod. sericea	0.88 ± 0.07 ab	400 ± 27.8 a	232 ± 47.5 a	1.28 ± 0.37 cde	36.57 ± 2.41 c	36.28 ± 6.85 b
	M. heisteria	0.92 ± 0.08 ab	347 ± 0.0 a	233 ± 96.0 a	1.20 ± 0.18 de	38.33 ± 0.00 bcd	27.38 ± 0.00 b
	L. laureolum	0.81 ± 0.08 ab	754 ± 640.1 a	2116 ± 1500.6 a	0.30 ± 0.00 a	8.57 ± 0.92 a	28.55 ± 3.06 b
	L. laureolum cluster	0.26 ± 0.19 a	128 ± 0.0 a	281 ± 0.0 a	-	-	-
F ratio		**2.89***	**5.81****	**1.97**	**44.57*****	**155.00*****	**50.97*****

2.3.5 Root morphology and root hair assessment

Out of the three species, *A. linearis* produced the most root hairs per mm of root (root hair density) while *Pol. myrtifolia* produced the least with a tenfold difference between the two species ($p < 0.001$; Figure 2.7.I). There were, however, no differences in root hair density for both *A. linearis* and *Pol. myrtifolia* between soils but there was for *Pod. calyptrata*, which produced almost three times more root hairs in Silvermine soils than in both Niewoudtville and Rhodes Memorial soils ($p < 0.001$; Figure 2.7.I). Root hair length for all three species showed no variation between soils (Figure 2.7.II), however, between species, there were significant differences where *Pod. calyptrata* had almost two and four times longer root hairs than *A. linearis* and *Pol. myrtifolia* respectively ($p < 0.001$; Figure 2.7.II). Similarly, there were no differences between soils for *A. linearis* and *Pod. calyptrata* with regards to root hair width, but there was for *Pol. myrtifolia* which had wider root hairs in Rhodes Memorial soils compared to Niewoudtville soils ($p < 0.01$; Table 2.3). Overall, *Pod. calyptrata* had the widest root hairs while *Pol. myrtifolia* had the narrowest ($p < 0.001$).

Polygala myrtifolia had the longest rooting system compared to *Pod. calyptrata* and *A. linearis* plants, having almost two and four times longer total root length than *Pod. calyptrata* and *A. linearis* respectively ($p < 0.001$; Figure 2.8.I). *Podalyria calyptrata* and *Pol. myrtifolia* had three times more total root surface area than *A. linearis* ($p < 0.001$; Figure 2.8.II). The average diameter of roots for all three species had no differences between the soil types. However, *Pod. calyptrata* plants had the greatest average root diameter compared to the other two species, while *Pol. myrtifolia* had the lowest average root diameter ($p < 0.001$; Figure 2.8.III). Among the three species, *Pol. myrtifolia* had two and three times higher SRL than *A. linearis* and *Pod. calyptrata* respectively ($p < 0.001$; Figure 2.8.IV). Both *Pod. calyptrata* and *Pol. myrtifolia* had similar total root surface areas (Figure 2.8.II). Total root length and total root surface area had the same pattern between soils for all three species; *A. linearis* had no differences for both among soils, *Pod. calyptrata* had a higher total root length and total root surface area in Niewoudtville soils compared to Rhodes Memorial and Silvermine soils, *Pol. myrtifolia*, however, had higher total root length and total surface area in Rhodes Memorial soils compared to Niewoudtville and Silvermine soils ($p < 0.001$; Figure 2.8.I and 2.8.II). There were no soil differences for *Pod. calyptrata* and *Pol. myrtifolia* but *A. linearis* had a higher SRL in plants grown in Niewoudtville soils compared to those grown in Rhodes Memorial soils ($p < 0.01$; Figure 2.8.IV).

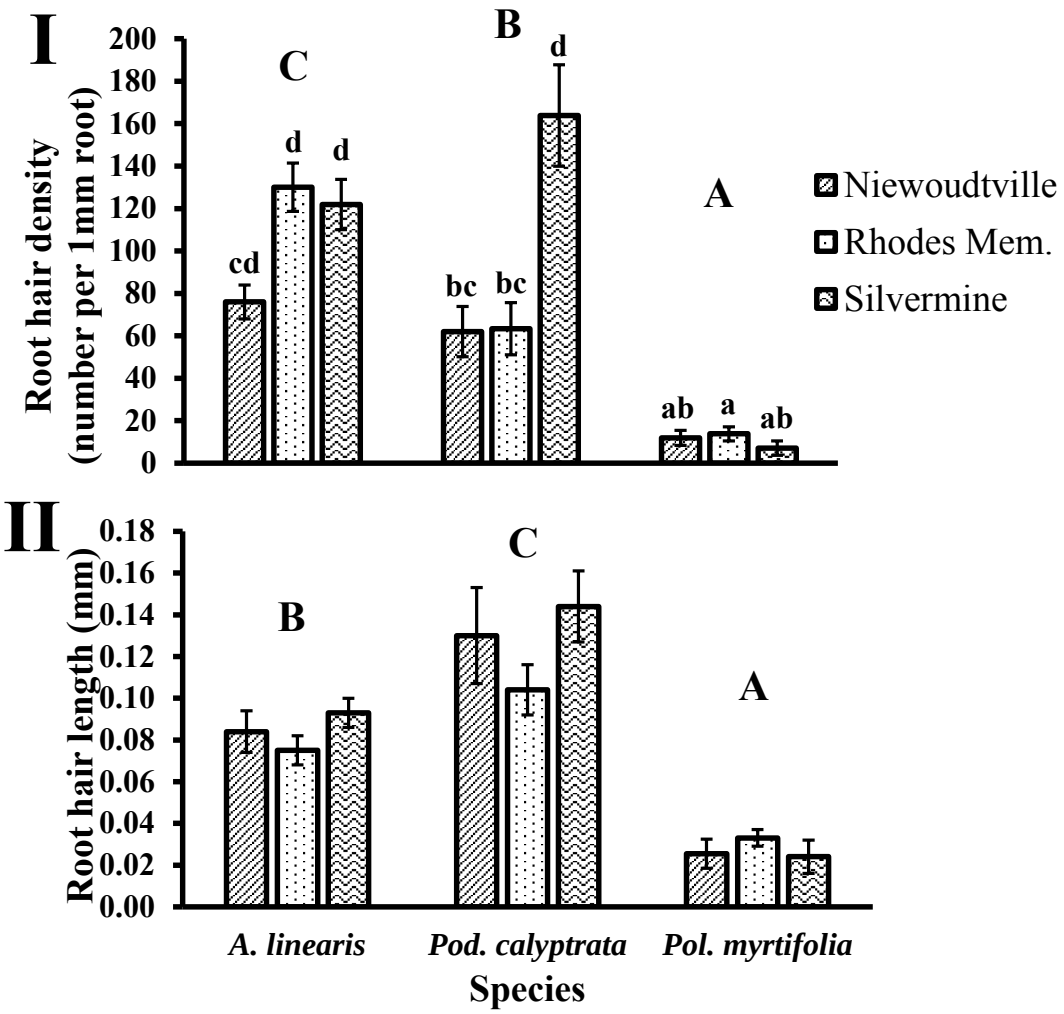

Figure 2.7: Root hair density (I) and root hair length (II) of *A. linearis, Pod. calyptrata* and *Pol. myrtifolia* grown in soil from Niewoudtville, Rhodes Memorial and Silvermine. Upper case letters indicate significant differences between species only and lower case letters indicate significant differences between soils by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

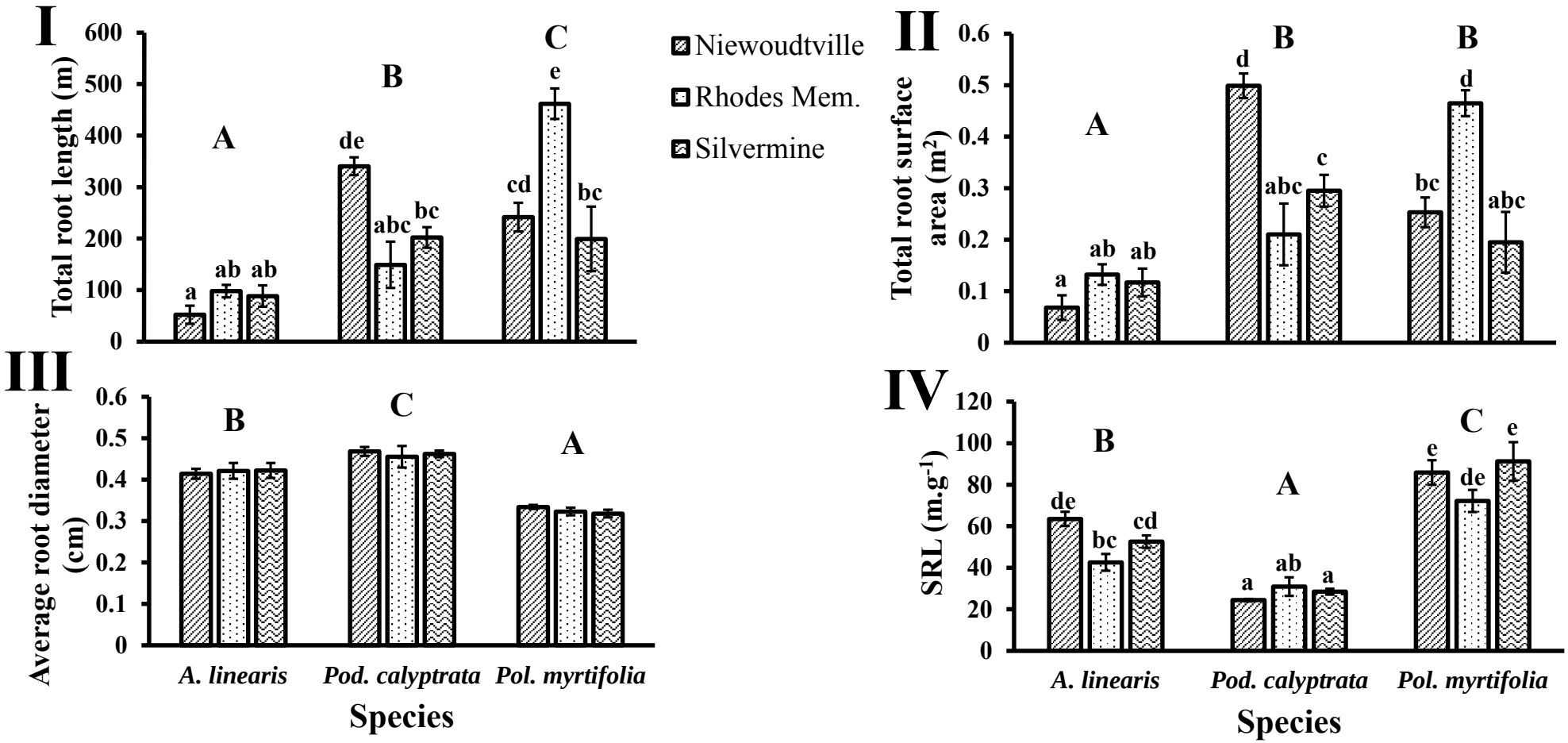

Figure 2.8: Total root length (I), average root diameter (II), total root surface area (III) and specific root length (SRL) (IV) of *A. linearis, Pod. calyptrata* and *Pol. myrtifolia* grown in Niewoudtville, Rhodes Memorial and Silvermine soils. Upper case letters indicate significant differences between species only and lower case letters indicate significant differences between soils by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

2.3.6 Tissue concentrations of N and P

In the glasshouse study, leaf and root P concentrations of *A. linearis* and *Pol. myrtifolia* were similar in that they were both significantly greater in plants grown in soil from Niewoudtville compared to plants grown in Silvermine soils ($p < 0.001$; Table 2.3). *Podalyria calyptrata*, however, showed no differences between soils. Overall, *Pod. calyptrata* had less leaf and root P compared to both *A. linearis* and *Pol. myrtifolia* while *Pol. myrtifolia* had the greatest leaf P. Both *A. linearis* and *Pol. myrtifolia* had similar and greater root P concentrations than that of *Pod. calyptrata* ($p < 0.001$).

There were no differences in leaf and root N concentrations for *Pol. myrtifolia* plants between soils while *A. linearis* had no difference in root N between soils. *A. linearis* had significantly greater leaf N in plants grown in soil from Niewoudtville compared to plants grown in Rhodes Memorial and Silvermine soils. Leaf N was greater in *Pod. calyptrata* plants grown in soil from Rhodes Memorial while leaf N was similar and less in *Pod. calyptrata* plants grown in Niewoudtville and Silvermine soils. Similarly, root N for *Pod. calyptrata* was significantly greater in Rhodes Memorial soils than in Niewoudtville soils ($p < 0.001$; Table 2.3). Overall, *A. linearis* had the greatest leaf N compared to the other two species ($p < 0.001$), while *A. linearis* and *Pod. calyptrata* had similar root N that were both greater than *Pol. myrtifolia* ($p < 0.001$). *Polygala myrtifolia* had the lowest leaf and root N. Both *A. linearis* and *Pol. myrtifolia* had lower leaf N:P ratios in Niewoudtville soils compared to Rhodes Memorial and Silvermine soils ($p < 0.05$) while *Pod. calyptrata* showed no differences between soil types but did have the greatest leaf N:P ratio, among the three species, followed by *A. linearis* and then *Pol. myrtifolia* ($p < 0.001$).

