# The secretion of acid phosphatase by Proteaceae as an adaptation to limited phosphate availability

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## Plagiarism declaration

I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own. Each contribution to this thesis from the works of other people has been cited and referenced. This document is my own work.

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# Abstract

The Proteaceae are a distinctive component of South Africa’s Fynbos and Australia’s Kwongan vegetation and display a number of adaptations to the soils of these nutrient-poor regions, where availability of phosphate (P) is particularly limited. The Proteaceae are notable for the virtually family-wide ability to form cluster roots. These cluster roots are specialised structures that enhance availability of P by exuding a range of P-solubilising compounds into the rhizosphere, including acid phosphatase (AP) enzymes, which are believed to hydrolyse P bound in stable organic P forms. The physiology of AP release and the importance for P acquisition has not been well studied in the Proteaceae. This study attempted to characterise the role of cluster root-secreted AP as part of a suite of adaptations to P-limited soils. The central hypotheses of this thesis are that 1) the secretion of AP is an adaptation to P-limited soils; 2) AP secretion is enhanced or suppressed in response to P-form and P-availability; 3) edaphic endemic Proteaceae are adapted to P-forms found in their respective acid, aeolian or calcareous sandy soils and 4) AP-solubilised P makes a similar contribution to P nutrition compared to inorganic P.

Secretion of AP was observed in all wild Proteaceae, but activity was highest in the most P-limited soils, suggesting that organic P sources are of greater importance to plant nutrition in P-deficient soils than in comparatively P-rich soils (e.g. species on relatively high-P soils invested less in AP secretion than calcifuge species in acidic low-P soils). The differences in AP activity between *Protea repens* specimens growing in dissimilar soils demonstrated the plasticity of AP secretion, which was negatively correlated with P availability. In hydroponics, a reversal of P availability induced changes in AP secretion within 48 h, demonstrating a regulatory mechanism sensitive to short-term changes in P availability. In plants grown in sand, AP secretion was highest in those plants supplied with the least available forms of P, including organic MgIHP. All species were able to utilise organic P similarly to inorganic P sources, except for *P. obtusifolia*, which was unable to produce cluster roots and therefore could not secrete sufficient AP to utilise MgIHP. It appears that the capacity to secrete AP does not differ between Proteaceae, but the suppression of AP activity in response to increasing P availability suggests that the contribution of AP to plant nutrition is highly variable between soils. All species studied appear capable of utilising organic P, provided plants have sufficient resources to form

cluster roots. The roles played by plant-secreted AP and organic P forms deserve more consideration in the study of Proteaceae nutrition in particular and P-limited ecosystems in general.

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# Chapter 1 - Introduction

## Proteaceae in Fynbos soils

The family Proteaceae is indigenous to Australia, Central and South America, Southeast Asia, the Southeast Pacific Islands, and South Africa (SA) (Rebelo, 1996), and are a distinctive part of the vegetation of SA's Fynbos and Western Australia's Kwongan heathlands (Bond and Goldblatt, 1984; Cowling and Lamont, 1998). Due to a Mediterranean climate of winter rainfall and relatively stable geology since the Pliocene (Goldblatt, 1997), the soils of these regions are highly weathered and nutrient-leached, where the majority of soil minerals remaining have very low solubility and are largely unavailable to plants (Beadle, 1966; Witkowski and Mitchell, 1987). Although generally considered to be nutrient-poor, spatial variations in rainfall, soil parent material and topography over relatively small areas of the Fynbos biome have resulted in a mosaic of spatially heterogenous soils (Bond and Goldblatt, 1984; Mitchell *et al.*, 1984), where soil chemistry is one factor determining species composition and distribution (Cowling *et al.*, 1992). Fynbos vegetation is characterised by a high degree of edaphic endemism, where species distribution is often clearly delineated and restricted by soil type (Cowling *et al.*, 1992). It has been suggested that the separation of edaphically restricted species of Proteaceae on neighbouring soils (e.g. *Protea obtusifolia* in calcareous soil and *P. susannae* in sandstone-based soil) is maintained by specific plant adaptations to native soil nutrition (Mustart and Cowling, 1993; Richards *et al*., 1997).