In the field work study, leaf P was highest in *Pol. myrtifolia*, ranging from 2-11 times greater than other species, while the lowest leaf P was observed in *L. laureolum* ($p < 0.001$; Table 2.2), however there were no overall differences in leaf P between soils. Leaf N, however, was greater in plants from Silvermine soils than on Rhodes Memorial soils. *Psoralea pinnata* from Silvermine, had the greatest leaf N with a high of 73.27 mg.g DW^{-1} while the lowest was observed in *Pol. myrtifolia* from Rhodes Memorial ($p < 0.001$; Table 2.2). Leaf N:P ratios were highest plants from Rhodes Memorial ($p = 0.017$) and *Pol. myrtifolia* and *A. macrantha* had the lowest (4.93) and highest (82.23) leaf N:P ratios respectively.

Table 2.3: Tissue concentrations of N and P, N:P ratios and root hair width of *A. linearis*, *Pod. calyptrata* and *Pol. myrtifolia* grown in Niewoudtville, Rhodes Memorial and Silvermine soils. Means and standard error followed by different letters in the columns are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001.

Treatment		Tissue Concentrations					Root Morphology
Species	Soil	Leaf P ($mg.g^{-1}$)	Leaf N ($mg.g^{-1}$)	Leaf N:P ratio	Root P ($mg.g^{-1}$)	Root N ($mg.g^{-1}$)	Root hair width (mm)
A. linearis							
	Niewoudtville	1.11 ± 0.08 cd	24.03 ± 0.65 e	22.06 ± 1.22 c	1.67 ± 0.48 cd	13.42 ± 1.47 cd	0.015 ± 0.001 cd
	Rhodes Mem.	0.77 ± 0.03 bc	20.22 ± 0.38 d	26.55 ± 0.85 cd	0.85 ± 0.11 bc	14.34 ± 0.91 cd	0.012 ± 0.001 bcd
	Silvermine	0.57 ± 0.07 b	19.52 ± 0.83 cd	36.06 ± 2.75 de	0.52 ± 0.11 ab	11.74 ± 1.35 c	0.010 ± 0.000 bc
Pod. calyptrata							
	Niewoudtville	0.35 ± 0.02 a	15.57 ± 0.47 b	45.51 ± 3.54 e	0.40 ± 0.00 a	10.99 ± 0.21 bc	0.016 ± 0.001 d
	Rhodes Mem.	0.54 ± 0.07 ab	23.01 ± 1.90 de	44.37 ± 4.15 e	0.76 ± 0.12 ab	16.76 ± 1.23 d	0.017 ± 0.001 d
	Silvermine	0.35 ± 0.02 a	16.74 ± 0.51 bc	48.60 ± 2.76 e	0.42 ± 0.03 a	13.19 ± 0.31 cd	0.017 ± 0.001 d
Pol. myrtifolia							
	Niewoudtville	1.46 ± 0.29 d	7.50 ± 0.21 a	5.87 ± 0.77 a	1.63 ± 0.12 d	7.26 ± 0.37 a	0.005 ± 0.001 a
	Rhodes Mem.	0.82 ± 0.04 bc	7.46 ± 0.18 a	9.22 ± 0.54 b	0.50 ± 0.04 ab	6.11 ± 0.15 a	0.011 ± 0.001 bc
	Silvermine	0.85 ± 0.10 bcd	8.76 ± 0.37 a	10.92 ± 1.83 b	0.75 ± 0.03 abc	7.90 ± 0.37 ab	0.007 ± 0.002 ab
F ratio		**7.45*** **	**20.07*** **	**3.50****	**13.21*** **	**6.49*** **	**4.20****

2.3.7 *Leucadendron argenteum*

Due to the *L. argenteum* plants being well established (>20 cm) at the time of planting, they were not compared with the other species. Their results will be discussed here.

All the *L. argenteum* plants had cluster roots present before planting into the soils and those that were planted in Silvermine soils struggled to grow, with half of them dying after a few weeks of being planted. This is most likely due to *L. argenteum* naturally occurring on granite and shale derived clay soils which are higher in nutrients (Notten and Walt 2008). A few plants from Niewoudtville (one plant) and Rhodes Memorial (two plants) soils also died out possibly caused by the root rot fungus *Phytophthora*, which *L. argenteum* is particularly susceptible to (Knox *et al.* 1986; Notten and Walt 2008). These plants exhibited leaf chlorosis and necrosis as well as premature leaf fall and eventual plant death and when dug up, had blackened roots. Out of a total of 18 plants, only 12, three in Silvermine, five in Niewoudtville and four in Rhodes Memorial soils, survived and were used in the analyses.

Overall there were no differences in total biomass, root:shoot ratios and cluster root:root ratios for *L. argenteum* between the three soils. Similarly, there were no differences in APase activity between soil types, however, cluster root APase activity differed between soils in that it was significantly higher in Rhodes Memorial soils than it was in Niewoudtville and Silvermine soils. There were also no significant differences in exudation of citric and succinic acids from cluster roots and non-cluster roots. There were no significant differences in leaf P and N, root N, leaf and cluster root N:P ratios between soils. There were differences in cluster root and non-cluster root P, as well as cluster root N between the soils, all of which were greater in plants grown in Rhodes Memorial soils. There were no significant differences in any of the root morphological traits between soils (Table 2.4).

Table 2.4: Biomass, chemical, tissue concentrations and root morphology data of *L. argenteum* grown in Niewoudtville, Rhodes Memorial and Silvermine soils. Means and standard error followed by different letters in the rows are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001. – denotes data not available.

	Variable	Niewoudtville	Rhodes Mem.	Silvermine	F-ratio
Biomass	Total biomass (g)	71.73 ± 2.97 a	81.77 ± 9.25 a	98.93 ± 23.92 a	1.52
	Root:shoot	0.80 ± 0.11 a	0.61 ± 0.09 a	1.26 ± 0.42 a	3.42
	Cluster root:root	0.41 ± 0.21 a	0.90 ± 0.37 a	1.03 ± 0.96 a	0.07
Chemical	Phosphatase activity (µmol p-NP.g root $DW^{-1}.hr^{-1}$)	0.18 ± 0.05 a	0.15 ± 0.03 a	0.37 ± 0.28 a	0.36
	Cluster root phosphatase activity (µmol p-NP.g root $DW^{-1}.hr^{-1}$)	0.03 ± 0.01 a	0.16 ± 0.05 b	0.02 ± 0 a	11.25**
	Citric acid (µmol.g DW^{-1})	291 ± 101.8 a	568 ± 101.2 a	207 ± 14.4 a	3.28
	Cluster root citric acid (µmol.g DW^{-1})	229 ± 113.2 a	486 ± 252.7 a	69 ± 45.4 a	1.26
	Succinic acid (µmol.g DW^{-1})	-	120 ± 31.3 a	88.1 ± 88.1 a	1.65
	Cluster root succinic acid (µmol.g DW^{-1})	16 ± 10.4 a	29 ± 5.1 a	108 ± 100.7 a	1.25
Tissue Concentrations	Leaf P ($mg.g^{-1}$)	0.34 ± 0.02 a	0.33 ± 0.03 a	0.33 ± 0.03 a	0.09
	Root P ($mg.g^{-1}$)	0.42 ± 0.02 ab	0.45 ± 0.05 b	0.30 ± 0 a	4.97*
	Cluster root P ($mg.g^{-1}$)	0.10 ± 0.03 a	0.25 ± 0.03 b	0.07 ± 0.03 a	8.90**
	Leaf N ($mg.g^{-1}$)	5.01 ± 0.31 a	6.04 ± 0.16 a	4.85 ± 0.51 a	3.91
	Root N ($mg.g^{-1}$)	4.54 ± 0.33 a	4.81 ± 0.42 a	4.53 ± 0.47 a	0.16
	Cluster root N ($mg.g^{-1}$)	1.25 ± 0.38 a	3.78 ± 0.76 b	-	16.54**
	Leaf N:P	14.99 ± 1.36 a	18.80 ± 0.93 a	14.72 ± 1.85 a	2.71
	Cluster root N:P	10.27 ± 2.87 a	15.12 ± 2.06 a	-	1.23
Root morphology	Total root length (m)	-	813.43 ± 58.46 a	1362.72 ± 360.94 a	4.69
	Average root diameter (cm)	-	0.31 ± 0.02 a	0.31 ± 0.05 a	0.03
	Total surface area (m^2)	-	0.79 ± 0.08 a	1.26 ± 0.15 a	9.22
	SRL ($m.g^{-1}$)	-	47.85 ± 5.53 a	46.19 ± 7.25 a	0.04
	Root hair abundance	183.75 ± 17.08 a	226.00 ± 23.49 a	-	2.36
	Root hair length (mm)	0.05 ± 0 a	0.06 ± 0 a	-	3.35
	Root hair width (mm)	0.01 ± 0 a	0.01 ± 0 a	-	3.08

2.3.8 Stepwise regression and Phosphorus Uptake Efficiency (PUpE)

A forward stepwise model identified total root surface area (m^2), root hair density, root hair width (mm) and cluster root:root ratio as major significant predictors of total biomass, with the variables yielding R^2=0.92 ($p < 0.001$). Phosphorus Uptake Efficiency (PUpE) differed between species with *A. linearis* showing the lowest PUpE while *Pol. myrtifolia* had the highest, almost three times more than that of *A. linearis* ($p < 0.05$; Figure 2.9).

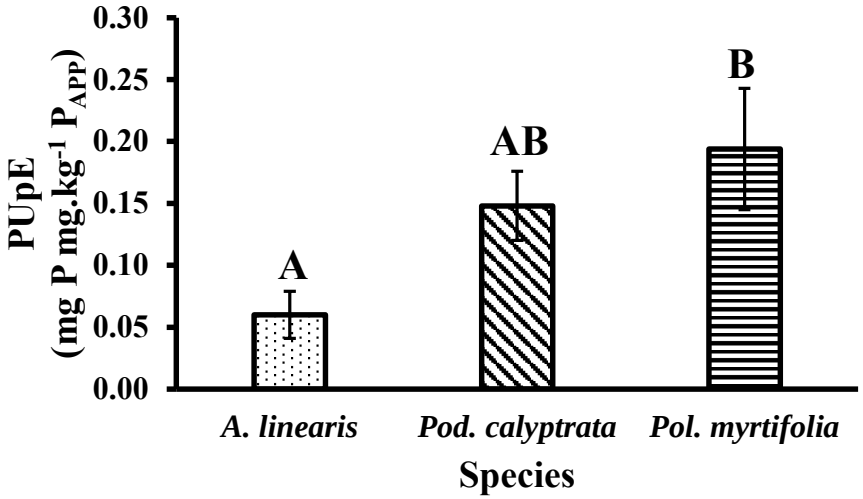

Figure 2.9: Phosphorus Uptake Efficiency of *A. linearis*, *Pod. calyptrata* and *Pol. myrtifolia*. Letters indicate significant differences by Tukey HSD post doc test ($p < 0.05$). Vertical bars denote standard errors.

2.4 Discussion

In contrast to the hypothesis, *Pol. myrtifolia*, both a non-legume and a non-cluster root forming species, recorded the highest PUpE indicating that other root traits besides cluster roots are important for enhancing P uptake in CCR plants. In both the glasshouse study and the field work, leaf P concentrations in *Pol. myrtifolia* were significantly higher than the other species, supporting that *Pol. myrtifolia* is efficient at P-uptake. This view was reinforced by the observations that *Pol. myrtifolia* was superior in the most number of traits for enhanced P-acquisition (total root length, SRL, total root surface area, root:shoot ratio, citric acid exudation), followed by *Pod. calyptrata* (root hair length, average root diameter, total root surface area and root:shoot ratio) and lastly *A. linearis* (root hair abundance, extracellular APase activity and exudation of succinic acid). Moreover, *Pol. myrtifolia* was superior in two important traits (total root length and SRL) that influence root surface area, one of the major significant predictors of total biomass linked with P uptake mechanisms. These results indicate that root morphological features are important drivers of P uptake as they are linked to the increased ability of roots to explore a high volume of soil thereby enhancing the scavenging potential of the roots for P in the soil. Several studies have also shown that the most important factors contributing to a plant's uptake efficiency were root morphological traits such as total root length, root surface area, root diameter, specific root length and root mass ratios (Sanginga *et al.* 2000; Gahoonia and Nielsen 2004; Lynch 2007; Hammond *et al.* 2009; Pang *et al.* 2010a; Fageria *et al.* 2014). In addition studies by Barraclough (1984) and Leon and Schwang (1992) found that total root length was positively correlated to the yield of grains, oats and barley. *Polygala myrtifolia* exhibited the most number of root traits for superior P acquisition, compared to *A. linearis* and *Pod. calyptrata*, which is consistent with its observed occurrence across many different vegetation and climate types across Southern Africa (Walt 2003), making it an excellent habitat generalist plant.

The root exudation of organic acids varied with species and type of organic acids. For instance, in the glasshouse study, *Pol. myrtifolia* was superior in terms of citric acid and exuded about two times more citric acid than *A. linearis* and *Pod. calyptrata,* whereas *A. linearis* was the superior in terms of succinic acid release. In contrast to this, in the field study, *A. macrantha* from Rhodes Memorial exuded more citric acid than any other species followed by *Pol. myrtifolia* on the same soil. It has been well documented that the rates and compositions of organic acids are highly variable and dependent on the plant species, plant age and soil conditions (Curl and Truegolve 1986; Ae *et al.* 1990; Dinkelaker *et al.* 1989,

1995; Hinsinger 2001; Veneklass *et al.* 2003; Nuruzzaman *et al.* 2005). For instance under P deficiency it has been reported that citric acid was the dominant organic acid released in species like white lupin (Johnson *et al.* 1994; Keerthisinghe *et al.* 1998; Neumann and Römheld 1999; Watt and Evans 1999) and alfalfa (Lipton *et al.* 1987) while malic acid was dominant in plants such as maize, wheat, oilseed, rape and tomato (Hoffland 1992; Hoffland *et al.* 1992; Jones and Darrah 1995; Jones 1998; Neumann and Römheld 1999) and oxalic acid was the major organic acid released by sugar beet (Gerke *et al.* 2000). Succinic acid, while not a dominant organic acid, has been found to occur in many species such as alfalfa (Lipton *et al.* 1987), chickpea (Ohwaki and Hirata 1992; Neumann and Römheld 1999) and various other Fabaceae species (Pang *et al.* 2010; Power *et al.* 2010) and Proteaceae species (Shane *et al.* 2004; Power *et al.* 2010). Noteworthy is the observation that the dominant organic acid exuded by the roots of the fynbos species studied here was citrate (86, 85, 80 and 75% for *Pol. myrtifolia, L. argenteum, Pod. calyptrata* and *A. linearis* respectively). Tricarboxylates, such as citrate^{3-}, are reported to be more effective at chelating cations than dicarboxylates such as succinic^{2-}, malate^{2-}, oxalate^{2-}, malonate^{2-} and fumarate^{2-} or monocarboxylates, such as acetate^{-} and lactate^{-} due to their higher affinity for the cations (Hinsinger 2001; Ryan *et al.* 2001) and they can mobilize P from a range of sparingly soluble inorganic P complexes (Jones 1998; Jones *et al.* 2003).

Each species in this study had a unique specialization in P traits which were a mix between morphological and physiological, implying that competition between species may not be very strong in P-limited soils of the CCR. There are two main reasons for the lack of competition in low P soils: the different chemical forms of P within soils allows the co-existence of species if they exploit different P fractions depending on which specific P-acquisition traits they exhibit (Kamh *et al.* 1999) and the competition for a resource that is not highly mobile is weaker than competition for a highly mobile resource (Huston and De Angelis 1994). This allows more species to co-exist in P-limited environments (Güsewell 2004). Similarly, studies by McCrea *et al.* (2004) and Güsewell *et al.* (2005) concluded that higher P concentrations were the main inhibitory reason for the establishment of species-rich assemblages and that P fertilization of areas is unlikely to raise species richness. Therefore plant competition is low in P-limited soils, since P is relatively immobile, plant species acquire their P from different sources and exhibit various and contrasting P acquisition mechanisms which allow for a higher plant diversity in P-limited environments (Janssens *et*

al. 1998; Güsewell 2004; McCrea *et al.* 2004; Güsewell *et al.* 2005; Sardens and Peñuelas 2014).

Cluster roots, which are also significant predicators of total biomass linked with P-uptake mechanisms, were produced by *A. linearis* only in Silvermine soils, which had the lowest concentrations of P. This confirms the view that cluster root formation is a response to P deficiency in the soil (Watt and Evans 1999; Lambers *et al.* 2003; Shane *et al.* 2004). The concentration of cluster root exudates were less than that from the 'normal' roots in this study, a finding that is not in agreement with the reports that cluster roots release large amounts of organic acids into the rhizosphere (Gardner *et al.* 1983; Dinkelaker *et al.* 1995; Neumann *et al.* 2000; Lambers *et al.* 2003, 2006). However, in a study by Shane *et al.* (2004) on the growth and development of cluster roots in *Hakea protstrata* (harsh hakea), cluster roots were found to have lower organic acid exudation than normal roots up until the point of their maturation, where they experience an exudative burst and thereafter their exudation drops down to almost zero. The observation in this study, that cluster roots exuded less than normal roots, could therefore be attributed to the time of collecting organic acids which did not match the exudative bursts associated with cluster roots (Shane *et al.* 2004).

The highest leaf N concentrations were observed in *A. linearis* and *Pod. calyptrata* in Niewoudtville and Rhodes Memorial soils respectively, indicating effective rhizobia in their native habitat soils. In the glasshouse, all four species experienced the same conditions yet the active nodules in *A. linearis* and *Pod. calyptrata* implied an ability to supplement needed N via atmospheric N fixation (Markham and Zekveld 2007) as shown by their greater tissue N concentrations. Similarly, in the field, the highest leaf N concentrations were found in the four legume species most likely owing to their symbiotic relationship with N_2-fixing bacteria which have been reported to lead to higher tissue N concentrations (Gebauer *et al*. 1988; Del Pozo *et al*. 2000; Tjoelker *et al*. 2005). Higher leaf N concentrations are associated with rapid-growth strategies (Wright *et al*. 2004) therefore, higher leaf N concentrations in legumes could facilitate a more rapid growth rate compared to other species in the area but a shorter life span which could explain their lack of persistence in mature fynbos stands (Power *et al.* 2010). In contrast to this, lower leaf N concentrations are associated with long leaf lifespans and nutrient conservation (Eckstein *et al*. 1999) as seen in *Pol. myrtifolia* which had the lowest leaf N concentrations across soils. Similarly, *A. linearis* and *Pod. calyptrata* had the highest root N concentrations overall while *Pol. myrtifolia* had the lowest, implying

increased longevity as shown in other studies (Eissenstat *et al.* 2000; Tjoelker *et al.* 2005). However, root life spans are highly variable, ranging from a few weeks to a few years (Eissenstat and Yanai 1997) and can vary within individual species (Eissenstat *et al.* 2000). Therefore in both leaves and roots, increased N concentrations were associated with higher rates of metabolic activities and a decline in tissue longevity. This reflects a trade-off between the rapid acquisition of resources and conservation of resources within tissues (Díaz *et al.* 2004).

The high APase activities in the two legumes, *A. linearis* and *Pod. calyptrata,* relative to *Pol. myrtifolia* and *L. argenteum*, is in agreement with several studies that show that N_2-fixing plants have significantly higher phosphatase activity in their rhizosphere compared to non-fixing plants (Zou *et al.* 1995; Houlton *et al.* 2008; Lv *et al.* 2013; Maseko and Dakora 2013). The addition of nitrogen greatly increases the production of APases (Zou *et al.* 1995; Treseder and Vitousek 2001) due to their large nitrogen requirement. The ability of the legume species to secrete higher quantities of APase compared to the non-legumes in the same soils, could allow them to access forms of organic P in the soil. However, Hayes *et al.* (1999) indicated that APase does not adequately act on all potential organic P substrates which are present in soil, particularly phytate which is a major component of soil organic P. The higher density of root hairs in the two legumes, *A. linearis* and *Pod. calyptrata* could also have facilitated the higher APase activity due to root hairs assisting in the release of APase (Gahoonia *et al.* 2001).

Plant growth is assumed to increase in soils that have higher soil nutrient concentrations (Lynch and Brown 2001; Kuang *et al.* 2005; Li *et al.* 2011). However, in this study, plant growth varied with species and soil types. For instance, biomass accumulation in *A. linearis* was similar for the three sites, *Pod. calyptrata* biomass was greatest in soil from Niewoudtville and *Pol. myrtifolia* was highest in soil from Rhodes Memorial. The better than expected performance of plants in soil from Silvermine could be associated with the adaptation of fynbos plants to growing in infertile soils but the mechanism for this adaptation is not yet fully understood. However, Maistry *et al.* (2013, 2015a) indicated that a balanced allocation of resources to processes, that are important at that point in time, enables plants to grow relatively well under low nutrient conditions. Noteworthy points are the growth of the plants in soils from areas where they do not naturally grow and the similar levels of nodulation of the two legumes in all three soils (Figure 2.4). These observations suggest that

soil chemical characteristics, per say, are not the most limiting factors for plant growth in those areas and that the rhizobia nodulating *A. linearis* and *Pod. calyptrata* are present throughout the CCR, consistent with the report by Lemaire *et al.* (2015, in press).
While *Pod. calyptrata* showed no differences in leaf P concentrations nor leaf N:P ratios between soils, both *A. linearis* and *Pol. myrtifolia* had higher leaf P concentrations when grown in Niewoudtville soils correlating to the higher P concentrations in these soils. Similar to other studies where plants exhibited lower N:P ratios in higher P soils (Aerts and Chapin 2000; Tessier and Raynal 2003; Güsewell 2004), both *A. linearis* and *Pol. myrtifolia* had lower leaf N:P ratios when grown in Niewoudtville soils indicating that both species were N-limited in their growth on this soil. Güsewell (2004) considered an N:P ratio in the range of 10-20, on a mass basis, to be optimal for plants, suggesting that plants need to absorb about 10 to 20 times more N than P to promote plant growth.

The field work results show an overall positive relationship with APase activity and soil P status, where the APase activity was generally higher in plants from Rhodes Memorial, which had higher P in the soils, and lower in plants from Silvermine, which had lower P in the soils, correlating with numerous studies (Juma and Tabatabai 1978; Spiers and McGill 1979; Sinsabaugh *et al*. 1993; Tadano *et al*. 1993) that support the idea that phosphatase production and activity are linked to substrate availability (Clarholm 1993). However, there are studies that, in contrast, have found little or no evidence for this link (Harrison 1983; Speir and Cowling 1991; Adams 1992) including the glasshouse study where APase activity was similar between soils but varied for species therefore indicating that phosphatase secretion is not influenced just by the available phosphate in the soil but also by genotype.

Organic acid exudation and SRL in plants did not differ across soil types which is in contrast to other studies that have found that plants were able to distinguish differences in the chemistry of their surrounding environment and adapt by exuding different carboxylates in response (Lambers *et al.* 2002; Veneklass *et al.* 2003) as well as increasing their SRL with decreasing P (Makita *et al.* 2009; Pang *et al.* 2010a; Fageria *et al.* 2014).

2.4.1 Conclusion

The differences between the rooting systems, both morphological and physiological, of *Pol. myrtifolia, Pod. calyptrata* and *A. linearis* were quite evident. It was observed that *Pol. myrtifolia* was superior in the most number of traits for enhanced P-acquisition (root:shoot

ratio, citric acid exudation, total root length, total root surface area and SRL), followed by *Pod. calyptrata* (root:shoot ratio, root hair length, average root diameter and total root surface area) and lastly *A. linearis* (extracellular APase activity, exudation of succinic acid and root hair abundance). These results were consistent with the calculated species PUpE where *Pol. myrtifolia* was found to be the most efficient with regards to phosphorus uptake relative to the other species. The superiority of *Pol. myrtifolia* could be associated with its wide distribution and persistence in mature fynbos across the CCR (Walt 2003). Soils influenced certain traits in certain species (root:shoot ratio, total root length, total root surface area, root hair density and root hair width) while some traits were not different between soils (organic acid exudation, APase activity, SRL, average root diameter and root hair length) which is consistent with a number of studies (Ae *et al.* 1990; Cowling *et al.* 1994; Lambers *et al.* 2002; Yan *et al.* 2002; Gahoonia and Nielsen 2003; Lamont 2003; Linder 2003; Veneklaas *et al.* 2003; Orians and Milewski 2007; Lambers *et al.* 2008). Furthermore, each species exhibited specializations in different P acquisition mechanisms which supports the view of co-existence of different species in the fynbos biome through a diversity of nutrient use and acquisition mechanisms (Sardans and Peñuelas 2014).

Chapter 3

Phosphorus efficiency of cluster root and non-cluster root species from the Core Cape Subregion

3.1 Introduction

The ability of plants to acquire and utilize nutrients for maximum yield differs between species and can be evaluated by measuring species nutrient use efficiency (NUE). Nutrient use efficiency is based on root and shoot parameters, such as influx rate of nutrients from soil, transport to leaves and remobilization of nutrients within a plant, which influence uptake and incorporation in plants (Baligar *et al.* 2001; Vandamme *et al.* 2013). In particular, phosphorus use efficiency (PUE) is the ability of plants to function well under low P availability in the soils (Shenoy and Kalagudi 2005) in that they are able to utilize P more efficiently in the shoot for the production of dry matter (Blair 1993; Korkmaz *et al.* 2009). There are several measures of PUE that are reported in literature (Hammond *et al.* 2009) and used in this study (Table 3.1). The following measures of PUE are commonly reported: agronomic P use efficiency (APE) which is the increase in plant yield per unit of P in the soil, P uptake efficiency (PUpE) which is the product of the increase in plant P content per unit of added P fertilizer, P utilization efficiency (PUtE) which is the increase in plant yield per unit increase in plant P content, P efficiency ratio (PER) which is the plant's yield divided by its P concentration, and physiological P use efficiency (PPUE) which is plant yield divided by tissue P concentration at a given P concentration in the rooting medium. Agronomic use efficiency is the product of PUpE and PUtE and for this reason genetic strategies to increase the yield of plants on low P soils have focused on improving P acquisition by roots and P utilization efficiency in plant tissues (White and Hammond 2008). Nutrient utilization and variations within and between species are known to be controlled genetically and physiologically, however, it can also be modified by plant interactions with environmental variables (Baligar *et al.* 2001). To understand plants' PUE requires studying traits within plants that enhance the uptake and utilization of P (Narang *et al.* 2000).

3.1.1 P acquisition and root adaptations

As has been previously established (Chapter 2), plant species have developed numerous adaptations and mechanisms for the acquisition of P, which included enhanced expression of acid phosphatase (APase), exudation of organic acids, higher root:shoot ratios, longer and thinner roots, higher specific root length (SRL), higher root hair density and the production of cluster roots. However, plants that produce cluster roots are associated with better capabilities to take up P from sparingly soluble P sources (Skene 1998; Pate *et al.* 2001; Vance *et al.* 2003; Lambers *et al.* 2006, 2008).