Fynbos and Kwongan soils are particularly limited by availability of phosphorus (P) (Lamont, 1982; Witkowski and Mitchell, 1987; Pate and Watt, 2002; Shane and Lambers, 2005a), where most P occurs in sparingly soluble forms (Mitchell *et al*., 1984) and available P ranges from 0.8 – 8 µg P $g^{-1}$ soil (Witkowski and Mitchell, 1987). Proteaceae have a number of adaptations to P-limited soils (Beadle, 1966); for example, plant P-use efficiency is enhanced by producing long-lived sclerophyllous leaves and serotinous seed cones (Cramer and Midgley, 2009) and by preferentially reallocating P to photosynthetically active leaves or developing seeds (Jongens-Roberts and Mitchell, 2006). The most well-known P-acquisition strategy of Proteaceae is the ability to produce cluster roots, which are dense lateral root structures comprising determinate tertiary rootlets formed as an adaptation to soils of low P availability (Purnell, 1960; Lamont, 1982; Skene, 1998). Cluster roots enhance plant P-availability by exuding P-solubilising compounds into the rhizosphere, in particular carboxylates (Lamont, 1982; Dinkelaker *et al.*, 1989; Roelofs *et al.*, 2001; Lambers *et al.*, 2002) and acid phosphatase (AP) enzymes (Gilbert *et al.*, 1999; Grierson and Comerford, 2000). Carboxylates increase the availability of phosphate to plants by competing for cation binding sites on metal-phosphate complexes and minerals, with subsequent release of phosphate ions (Jones *et al.*, 2003).

The majority of studies of cluster root exudates have concentrated on white lupin, *Lupinus albus* (Fabaceae) e.g. (Gardner *et al.*, 1982; Adams and Pate, 1992; Johnson *et al.,* 1996; Gilbert *et al.*, 1999; Neumann *et al.*, 1999; Watt and Evans, 1999, 2000; Li *et al.*, 2003, 2005; Shane *et al.*, 2003b; Wasaki *et al.*, 1999, 2003 and Shu *et al.*, 2007). There have been several studies of cluster root exudation of carboxylates by Proteaceae (e.g. Roelofs *et al.*, 1999; Lambers *et al.*, 2002, 2003; Shane *et al.*, 2004, 2005). By comparison, the contribution of root-secreted acid AP and soil organic matter to Proteaceae, and indeed all plant nutrition, is poorly understood (Raghothama, 1999). Root AP activity increases plant-available P by hydrolysing P from organic compounds in soil (Tarafdar and Jungk, 1987; Adams and Pate, 1992). While cluster root AP activity in *Banksia sessilis* has been recorded (Grierson and Comerford, 2000), the cluster root-secretion of AP and the contribution thereof to the P-nutrition of Proteaceae have not been studied.

*Phosphorus availability*

Availability of P, rather than total soil P, is commonly a limitation to plant productivity. The most important factor in inorganic P fixation is soil pH (Marschner, 1995). Soil phosphorus may be divided into the following four categories (Barber, 1984):

*i) Plant-available phosphorus*.

Phosphorus is primarily absorbed by plants in the form of orthophosphate ions ($HPO_4^{2-}$ and $H_2PO_4^-$) in the soil solution. The predominant form varies with pH. Below pH 7.2, $H_2PO_4^-$ predominates to *ca.* pH 4.8, below which $H_2PO_4^-$ is converted to $H_3PO_4$. Above pH 7.2, $HPO_4^{2-}$ is the dominant form until *ca.* pH 9.7, above which $HPO_4^{2-}$ is converted to $PO_4^{3-}$. A pH of *ca*. 6.5 is generally optimum for inorganic P availability to plant roots (Tisdale *et al.*, 1993).

*ii) Phosphorus adsorbed on the surfaces of minerals*.

Phosphate ($PO_4^{3-}$) readily binds with metal cations and soil particles, forming sparingly soluble complexes (Tisdale *et al.*, 1993) which are largely unavailable to plants (Marschner, 1995). Mineral surfaces donate protons to the phosphate anion, making it more positive and resulting in specific adsorption to the mineral, coordinating the anion with the mineral's metal cation. The degree of adsorption increases with pH (Barber, 1984).

*iii) Phosphorus minerals*.