Of great significance to plant nutrition is the release of organic anions and phosphatase enzymes from the roots of plants (Marschner *et al.* 1986; Hocking 2001; Ryan *et al.* 2001; Richardson *et al.* 2005). There is limited information on intraspecific variation in organic acid exudation in legumes with the exception of studies on *Lupinus albus* (white lupin; Pearse *et al.* 2008) and *Cajanus cajan* (pigeon pea; Subbarao *et al.* 1997; Ishikawa *et al.* 2002) that have shown that these species had differences in organic acid exudation and a relative capacity to access different forms of mineral P. The difficulty lies in establishing whether it is worthwhile to pursue these genotypes with increased organic acid exudation and whether these differences can be exploited commercially to enhance the uptake of P for all plants (White and Hammond 2008). Significant genotypic variation across and within species has been found with regards to extracellular phosphatase activities of plant roots (Tadano *et al.* 1993; Asmar 1997; Li *et al.* 1997; Gaume *et al.* 2001; George *et al.* 2008) and as such it has been proposed that variation in root phosphatase activity could be useful in determining genotypes for greater utilization of organic soil P (Asmar *et al.* 1995).

3.1.2 Cluster roots

From the time of their first observation, by Engler (1894), cluster roots have since become widely studied and regarded as one of the major adaptations plants have to nutrient acquisition along with mycorrhizae and N_2-fixing nodules (Skene 1998; Vance *et al.* 2003; Lambers *et al.* 2006). Since being described by Purnell (1960) in the Proteaceae, cluster roots have now been found to occur in almost all 1800 species of Proteaceae with the basal genus, *Persoonia*, being the only exception (Lamont 1982). Cluster roots are densely packed, short rootlets that are produced off the parent root and have the appearance of a bottle-brush (Purnell 1960). At maturity, cluster roots produce a superabundance of root hairs which results in an over 100-fold increase in surface area compared to normal roots (Raven and

Sprent 1993; Vance *et al.* 2003). Coupled with this increase in surface area, cluster roots can chemically modify their surroundings by exuding carboxylate organic anions, APases, phenolics, mucilages and water which facilitate the mobilization of nutrients in the soil (Gardner *et al.* 1982; Reddell *et al.* 1997; Watt and Evans 1999, 2003). Although, cluster roots can only explore a small volume of soil (Pate and Watt 2001), their ability to concentrate root exudates in localized patches enable them to more effectively mobilize sparingly soluble inorganic P (Gardner *et al.* 1983; Reddell *et al.* 1997) than non-cluster roots. Exudation events from cluster roots occur as an 'exudative burst' where, over a course of two to three days, they exude large amounts of organic acids (Gardner *et al*. 1983; Dinkelaker *et al.* 1989, 1995; Johnson *et al*. 1996a, b; Skene *et al.* 1996). Cluster roots have also shown increased P absorption rates compared to normal roots on a dry weight and per unit area root basis (Vorster and Jooste 1986).

Similar structures, clusters of swollen, short, lateral roots, occur in species of Cyperaceae and Restionaceae (Davies *et al*. 1973; Lamont 1974). These root clusters were first described by Russian scientists Selivanov and Utemova (1969) and later named 'dauciform roots' due to their carrot-shape (Lamont 1974). Dauciform roots do not make a formation of dense clusters of short rootlets, instead the dauciform root is covered with a dense layer of long root hairs approximately 2mm long and they can occur in groupings of 20-30 individuals (Lamont 1974). Dauciform roots in Cyperaceae are similar to cluster roots in that they also release carboxylates (citrate) in large quantities during an exudative burst (Playsted *et al*. 2006; Shane *et al.* 2006) and their development is suppressed when there are higher levels of P in the environment (Lambers *et al.* 2006).

3.1.3 Problem statement and objective

The soils in the CCR are highly weathered and nutrient-leached resulting in extremely low nutrient concentrations (especially N and P) which are mostly unavailable to plants either due to low content in the parent substrate or due to being fixed by cations in the soils (Witkowski and Mitchell 1987). Plants living in these depleted soils have to be able to acquire and utilize P more efficiently (Lambers *et al.* 2006; Lambers and Shane 2007). Plants in the Fynbos have evolved adaptations to these low P conditions (Lambers and Shane 2007) therefore making them the perfect plants to study with regards to efficiency of uptake and use of P. Cluster roots are long thought of as a superior P acquisition trait therefore plants that produce cluster roots would be able to better access the sparingly soluble P in the soils (Skene 1998; Vance *et*

al. 2003; Lambers *et al.* 2006; Lambers *et al.* 2008). The aim of this chapter was to compare the P-uptake and use efficiency in cluster root and non-cluster root forming Fynbos species under glasshouse conditions. It is hypothesized that the cluster root forming species, *Aspalathus linearis, Leucadendron salignum* and *Leucadendron coniferum* would be superior in PUE.

3.2 Materials and Methods

3.2.1 Species and growth conditions

Seven fynbos species were grown in a glasshouse experiment, consisting of two Fabaceae species, with a cluster root forming species; *Aspalathus linearis* (Burm. f.) R. Dahlgreen and non-cluster root forming species; *Podalyria calyptrata* (Retz.) Willd., two cluster root forming Proteaceae species; *Leucadendron coniferum* (L.) Meisn. and *Leucadendron salignum* P.J. Bergius, and non-cluster root forming species from Polygalaceae; *Polygala myrtifolia* L., Cyperaceae; *Ficinia trispicata* (L.f.) Druce and Juncaceae; *Juncus kraussii* Hochst. This experiment was aimed at observing the growth of plants and phosphorus acquisition to two concentrations of phosphorus, 10 and 25 $mg.kg^{-1}$ P, in a potted sand experiment. These concentrations were established as low and high P treatments respectively, from preliminary experiments. Plants of *A. linearis* and *Pod. calyptrata* were grown from seed (Silverhill Seeds and Books, Cape Town, South Africa). Seeds of *A. linearis* were mechanically scarified while seeds of *Pod. calyptrata* were soaked in boiled water over night to break dormancy before planting. The five non-legume species were bought as seedlings from nurseries; *F. trispicata* and *J. kraussii* from New Plant Nursery (George, South Africa), *Pol. myrtifolia* and *L. coniferum* from Veld and Fynbos Propagation Nursery (Malmesbury, Cape Town, South Africa) and *L. salignum* from Good Hope Gardens Nursery (Cape Point, Cape Town, South Africa). Both *L. salignum* and *L. coniferum* had a few very young cluster roots present at transplanting.

Sand was acid washed and left over a few days to dry completely. An amount of 24 kgs of sand was weighed out and loaded into the cement mixer along with either 1.23 g Fe-P and 0.41 g Ca-P for the low P treatment, or 3.08 g of Fe-P and 1.03 g of Ca-P for the high P treatment, which were supplied in the form of ferric phosphate, $FePO_4$, and hydroxyapatite, $Ca_5 (PO_4)_3(OH)$, mixed at a ratio of 3:1. The sand and phosphorus was mixed for 20 minutes in the cement mixer and thereafter 3 kg of the sand mixture was put into 18 cm pots for the

plants to be transplanted into. Two seedlings of each species were planted into eight replicated pots and after a month of growth, the plants were thinned down to one per pot. Plants were watered twice a week with an University of Western Australia (UWA) nutrient solution, which contained 321 mM $Ca(NO_3)_2$, 200 mM K_2SO_4, 108 mM $MgSO_4$, 0.8 mM $MnSO_4$, 0.34 mM $ZnSO_4$, 0.06 mM $CuSO_4$, 8 mM H_3BO_3, 0.1 mM Na_2MoO_4 and 16.2 mM FeEDTA. Pots were arranged randomly on trolleys in a well-ventilated glasshouse situated at the University of Cape Town (S 33° 57.353'; E 018° 27.742'). Trolleys were re-arranged every two weeks and plants were left to grow for seven months, from April to October 2012, at an average temperature of 14.5°C and a range of 6-25°C.

3.2.2 Plant biomass

At the time of harvest, plants were separated into leaves, stem, roots, nodules and cluster roots (if present). Root samples were taken as explained below for extracellular root phosphatase activity and organic acid exudation analysis and the remainder of the biomass was weighed and oven dried at 60°C for three days. After drying, the organs were re-weighed and milled using a Hammer Mill (United Scientific Pty Ltd, South Africa) for nutrient analysis.

3.2.3 Extracellular acid phosphatase activity

Root biomass between 300-600 mg was collected fresh at harvesting, weighed for fresh weight and put in vials and on ice until further assay for phosphatase activity. The method of assay is described in Tabatabai and Bremner (1969) and modified by Hedley *et al.* (1982). The assay involved colourimetric estimation of the p-nitrophenol released by phosphatase activity after the root sample was incubated with 4 ml of MES 15 mM/ $CaCl_2$ 500 µm buffer (pH 5.5) at 28°C for 30 minutes. The reaction was stopped with 0.5 M NaOH and the absorbance of the solution was measured spectrophotometrically at 412 nm. The detection limit for absorbance was < 0.001nm. One unit of acid phosphatase activity was defined as the activity per gram of root which produced 1 µmol p-nitrophenol per hour. The roots were recovered and then oven dried at 60°C for three days and re-weighed for dry weight.

3.2.4 Organic acid exudation

Root samples between 300-600 mg were collected and transferred to vials containing 15 ml of 200 µM $CaCl_2$ to ensure cell integrity (Pearse *et al.* 2006) for determination of root exudation of organic acids. The samples were gently shaken on an orbital shaker (Laboratory

Marketing Services, Roodepoort, South Africa) at 160 rpm for one hour to collect organic acid exudates. The solutions from the vials were then filtered through filter paper and stored at -20°C while the root material was recovered and oven dried at 60°C for three days and re-weighed for dry weight. The filtrates were filtered again through 20 µm syringe filters, freeze dried and then re-suspended in 1 ml of sterilized water. The samples were analysed at the Central Analytical Facility of Stellenbosch University (Stellenbosch, South Africa) by means on enzymatic test kits in an Arena 20XT Enzyme Robot (Thermo Electron, Oy, Finland). The concentrations of acetic, lactic and malic acids were determined photometrically by measuring the increase in absorbance at 340 nm (Enzytec^{TM} Fluid enzyme kits, Thermo Fisher Scientific, Oy, Finland). The concentrations of citric and succinic acids were determined by the decrease in absorbance associated with the oxidation of NADH using Yellow line enzyme kits (Roche, R-Biopharm AG, Darmstadt, Germany). Detection limits for the organic acids were as follows; < 0.003 $g.l^{-1}$ for acetic acid, <0.0005 $g.l^{-1}$ for citric acid, < 0.0021 $g.l^{-1}$ for lactic acid, < 0.0025 $g.l^{-1}$ for malic acid and < 0.00015 $g.l^{-1}$ for succinic acid.

3.2.5 Nitrogen and phosphorus analysis in plant tissue

Milled leaf and root samples were sent to the Plant Sciences Laboratory, Department of Agriculture Western Cape (Elsenburg, Stellenbosch, South Africa) for analysis of phosphorus concentration using the dry ashing method (ALASA 1998). Concentration of nitrogen in leaf and root samples were measured at the Archaeology Department, University of Cape Town using mass spectrometry. The samples were weighed to 2.5 – 2.7 µg on a Sartorius (AG, Göttingen, Germany) micro balance. Samples were then combusted in a Flash EA 1112 series elemental analyser (Thermo Electron, Milan, Italy). The gases were passed to a Delta Plus XP isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) via a Conflo III gas control unit (Thermo Finnigan, Bremen, Germany) for the analysis of nitrogen concentration.

3.2.6 Phosphorus Use Efficiencies

Equations for the calculation of phosphorus use efficiency were adapted from Hammond *et al*. (2009) and are summarized below in Table 3.1.

Table 3.1: Phosphorus use efficiency calculations reproduced from Hammond *et al.* (2009) where Y_{HIGH} and Y_{LOW} are plant biomass on high and low P treatments respectively; P_{HIGH} and P_{LOW} are tissue P concentrations on high and low P treatments respectively; ΔP_{APP} is the difference in amount of P applied between high and low treatments; P_{APP} is applied P; DW is dry weight.

Name	Abbreviation	Calculation	Units
Agronomic P use efficiency	APE	$\frac{(Y_{HIGH} - Y_{LOW})}{\Delta P_{APP}}$	g DW kg^{-1} P_{APP}
P uptake efficiency	PUpE	$\frac{(P_{HIGH} \times Y_{HIGH}) - (P_{LOW} \times Y_{LOW})}{\Delta P_{APP}}$	mg P kg^{-1} P_{APP}
P utilization efficiency	PUtE	$\frac{Y_{HIGH} - Y_{LOW}}{(P_{HIGH} \times Y_{HIGH}) - (P_{LOW} \times Y_{LOW})}$	g DW mg^{-1} P
Physiological P use efficiency	PPUE	$\frac{Y_{LOW}}{P_{LOW}}$	g^2 DW mg^{-1} P
P efficiency ratio	PER	$\frac{Y_{LOW}}{P_{LOW} \times Y_{LOW}}$	g DW g^{-1} P

3.2.7 Statistical analysis

Variables were log transformed where necessary before analysis using Statistica 12 (StatSoft, Inc., Tulsa, U.S.A.). A two-way factorial ANOVA was used to determine the interaction between species and P treatments for total plant biomass and tissue nutrient concentrations, and one-way ANOVA was used on all other factors to test for significant differences between species. Where significance was found, a Tukey HSD test was performed to establish homogenous groups. A forward stepwise regression was used to determine which variables; root:shoot ratios, total cluster root (g), cluster root:root ratios, APase activity (µmol p-NP. g root DW^{-1} hr^{-1}), citric or succinic acid exudation (µmol. g root DW^{-1}) contributed significantly towards the biomass of plants.

3.3 Results

The phosphorus acquisition mechanisms, root: shoot ratio, APase activity and organic acid exudation had no significant differences between the two P concentrations and therefore only the low-P treatment results were presented while the high-P results were collated into Table 3.4 in Appendix 3.1. Biomass and nutrient concentrations were significantly different

between species and P treatments and the results for both treatments were presented. All plants grew well and healthy in both P treatments.

3.3.1 Biomass accumulation and allocation

Biomass ranged from 0.75 g to 27.33 g with *A. linearis* producing the least and *J. kraussii* producing the most biomass respectively. Among the seven species; *A. linearis, Pod. calyptrata, Pol. myrtifolia* and *L. coniferum* showed significant differences between P treatments for biomass ($p < 0.001$) where they all produced more biomass under higher P (Figure 3.1.I). However, plants of *L. salignum, F. trispicata* and *J. kraussii* exhibited no differences between P treatments in their biomass production. There were differences in root:shoot ratios between species. Root:shoot ratios ranged from 0.33 to 2.18 with *F. trispicata* and *L. salignum* having the lowest and highest respectively ($p < 0.001$; Figure 3.1.II). The legume species: *A. linearis* and *Pod. calyptrata,* nodulated with *Pod. calyptrata* recording significantly higher nodule biomass than *A. linearis* ($p < 0.001$; Figure 3.2.I). However, there were no significant differences in nodule: root ratios between the two species ($p > 0.05$; Figure 3.2.II). The cluster root forming species, *A. linearis, L. salignum* and *L. coniferum* produced cluster roots during the experiment (Figure 3.3) but while *A. linearis* produced cluster roots under low P conditions only, *L. salignum* and *L. coniferum* retained and produced cluster roots at both low and high P conditions. Cluster root biomass ranged from 0.03 g to 2.26 g with *A. linearis* producing the least and *L. salignum* producing the most ($p < 0.001$; Figure 3.2.III). Both *L. coniferum* and *L. salignum* had similar and higher cluster root:root ratios compared to that of *A. linearis* ($p < 0.001$; Figure 3.2.IV).

3.3.2 Acid phosphatase activity

The activity of APase differed between species ($p < 0.001$; Figure 3.4.I) and ranged from 0.06 to 9.53 μmol p-NP.g root DW^{-1} with *J. kraussii* showing the least activity and *A. linearis* with the most activity respectively. Acid phosphatase activity differed between cluster and non-cluster roots of *L. coniferum* and *L. salignum* (Figure 3.4.II) where *L. salignum*'s cluster roots and *L. coniferum's* non-cluster roots had similar and higher APase activity while *L. salignum*'s non-cluster roots and the cluster roots of *L. coniferum* both had the lowest APase activity ($p < 0.001$; Figure 3.4.II).

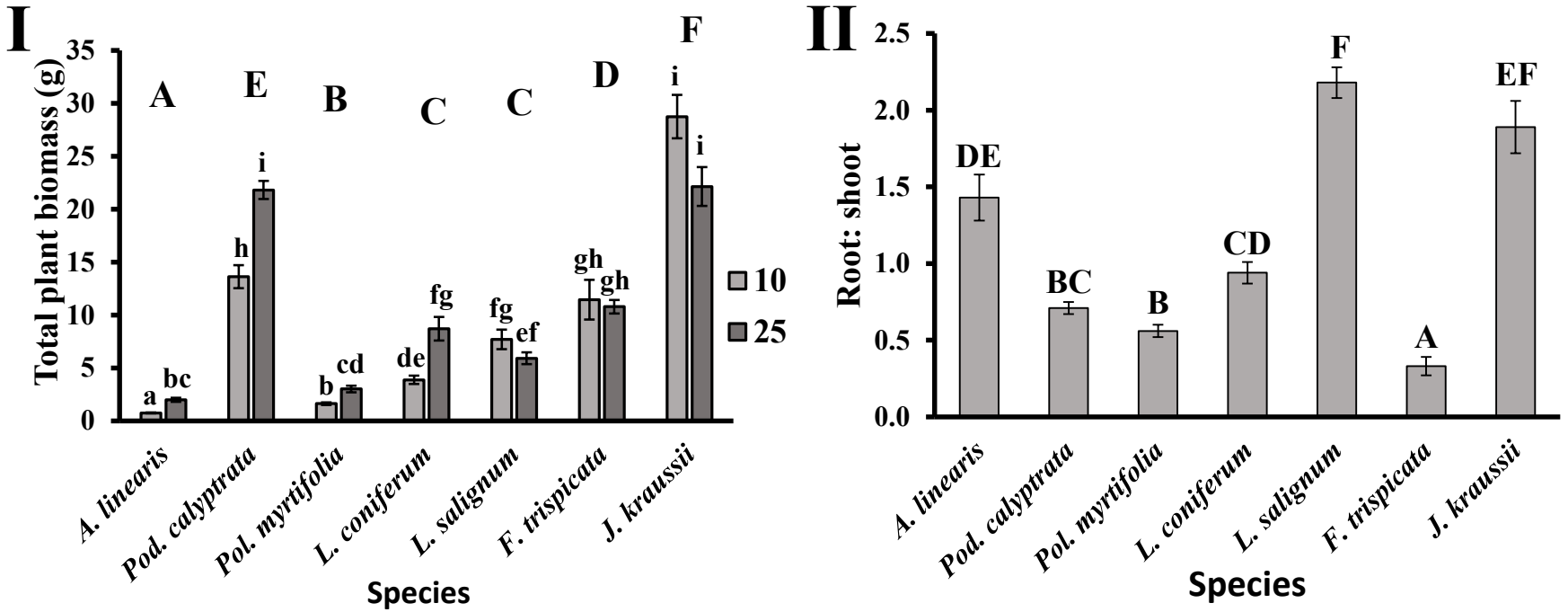

Figure 3.1: Total plant biomass (I) and root:shoot ratio at 10 mg P kg^{-1} sand (II) of the seven species grown in a potted sand experiment. Upper case letters indicate significant differences between species and lower case letters indicate significant differences between P treatments by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard error.

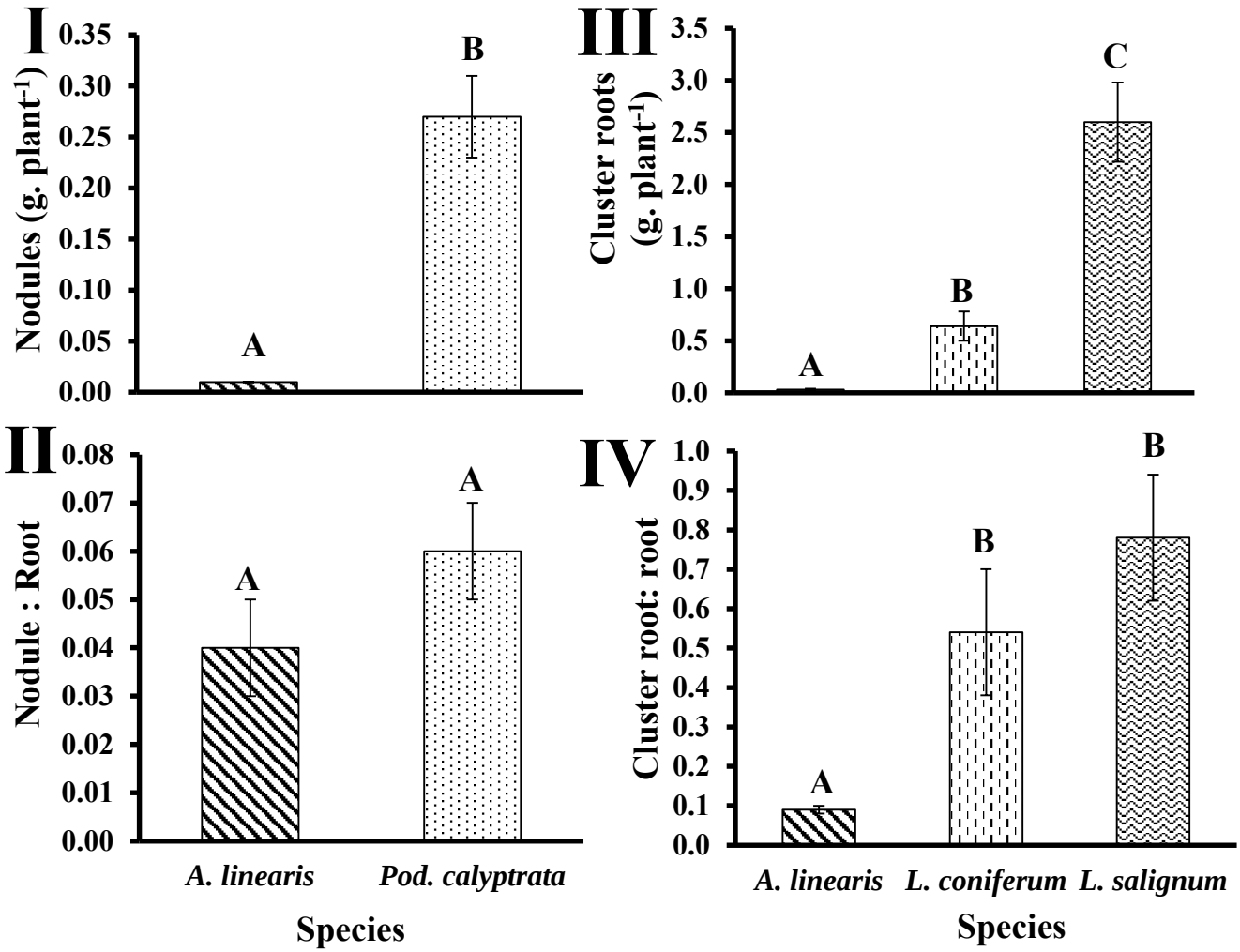

Figure 3.2: Nodules per plant (I) and nodule: root ratio (II) in *A. linearis* and *Pod. calyptrata* grown at 10 mg P kg^{-1} sand. Cluster root biomass (III) and cluster root:root ratio (IV) for *A. linearis, L. coniferum* and *L. salignum* grown at 10 mg P kg^{-1} sand. Letters indicate significant differences between species by Tukey's HSD post hoc test ($p < 0.05$). Vertical bars denote standard error.

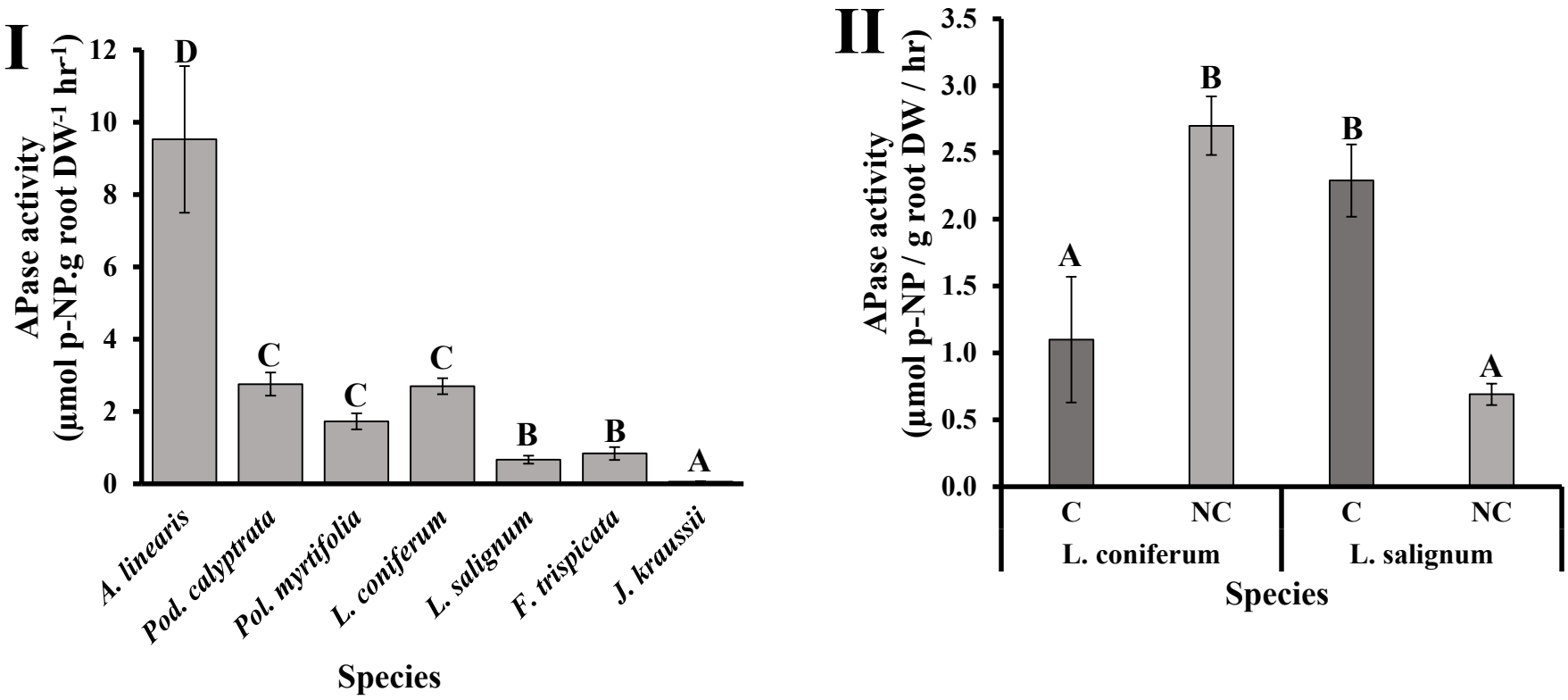

Figure 3.4: Acid phosphatase (APase) activity in the roots of the seven species (I) and in cluster (C) and non-cluster (NC) roots of *L. coniferum* and *L. salignum* (II) grown at 10 mg P kg^{-1} sand. Letters indicate significant differences between species by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