In acid soils, inorganic P precipitates as Fe/Al minerals such as variscite ($AlPO_4.2H_20$) or strengite ($FePO_4.2H_2O$). In neutral and calcareous soils, inorganic P precipitates as Ca minerals such as hydroxyapatite ($Ca_5(PO_4)_3OH$), fluorapatite ($Ca_5(PO_4)_3F$), dicalcium phosphate dehydrate ($Ca(HPO_4).2H_20$) and dicalcium phosphate ($CaHPO_4$) (Nriagu, 1972).

*iv) Phosphorus as a component of soil organic matter*.

Most organic P compounds are esters of orthophosphoric acid, primarily inositol phosphates (10 to 50%), phospholipids (1 to 5%) and nucleic acids (0.2 to 2.5%) (Barber, 1984).

*Phosphorus availability in Fynbos soils*

The low availability of P in soils of Mediterranean ecosystems such as the Fynbos and the Kwongan is considered a major constraint to plant growth (e.g. Kruger *et al.*, 1983). In leached acid soils such as those of the Fynbos, inorganic P rapidly precipitates as stable iron (Fe) or aluminium (Al) secondary minerals, or is adsorbed to surfaces of Fe/Al oxide and clay minerals (Tisdale *et al.*, 1993). Most of the inorganic P (70%) in fynbos is bound to Fe, and *ca.* 5% is bound to calcium (Ca) and 5% to Al (Mitchell *et al.*, 1984). Plant-available P concentrations in these soils typically range from 0.8 to 8.0 μg P $g^{-1}$ soil (Witkowski and Mitchell, 1987). When added in small quantities (i.e. less than 10 μg/g), P is rapidly adsorbed by oxide surfaces, whereas additions of higher concentrations of P, such as at the point of litter or ash deposition, are thought to precipitate primarily as Fe-, Ca- and Al- phosphates (Barber, 1984).

Soil organic matter, though of highly variable composition, often accounts for a significant proportion of P in fynbos soils (≤ 60% - Mitchell *et al.*, 1984; ≤ 77%, Witkowski and Mitchell, 1987). The P content of soil organic matter (SOM) is variable (1 - 3%) (Tisdale *et al.*, 1993). SOM content generally increases with soil age as mineral phosphate is taken up by plants and microbes and re-deposited in organic forms (Dalal, 1977), primarily in the form of litter fall and ash in the case of fynbos (Mitchell *et al.*, 1986). While much organic matter is re-mineralised into inorganic forms in a flux of nutrient cycling, largely mediated by microfaunal degradation, stable decomposition by-products persist in soil, thereby gradually increasing SOM content with soil age (Dalal, 1977). As deposition of plant litter and ash (Mitchell *et al.*, 1984), and sometimes dust and ocean spray (Brown *et al.*, 1984; Soderberg and Compton, 2007) are the primary inputs of nutrients in fynbos, the majority of SOM and organic P is concentrated in the uppermost soil layer, decreasing with depth (Syers and Walker, 1969; Walker and Syers, 1976; Lamont, 1982; Witkowski and Mitchell, 1987; Mitchell *et al*., 1986).

Soil organic matter may be divided into two fractions, humic and non-humic. Non-humic substances are comprised of carbohydrates, proteins, amino acids, lipids and low-molecular-weight carboxylates. They are unstable and are rapidly degraded and mineralised by soil micro-organisms (Barber, 1984). Humic substances are stable by-products of degraded organic matter and comprise the majority of SOM, primarily occurring as aromatic-phenolic polymers such as phytic acid (myoinositol heaxaphosphate, IHP), which accounts for up to 50 % of total organic P in soils (Barber, 1984, Tisdale *et al.*, 1993). The predominance of IHP in soils is attributable to its relative stability, being able to form strong complexes with proteins and insoluble salts with $Fe^{3+}$ and $Al^{3+}$ under acidic conditions and with $Ca^{2+}$ in alkaline conditions. Metal oxides and clay minerals such as montmorillonite may also strongly absorb IHP, the degree of absorption increasing with the number of phosphate groups attached to the phytate molecule (Tisdale *et al.*, 1993). Fynbos soil P is largely held in sparingly soluble forms, both inorganic and organic, which are not readily available for plant uptake, leaving very little available P to sustain plant growth. As a result, the Proteaceae have developed a number of adaptations to the limitations of their native soils, notably the formation of cluster roots, discussed further below.