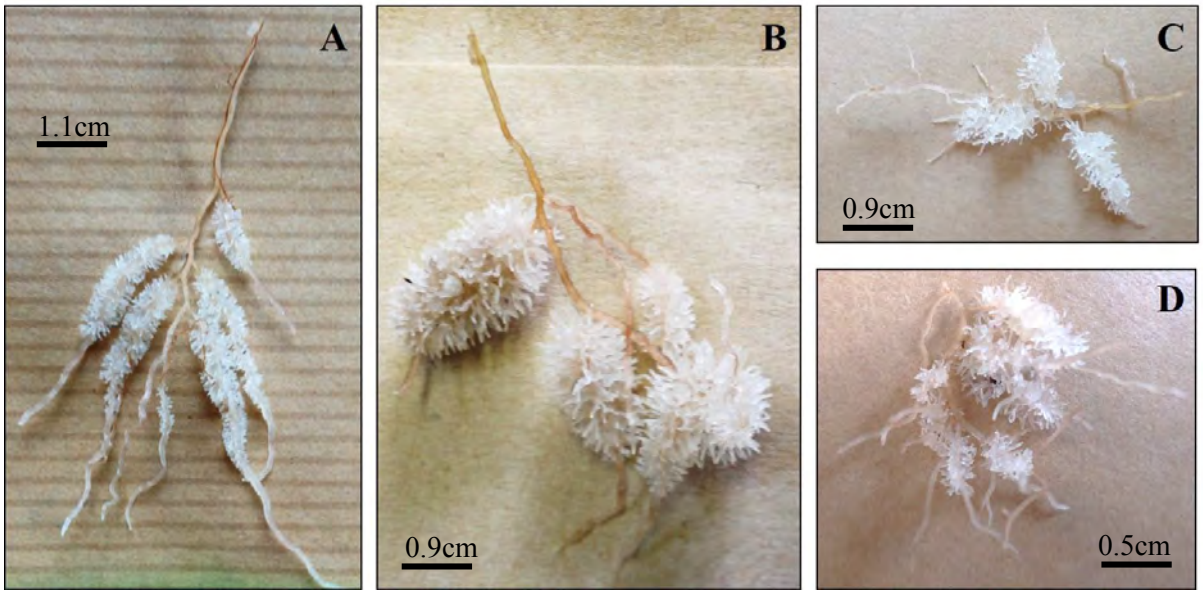

Figure 3.3: Cluster roots of *Leucadendron salignum* (A - B) and *Leucadendron coniferum* (C - D) grown at 10 mg P kg^{-1} sand.

3.3.3 Organic acid exudation

Exudation of organic acids varied greatly between species ranging from 151 to 5358 µmol.g root DW^{-1} and 16 to 792 µmol.g root DW^{-1} for citric and succinic acid respectively ($p < 0.001$; Figure 3.5.I and II). *Polygala myrtifolia* had the highest citric acid exudation along with *Pod. calyptrata*, while *L. coniferum, F. trispicata* and *J. kraussii* had similar and lowest citric acid exudation ($p < 0.001$; Figure 3.5.I). *Aspalathus linearis, Pod. calyptrata, Pol. myrtifolia* and *L. coniferum* all had similar and greater succinic acid than *L. salignum, F. trispicata* and *J. kraussii* ($p < 0.001$; Figure 3.5.II). Overall, plants exuded almost four times more citric acid than succinic acid ($p < 0.001$; Figure 3.6.I). Non-cluster roots exuded ten times more citric acid than cluster roots ($p = 0.024$; Figure 3.6.II) and exuded seven times more organic acids than cluster roots overall ($p < 0.001$).

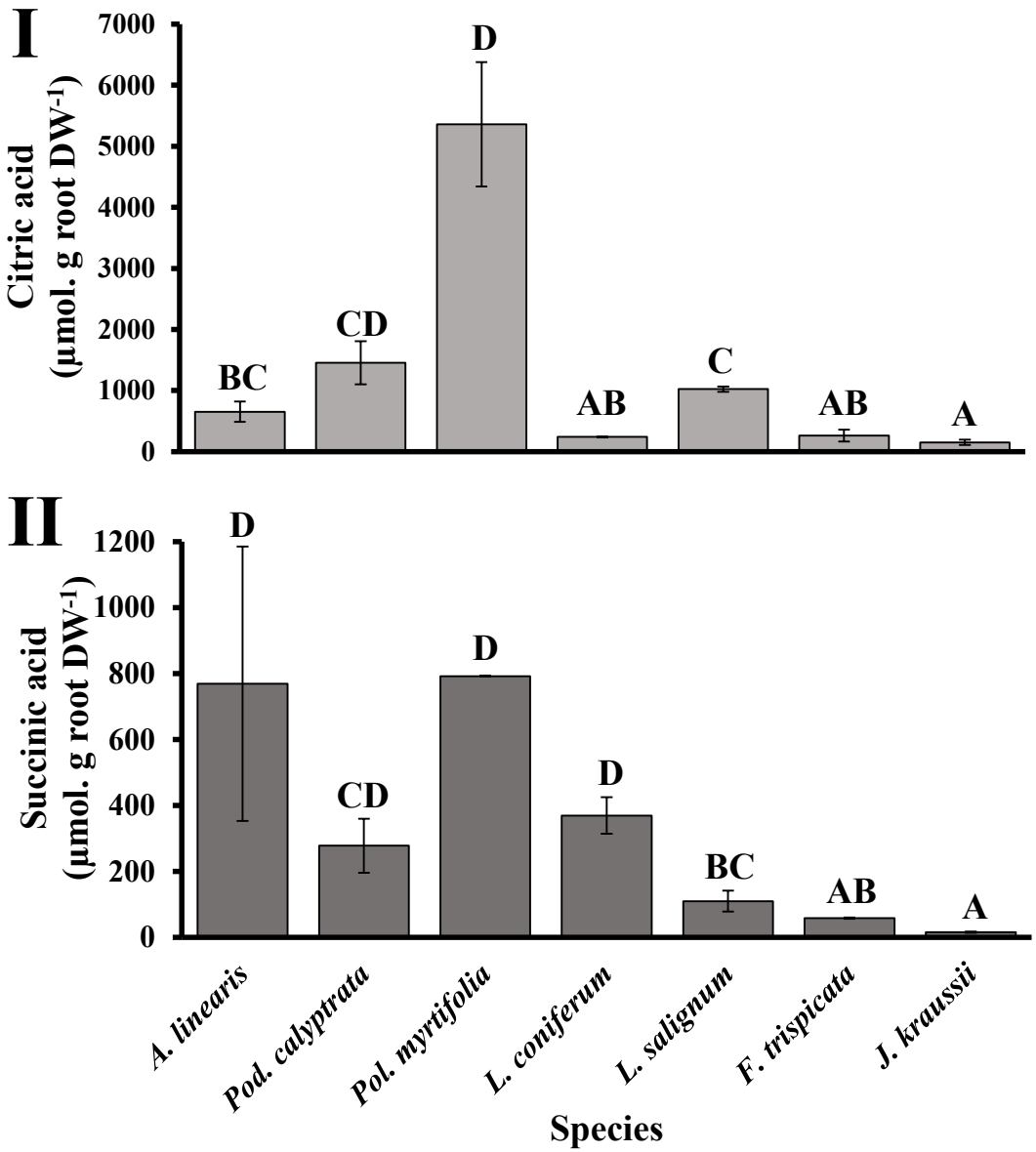

Figure 3.5: Root exudation of citric acid (I) and succinic acid (II) of the seven species grown at 10 mg P kg^{-1} sand. Letters indicate significant differences between species by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

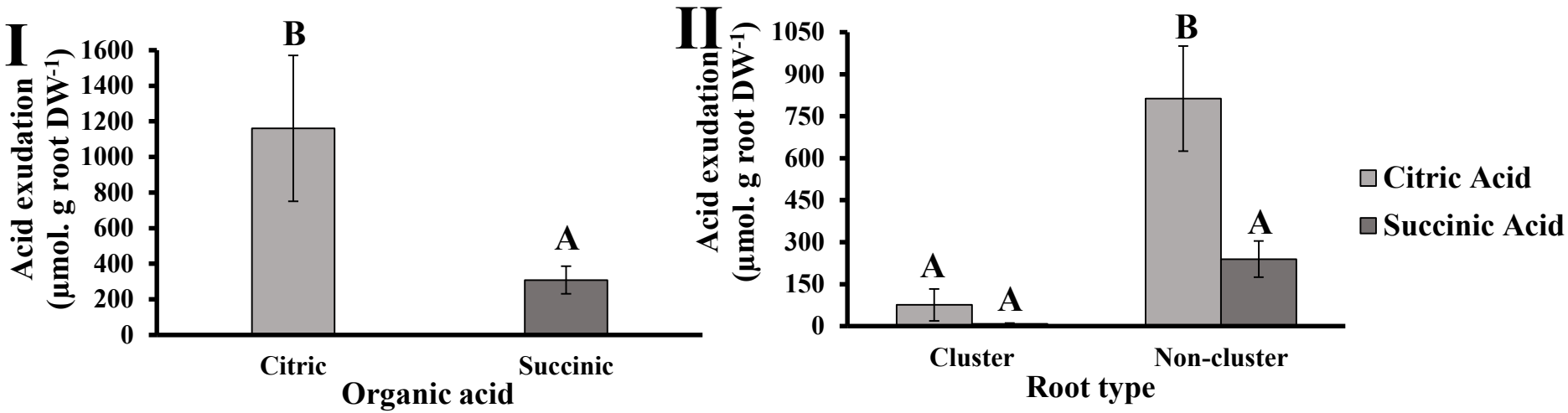

Figure 3.6: Acid exudation of citric and succinic acid of the seven species (I) and total acid exudation of cluster and non-cluster roots grown at 10 mg P kg^{-1} sand. Letters indicate significant differences between acids by Tukey HSD post hoc test ($p < 0.05$). Vertical bars denote standard errors.

3.3.4 Tissue N and P concentrations

Cluster root species, *Aspalathus linearis* and *L. coniferum* were the only species to exhibit differences in leaf P concentrations between P treatments, where in both cases, they had greater P concentrations at the higher P treatment ($p < 0.001$). Only one species, *F. trispicata*, showed differences in leaf N concentrations between treatments, where the leaf N was two times greater at the high P treatment ($p < 0.001$). Similarly, only *F. trispicata* showed differences in leaf N:P between P treatments, where the N:P ratio was almost three times higher at the high P treatment than the low P treatment ($p < 0.001$; Table 3.2). Overall, leaf N and P concentrations were significantly greater at the higher P treatment ($p < 0.001$), while there were no differences for root N ($p > 0.05$) and leaf N:P ($p > 0.05$) between treatments. *Leucadendron salignum*, also a cluster root forming species, had, on average, the highest leaf P concentration, similar to that of *F. trispicata,* compared to the other species. Both legumes, *A. linearis* and *Pod. calyptrata,* had the lowest leaf P concentrations with 1.41 and 0.74 mg P. g DW^{-1} respectively ($p < 0.001$). Leaf N concentration was highest in *A. linearis* plants, 25.06 mg P.g DW^{-1}, and lowest in the Juncaceae species, *J. kraussii*, 5.44 mg P.g DW^{-1} ($p < 0.001$). Root N concentrations were dominated by the two legumes, *Pod. calyptrata* and *A. linearis* with 10.89 and 8.03 mg P.g DW^{-1} respectively, while the lowest was in the cluster root species, *L. salignum* with 2 mg P.g DW^{-1} ($p < 0.001$). Similarly, leaf N:P ratios were highest in *Pod. calyptrata* (22.22) and *A. linearis* (18.19) and lowest in *L. salignum* (1.64; $p < 0.001$).

3.3.5 Phosphorus use efficiencies

Species differences were evident for all the phosphorus efficiency variables. However, the two legumes dominated in most of the PUE values where *Pod. calyptrata* was highest in PER with a value of 2,222.05 g $DW.g^{-1}$ P which was two to eight times higher than all the other species, PUtE with a value of 1,205.21 g $DW.mg^{-1}$ P, which was two to three times higher than the other species, and was similar to *F. trispicata* with regards to APE with a value of 258.89 g DW kg^{-1} P_{APP}. *Aspalathus linearis,* one of the cluster root forming species, had the highest PPUE with 3.82 g^2 $DW.mg^{-1}$ P while in this case *Pod. calyptrata* had the lowest with 0.07 g^2 $DW.mg^{-1}$ P. However, *F. trispicata*, which was neither a legume species nor did it produce dauciform roots, was highest in both PUpE with a value of 1015.49 mg P kg^{-1} P_{APP} which was four to fifteen times higher than all the other species, and APE which was similar to *Pod. calyptrata* with a value of 400.79 g DW kg^{-1} P_{APP} ($p < 0.001$; Table 3.3). A forward stepwise model identified total cluster roots ($R^2 = 0.22$; $p = 0.002$), root:shoot ratios ($R^2 =$

0.024; p = 0.284), citric acid exudation (R^2 = 0.029; p = 0.229) and cluster root:root ratios (R^2 = 0.032; p = 0.204) as the major predictors of plant total biomass, however only total cluster root was found to be a significant variable.

Table 3.2: Tissue concentrations of P and N of the seven species grown at 10 and 25 mg P kg^{-1} soil. Means and standard error followed by different letters in the columns are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001. – denotes data not available.

Species	P treatment ($mg.kg^{-1}$)	Leaf P (mg. g DW^{-1})	Leaf N (mg. g DW^{-1})	Root N (mg. g DW^{-1})	Leaf N:P
A. linearis	10	1.16 ± 0.06 c	23.20 ± 1.06 e	8.13 ± 0.92 fg	20.63 ± 0.84 ef
	25	1.63 ± 0.06 de	26.91 ± 1.09 e	7.92 ± 1.42 fg	16.06 ± 0.47 e
Pod. calyptrata	10	0.68 ± 0.03 a	15.68 ± 0.88 d	10.85 ± 0.46 g	25.78 ± 2.16 f
	25	0.80 ± 0.04 ab	15.42 ± 0.44 d	10.93 ± 0.76 g	18.66 ± 1.61 ef
Pol. myrtifolia	10	1.45 ± 0.25 cd	-	5.59 ± 0.31 ef	-
	25	1.56 ± 0.15 cd	-	5.12 ± 0.24 ef	-
L. coniferum	10	1.08 ± 0.10 bc	-	4.04 ± 0.32 cde	-
	25	1.73 ± 0.12 de	-	2.62 ± 0.18 bc	-
L. salignum	10	3.75 ± 0.07 f	6.28 ± 0.17 ab	1.67 ± 0.14 a	1.78 ± 0.09 ab
	25	4.54 ± 0.27 f	6.76 ± 0.13 b	2.33 ± 0.15 ab	1.5 ± 0.08 a
F. trispicata	10	4.18 ± 0.47 f	9.46 ± 0.79 c	4.59 ± 0.71 de	2.33 ± 0.21 bc
	25	3.64 ± 0.35 f	22.27 ± 0.44 e	3.85 ± 0.14 cde	6.44 ± 0.74 d
J. kraussii	10	2.15 ± 0.12 e	5.07 ± 0.13 a	3.05 ± 0.24 bcd	2.48 ± 0.15 c
	25	2.02 ± 0.09 de	5.81 ± 0.37 ab	3.67 ± 0.14 cde	2.66 ± 0.15 c
F ratio		**4.27*** **	**29.43*** **	**3.57** **	**26.77*** **

Table 3.3: Phosphorus efficiency results of the seven species grown at 10 and 25 mg P kg^{-1} sand. Means and standard error followed by different letters in the rows are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001. – denotes data not available

	A. linearis	*Pod. calyptrata*	*Pol. myrtifolia*	*L. coniferum*	*L. salignum*	*F. trispicata*	*J. kraussii*	***F* ratio**
Phosphorus Uptake Efficiency (mg P kg^{-1} P_{APP})	132.61 ± 17.49 ab	217.61 ± 49.35 ab	66.53 ± 18.48 a	234.71 ± 29.25 b	146.39 ± 26.55 ab	1,015.49 ± 305.15 c	-	**9.39*** **
Agronomic P use efficiency (g DW kg^{-1} P_{APP})	48.22 ± 8.38 ab	258.89 ± 47.97 cd	35.51 ± 14.23 a	104.33 ± 12.26 bc	-	400.79 ± 9.51 d	-	**18.56*** **
P utilization efficiency (g DW g^{-1} P)	356.59 ± 19.82 a	1,205.21 ± 196.68 b	398.58 ± 207.24 a	407.12 ± 66.91 a	464.43 ± 143.97 a	455.45 ± 140.33 a	568.22 ± 19.43 a	**6.35*** **
Physiological P use efficiency (g^2 DW mg^{-1} P)	3.82 ± 0.31 d	0.07 ± 0.01 a	1.17 ± 0.19 c	0.56 ± 0.05 c	1.26 ± 0.12 c	1.13 ± 0.63 c	0.2 ± 0.02 b	**49.55*** **
P efficiency ratio (g DW g^{-1} P)	740.00 ± 39.65 bc	2,222.05 ± 169.93 d	891.21 ± 124.89 c	1,003.85 ± 82.81 c	277.55 ± 7.03 a	266.29 ± 30.53 a	547.99 ± 25.71 b	**82.30*** **

3.4 Discussion

The highest PUpE, a measure of the aggregate effect of P acquisition mechanisms, was interestingly observed in *F. trispicata* which corresponded with its higher leaf P concentrations despite having the lowest recorded root:shoot ratio as well as very low APase activity and organic acid exudation and no dauciform root production. This suggests that other factors, not measured in this chapter, contributed to its superior P acquisition. These factors could include a root system with longer and finer roots, root hair production, and symbioses with mycorrhizae or enhanced expression of phosphate transporters (Vance 2001). In Chapter 2 and other studies, it was found that total root length, root surface area, root diameter, specific root length and root mass ratios were factors that contributed largely to high PUpE (Sanginga *et al.* 2000; Gahoonia and Nielsen 2004; Hammond *et al*. 2009; Pang *et al*. 2010a; Fageria *et al*. 2014). The superiority of *F. trispicata* in PUpE and the lack of separation among the other species, including legumes and cluster root forming species, indicates that nodulation and cluster root production do not always guarantee the highest aggregate P acquisition in a plant. In this study, legumes had significantly lower root:shoot ratios, similar to the report by Power *et al*. (2010), associated with their capacity to fix N which alleviates N deficiency and reduces the investment into below-ground biomass (Markham and Zekveld 2007).

However, the two legumes, *A. linearis* and *Pod. calyptrata* exhibited the best phosphorus use efficiencies (PPUE, APE, PUtE and PER) implying that the increased N availability, via nodulation and subsequent N_2-fixation, which was evident in their greater leaf and root N concentrations, enhanced their use of P. Nitrogen and phosphorus are positively correlated in plants and have been found to co-limit plant growth (Elser *et al.* 2007; Craine and Jackson 2010; Harpole *et al.* 2011; Maistry *et al.* 2013). Due to the low P supply and subsequent low leaf P concentrations, N:P ratios in the legumes were higher than non-legume species which implies an N-induced demand for P (Maistry *et al.* 2015a, b). To satisfy this demand, plants are likely to allocate their resources on the acquisition of P (Bloom *et al.* 1985) which was evident in the production of cluster roots by *A. linearis* in the low P treatment but was suppressed at the high P treatment. These observations are similar to studies conducted on *L. albus* (Dinkelaker *et al.* 1989; Li *et al.* 2008) and *A. linearis* (Maistry *et al.* 2015a). In addition, the N-induced demand for P is also evident in studies that report that legumes respond better to P supplies than non-legumes, for example in the study by Ae *et al.* (1990)

on pigeon pea, *Cajanus cajan* and a similar result was found in peanut, *Arachis hypogaea,* by Wissuwa and Ae (1999).

Similar to the previous chapter and other studies (Zou *et al.* 1995; Houlton *et al.* 2008; Lv *et al.* 2013; Maseko and Dakora 2013), the two legumes also had the highest APase compared to the other six species, *A. linearis* with at least three fold more APase activity than all other species. Houlton *et al.* (2008) found that non-fixing plants had to rely solely on existing soil N therefore limiting them in their capacity for investing in APase enzymes while N_2-fixers were able to utilize their newly fixed nitrogen to support greater phosphatase activity in the surrounding soils. Due to the higher nitrogen requirement of APases, addition of extra nitrogen increased the production of APases (Zou *et al.* 1995; Treseder and Vitousek 2001).

As mentioned previously, cluster roots are also efficient at releasing APases in addition to carboxylates (Shane and Lambers 2005) which aid plants in their acquisition of P as seen in *Casuarina cunninghamiana* (Reddell *et al.* 1997), *Dryandra sessilis* (Grierson and Comerford 2000), *Hakea undulata* (Dinkelaker *et al.* 1997) and *L. albus* (Adams and Pate 1992; Ozawa *et al*. 1995; Neumann *et al*. 1999, 2000; Wasaki *et al*. 2003). In this chapter, *A. linearis, L. coniferum* and *L. salignum* produced cluster roots with *L. salignum* and *A. linearis* producing the most and least respectively. The cluster roots of *L. salignum* had three times more APase activity than the non-cluster roots on the same plant but the reverse was seen in *L. coniferum* where its non-cluster roots had higher APase activity than the cluster roots. The pattern seen in *L. coniferum* could be due to the cluster roots being older therefore not as physiologically active as the cluster roots in *L. salignum*. The exudation of both organic acids and APases simultaneously is an important combination as inorganic P that has been liberated by APases is more likely to get absorbed in the presence of citrate which suppresses re-adsorption and precipitation of inorganic P (Gerke *et al*. 1994; Braum and Helmke 1995). Further support comes from work showing that citrate accumulation around the rhizosphere of cluster roots is particularly effective in the solubilization of organic forms of P, which then serves as a substrate for APases (Hens *et al.* 2003). Under these conditions the mobilization of organic P is greatly enhanced since the low solubility of organic P and APase enzyme proteins in soils are the major factors that limit the acquisition of organic P by roots (Adams and Pate 1992; Neumann and Römheld 2000).

Cluster roots in this study, however, exuded significantly less organic acids than did non-cluster roots which could be due to the ephemeral nature of cluster roots and their short span of being physiologically active after maturation which did not match the sample collection time (Shane *et al*. 2004; Playsted *et al.* 2006). A comparative study by Lambers *et al.* (2006) in a number of crop species found that species that combined cluster root formation and organic acid release out-performed other species when grown under P deficient conditions. Field experiments carried out by Bolland *et al.* (2000) using three high-exuding *Lupinus* species, two with cluster roots (*L. albus* and *L. luteus*) and one without cluster roots (*L. angustifolius*) confirmed that plants with cluster roots had a distinct advantage over plants without these specialized structures in low P soils.

A plant's capacity to solubilize P does not just depend on the concentrations of the organic acids exuded but also on the composition and rate at which they are exuded, both of which are highly variable and influenced by, among other factors, species, plant age and soil conditions (Curl and Truegolve 1986; Dinkelaker *et al.* 1989; Ae *et al.* 1990; Veneklass *et al.* 2003; Nuruzzaman *et al.* 2005). From this chapter, and chapter 2, it was observed that a major organic acid exuded by fynbos plants was citrate which has been found to be the most superior organic acid in terms of mobilizing bound P, followed by malate, succinate, acetate and lactate (Jones 1998; Hinsinger 2001; Ryan *et al.* 2001; Jones *et al.* 2003). Similar to the previous chapter, *Pol. myrtifolia* exuded the most citric acid, followed by *Pod. calyptrata*, *L. salignum* and *A. linearis*. Furthermore, *Pol. myrtifolia* and *A. linearis* exuded the most succinic acid, followed by *L. coniferum* and *Pod. calyptrata.* The plants in this study therefore, while not exuding the full spectrum of organic acids, did exude the organic acids most efficient at mobilizing P from the sand. Many species, such as *Fagopyrum esculentum* (buckwheat), *Brassica napus* (oilseed rape) and various legumes have been found to be very efficient at utilizing rock phosphate as a source of inorganic P due to the release of organic acids (Hinsinger 2001). In a study on *Medicago sativa* (alfalfa), it was found that plants increased their exudation of citrate into the rhizosphere two-fold as a result of P deficiency (Lipton *et al.* 1987). Similarly, in a study by Pang *et al.* (2010a) it was found that there were more organic acids present in the rhizosphere of plants grown under P deficient (6 μg P g^{-1} dry soil) conditions and citric acid was predominantly exuded, though the exudation range peaked at about 130 μmol g^{-1} root DW whereas the plants in this study peaked at over 5000 μmol g^{-1} root DW at 10 mg P kg^{-1} sand. This is most likely due to the different methods used in collection of organic acids.

The growth of plants is influenced by, among other factors, the nutrient content of soils, where plant growth increases in soils with higher nutrient concentrations (Lynch and Brown 2001; Kuang *et al.* 2005; Li *et al.* 2011). Of the seven species studied in this chapter, *A. linearis*, *Pod. calyptrata*, *Pol. myrtifolia* and *L. coniferum* showed increases in biomass production at high P compared to low P. Previous studies looking at the growth of legumes at different P concentrations found that they grew better and produced more biomass under higher P (Kuang *et al.* 2005; Power *et al.* 2010; Maistry *et al.* 2013). This is also holds true for other species (Chapin *et al.* 1986, Ågren 1988; Güsewell 2004). However, most of the species showed no differences in their leaf P concentrations between P treatment except for *A. linearis* and *L. coniferum* both of which had higher leaf P concentrations when grown at a higher P. Similarly, none of the species exhibited differences in their root N concentrations nor their leaf N:P ratios between P treatments except *F. trispicata* which had a higher N:P ratio at the higher P treatment indicating that even though it was being grown at higher P it was still P deficient compared to when it was grown at lower P. This is in contrast to the previous chapter and other studies where plants had lower N:P ratios in higher P soils (Aerts and Chapin 2000; Tessier and Raynal 2003; Güsewell 2004).

Both *L. salignum* and *J. kraussii* had the lowest leaf N concentrations, which has been associated with longer leaf longevity and better nutrient conservation (Eckstein *et al.* 1999). This seems to be correlated with the fact that *Leucadendron* species are sclerophyllous and can retain their leaves for up to seven years (Bond and Midgley 1988).

A typical response plants have to P deficiency is to increase their root:shoot ratios (Lynch *et al.* 1991). The cluster root forming species, *L. salignum*, had the highest root:shoot ratio, compared to the rest of the species, at low P conditions which could be due to its ecology. It is one of the most widespread Proteaceae species, occurring over a large part of Southern Africa and a wide range of soils (Jamieson 2000) therefore having a naturally high root:shoot ratio would be an advantage for the plant in its distribution across the CCR to enable better foraging of soils. *Juncus kraussii*, which is found commonly in saline marshes (Manning and Goldblatt 2012), also had a high root:shoot ratio similar to that of *L. salignum* under low P conditions. In a study by Atkinson (1983), *Juncus squarrosus* had increased root growth under low P conditions which was assumed to be less important than its root morphology as *J. squarrosus* was able to produce an abundance of long root hairs (Atkinson 1982) which are able to exploit a greater soil volume. Interestingly, *A. linearis*, which produced the lowest

overall biomass, had the third largest root:shoot ratio indicating that root biomass may be important to the species' survival in low P soils.

3.4.1 Conclusion

All the species differed in their absorption and utilization of phosphorus due to differences in their morphology, physiology and biochemistry as well as their interactions with the environment. The two legume species, *A. linearis* and *Pod. calyptrata* were superior at phosphorus use efficiency while *F. trispicata* was the most efficient in phosphorus uptake efficiency even though it had the lowest APase activity, low organic acid exudation, low root:shoot ratio and no dauciform root production. This suggest that other factors such as root hair production, mycorrhizal associations, root morphological changes or enhanced expression of phosphate transporters could have contributed to its superior phosphorus uptake (Vance 2001).

Chapter 4

General discussion and conclusion

In a world of dwindling resources it is essential to find sustainable ways for utilizing these resources. Phosphorus (P) is a major nutrient for plants, crucial in their growth and development (Vance *et al.* 2003), and is one of the most unavailable and inaccessible macronutrients in soils due to its occurrence in organic forms and adsorption to cations in the soils (Gahoonia and Nielsen 2004). The use of P fertilization in P-deficient areas has risen in attempt to alleviate the poor plant growth; however, this is uneconomical and ecologically flawed as the efficiency of these fertilizers is very low (Russell 1973; Werft and Dekkers 1996). It has also been predicted that rock phosphate reserves will be completely depleted within the next two centuries (Koppelaar and Weikard 2013; Ulrich *et al.* 2013) indicating that conservation is vital. A viable alternative to the addition of more P fertilizers would be to target and enhance traits that increase the efficiency and utilization of P in plants (Lambers and Shane 2007). The plants in the Cape Core Subregion (CCR) provide an excellent opportunity to study Fynbos species for superior P acquisition traits in roots as they have, through natural evolutionary processes, adapted to low P conditions (Power *et al.* 2010). Studying the variation in these root traits and their physiological functions with regards to enhanced P acquisition is fundamental in identifying species in the CCR as a possible germplasm source for the selection and breeding of root traits for increasing P acquisition efficiency and improving the production of economically important species in low P areas (Lambers and Shane 2007).

Consistent with a number of studies (Ae *et al.* 1990; Cowling *et al.* 1994; Lambers *et al.* 2002; Yan *et al.* 2002; Gahoonia and Nielsen 2003; Lamont 2003; Linder 2003; Veneklaas *et al.* 2003; Orians and Milewski 2007; Lambers *et al.* 2008) P acquisition mechanisms observed in this study varied with species and soil types. As was observed in chapter 2, plants had varying root:shoot ratios and the root:shoot ratios in *A. linearis* were higher in plants grown in Silvermine soils compared to plants grown in Niewoudtville and Rhodes Memorial soils (Section 2.3.2). Organic acid exudation varied between species as was seen in the glasshouse experiment (Section 2.3.3) where *Pol. myrtifolia* was superior in terms of citric acid exudation and *A. linearis* was superior in terms of succinic acid exudation, while in the

field study (Section 2.3.4) *A. macrantha* was superior in terms of citric acid exudation followed by *Pol. myrtifolia* in Rhodes Memorial soils. Acid phosphatase activity in the field study (Section 2.3.4) varied between soils, showing a negative relationship between activity and soil P availability, however, the opposite was true in the glasshouse study (Section 2.3.3) where APase activity was similar between soils but varied between species. Similarly, in chapter 3, root:shoot ratios (Section 3.3.1), APase activity (Section 3.3.2) and organic acid exudation (Section 3.3.3) varied greatly between the species studied.

When there is a deficiency in P, plants allocate more photosynthate into root production which allows the root system to explore greater volumes of soil (Lynch *et al.* 1991). A commonly observed occurrence in P deficient plants is an increase in their SRL which gives them longer and more branched roots per unit of root dry weight (Makita *et al.* 2009; Pang *et al.* 2010a; Fageria *et al.* 2014). Plant available P is generally concentrated in the top layers of soils and plants that have an abundance of roots in the surface layers (Lynch 1995; Manske *et al.* 2000; Lynch and Brown 2001) as well as the ability to proliferate roots within nutrient-rich patches (Hodge 2010) are more effective in P acquisition. In addition to this, the production of dense rooting systems is an effective means of increasing P acquisition as well as adjusting to low P soils. This can be observed in chapter 2 where a greater root:shoot ratio, higher total root length, SRL and total root SA, together with the exudation of organic acids, especially citric acid, into the rhizosphere were the main traits required for a more superior P uptake, as was observed in *Pol. myrtifolia*, which is a widely distributed species in the CCR (Walt 2003). However, in chapter 3, where more species were studied, it was found that *F. trispicata*, which had the lowest APase activity, low organic acid exudation, low root:shoot ratio and no dauciform root production, was the superior in P uptake therefore suggesting that other factors, which were not looked at in the chapter, such as root hair production, mycorrhizal associations and root morphological changes (Vance 2001, Pearse *et al.* 2007) could have contributed to its superior P uptake.

Legumes are generally considered to be poorly adapted for acquiring P from sparingly soluble sources due to their lack of root biomass accumulation (Power *et al.* 2010) and this assertion is supported by findings in chapter 2 and 3 where the legumes had significantly lower root:shoot ratio, relative to non-legumes, possibly associated with their ability to fix N which lessens N deficiency and reduces below-ground biomass investment (Markham and Zedeveld 2007). However, in chapter 3, it was observed that the legumes, both *A. linearis* and

Pod. calyptrata, were superior in phosphorus use therefore implying that the increased availability of N, as a result of their N_2 fixing capabilities, enhanced their use of P. Nitrogen and phosphorus are positively correlated therefore co-limit plant growth (Elser *et al.* 2007; Craine and Jackson 2010; Harpole *et al.* 2011; Maistry *et al.* 2013). In chapters 2 and 3 it was also observed that legumes were superior in APase activity, in both cases having higher activity compared to the non-legumes studied which is in accordance with numerous other studies (Zou *et al.* 1995; Houlton *et al.* 2008; Lv *et al.* 2013; Maseko and Dakora 2013). This again, is most likely attributed to their ability to fix N which allows them to support greater phosphatase activity (Houlton *et al.* 2008) which inherently has a high N requirement (Zou *et al.* 1995; Treseder and Vitousek 2001).

Cluster roots are an important significant predictor of total biomass linked with P-uptake mechanisms and form in response to P deficiency in soils as seen in chapter 2 and 3 and other studies (Watt and Evans 1999; Lambers *et al.* 2003; Shane *et al.* 2004). Cluster roots are also known to be highly efficient at exuding large quantities of organic acids into the rhizosphere (Gardner *et al.* 1983; Dinkelaker *et al.* 1995; Neumann *et al.* 2000; Lambers *et al.* 2003, 2006), but this was not the case in this study most likely due to the collection time of organic acids not coinciding with the exudative bursts for which they are known for (Shane *et al.* 2004). In addition to the release of organic acids, cluster roots are also efficient at releasing APases (Shane and Lambers 2005) as was seen in the cluster roots of *L. salignum* in chapter 3 and in a number of species in other studies (Adams and Pate 1992; Ozawa *et al*. 1995; Dinkelaker *et al.* 1997; Reddell *et al.* 1997; Neumann *et al*. 1999, 2000; Grierson and Comerford 2000; Wasaki *et al*. 2003). Inorganic P, which when liberated from cations by APases, is more likely to get absorbed by plants in the presence of citrate, which suppresses the re-adsorption and precipitation of inorganic P, making the simultaneous exudation of organic acids and APases an effective combination (Gerke *et al*. 1994; Braum and Helmke 1995). Previous studies have shown that plant species that combine both cluster root production and organic acid release out-perform other species when grown under P-deficient conditions (Bolland *et al.* 2000; Lambers *et al.* 2006).

Organic acids are highly effective at chelating not only calcium but also iron and aluminum and therefore displacing inorganic P which then becomes available to plants (Braum and Helmke 1995; Jones 1998; Hinsinger 2001; Ryan *et al.* 2001). There are a variety of organic acids which are exuded by plants, however, both the composition and rate at which these are

exuded are highly variable and influenced by the species itself, the age of the plant and conditions in the soil (Curl and Truegolve 1986; Dinkelaker *et al.* 1989; Ae *et al.* 1990; Veneklass *et al.* 2003; Nuruzzaman *et al.* 2005). Citric acid, a tricarboxylate, has been found to be the most efficient at solubilizing unavailable P, followed by malic, succinic, acetic, lactic and oxalic acid (Hinsinger 2001; Ryan *et al.* 2001; Jones 1998; Jones *et al.* 2003) due to their rapid complexation with metals (Zhang *et al.* 1997; Hinsinger *et al.* 2006). In this study it was observed that citrate was the major organic acid exuded by all the species followed by succinate and trace amounts of malate. Fynbos plants therefore, while not exuding the full array of organic acids, do produce and exude the most efficient ones.

A noteworthy find in chapter 2 was that plants were able to grow in soil from areas where they do not naturally occur in the CCR and the two legume species had similar levels of nodulation in all three soils. These observations suggest that the most limiting factors for plant growth in those areas were not soil chemical characteristics and that the rhizobia responsible for nodulating *A. linearis* and *Pod. calyptrata* occur throughout the CCR, agreeing with Lemaire *et al.* (2015, in press). Plants grew reasonably well in Silvermine soils, even though it had the lowest plant available P, which could be due to the fynbos plants' adaptation to growing in infertile soil, through mechanisms that are not yet fully understood. In this regard, Maistry *et al.* (2015a, b) suggested a balanced allocation of resources for nutrient acquisition in response to demand as a physiological mechanism in the Cape legumes for growth in the nutrient poor fynbos soils.

4.1 Conclusion

Cluster roots, while considered one of the foremost adaptation for plants living in P deficient conditions, did not seem to give plants which produced them an advantage in P uptake or utilization in this study. The most efficient species at P uptake were *Pol. myrtifolia* (chapter 2) and *F. trispicata* (chapter 3) most likely due to their rooting morphologies, while the most efficient plants at P utilization were the two legumes, *A. linearis* and *Pod. calyptrata* (chapter 3) most likely due to the addition of N they receive through atmospheric fixation. It was shown that root morphological traits such as total root length, total root SA, SRL, root diameter and root:shoot biomass ratios, along with organic acids exudation are prime components in P acquisition in fynbos species. There is however, substantial genetic variation in all these traits.

4.2 Future Research

Although this study has helped our understanding of which plant root mechanisms are most beneficial with regards to P-uptake in Fynbos species, there is still more that can be done. Studies can be performed on a wider range of species in the Fynbos biome with a wider variety of soil types while measuring all the phosphorus efficiency indices, including conservation of P, alongside the mechanisms of P uptake and conservation.

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Appendix 3.1

Table 3.4: Results for all seven species grown under 25 mg P. kg^{-1} sand. Means and standard errors followed by different letters in the rows are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001. – denotes no available data.

	A. linearis	***Pod. calyptrata***	***Pol. myrtifolia***	***L. coniferum***	***L. salignum***	***F. trispicata***	***J. kraussii***	*F* ratio
Root:Shoot	0.53 ± 0.04 ab	1.01 ± 0.12 cd	0.89 ± 0.07 bc	1.62 ± 0.18 de	1.16 ± 0.10 cd	0.38 ± 0.08 a	2.18 ± 0.30 e	24.02***
Nodules (g)	0.041 ± 0.006 a	0.147 ± 0.022 b	-	-	-	-	-	33.58***
Nodule : Root	0.065 ± 0.007 b	0.015 ± 0.002 a	-	-	-	-	-	57.03***
Total cluster roots (g)	-	-	-	0.60 ± 0.15 a	1.13 ± 0.21 a	-	-	3.30
Cluster root:Root	-	-	-	0.19 ± 0.06 a	0.82 ± 0.14 b	-	-	16.99**
APase activity (µmol p-NP. g root DW^{-1}. hr^{-1})	5.90 ± 1.30 e	1.97 ± 0.3 d	1.82 ± 0.17 d	1.11 ± 0.13 cd	0.86 ± 0.04 bc	0.59 ± 0.05 b	0.04 ± 0 a	140.01***
Cluster root APase activity (µmol p-NP. g root DW^{-1}. hr^{-1})	-	-	-	1.15 ± 0.19 a	3.24 ± 0.70 b	-	-	12.04**
Non cluster root APase activity (µmol p-NP. g root DW^{-1}. hr^{-1})	-	-	-	1.05 ± 0.11 a	0.82 ± 0.04 a	-	-	
Citric acid exudation (µmol. g root DW^{-1})	7,021 ± 0.0 d	569 ± 149.3 abc	3,360 ± 671.8 d	1,659 ± 101.1 cd	1,162 ± 378.2 bc	448 ± 6.0 ab	262 ± 14.6 a	20.57***
Succinic acid exudation (µmol. g root DW^{-1})	883 ± 175.1 ab	323 ± 254.1 ab	2,220 ± 608.5 b	174 ± 84.5 a	970 ± 0.0 ab	45 ± 14.8 a	75 ± 27.2 a	5.18**