*Cluster roots as an adaptation to nutrient-poor soils*

Cluster roots are terminal, tertiary lateral roots occurring in clusters on root systems of certain species (Lamont, 1982) found in a wide range of plant families, including the Fabaceae, Proteaceae, Casuarinaceae, Myricaceae, Eleagnaceae, and Betulaceae (Skene, 1998). Cluster roots, once termed proteoid roots when first described as a feature of the Proteaceae (Purnell, 1960), are associated with the Proteaceae of South Africa and Western Australia, most notably in the highly leached sands, laterites and sandstones of these regions (Lamont, 1982). Cluster root formation is typically induced in response to P deficiency, and in some cases Fe deficiency, while their development is generally suppressed by high nutrient availability (Wasaki *et al.*, 2003; Lambers *et al*., 2006). Cluster roots typically proliferate close to soil surface, often in association with discrete nutrient-rich patches and litterfall (Lamont, 1982; Skene, 1998; Pate and Watt, 2002).

Cluster roots were first thought to allow enhanced nutrient acquisition by increasing root surface area (Lamont, 1982). Cluster roots are now known to increase mobilisation of P from nutrient-rich soil patches by exuding P-solubilising compounds, primarily carboxylates, but also phenolics, mucilage and acid phosphatase enzymes, into the rhizosphere (Lambers *et al.*, 2003). Carboxylates, phenolics and mucilage, in combination with rhizosphere-acidifying protons and other cations, mobilise inorganic and organic P by displacing bound phosphate from metal cations (Dinkelaker *et al.*, 1997; Hinsinger, 200; Dakora and Philips, 2005; Lambers *et al.*, 2006), through both desorption of P from metal oxide surfaces and by the chelation of iron and aluminium from Fe-phosphates and Al-phosphates (Smith *et al.*, 2003). Carboxylates such as malate and citrate are typically released in very high quantities in a brief exudative 'burst' over 2 -3 d rather than exuded continuously in small amounts. A brief coordinated release of exudates is of adaptive significance, minimising the readsorption of solubilised P to soil constituents and loss of carboxylates to microbial predation (Dinkelaker *et al.*, 1997; Shane *et al.* 2004a). Acid phosphatases serve to hydrolyse organic P compounds in order to release orthophosphate ions ($P_i$) for plant uptake (Tarafdar and Jungk, 1987; Adams and Pate, 1992; Raghothama, 1999). It is the combination of their specialised root morphology and biochemistry which allows cluster root-forming species to survive on some of the world's most impoverished soils (Lambers *et al.*, 2006). The role of carboxylates in solubilising poorly available inorganic and organic P is well known, however the role of cluster root-secreted AP in acquiring organic P is far less understood.

*Phosphatases*

*The role of acid phosphatases in phosphate solubilisation*

Acid phosphatases (AP) are a group of enzymes associated with hydrolysis of a variety of phosphate esters, the majority of the enzymes showing no clear substrate specificity (Olczak *et al*., 1997). Alkaline phosphatases catalyse optimally above pH 7.0 and tend to be highly specific, whereas acid phosphatases tends to be less substrate-specific and catalyse optimally below pH 7.0, and are the dominant form in plant roots (Duff *et al*, 1994). Many phosphatase enzymes, whether of plant or microbial origins, are capable of catalysing the mineralisation of P from organic compounds in soils (Tarafdar and Jungk, 1987; Adams and Pate, 1992). A number of plant species, to varying degrees, are capable of secreting phosphatase enzymes, generally increasing in response to P-deficiency. The root secretion of AP has been studied most extensively in the cluster roots of *Lupinus albus* (Adams and Pate, 1992; Gilbert *et al*., 1999; Wasaki *et al.,* 2003), but other species noted for root-secretion of AP include *Spirodela oligorrhiza* (Reid and Bieleski, 1970), *Lycopersicon esculentum* (Goldstein *et al*., 1988), *Zea mays* (Gaume *et al*., 2001), *Cicer arietium* (Li *et al*., 2003) and *Caustis blakei* (Playsted *et al*., 2006).

Enhanced root secretion of AP generally confers an advantage in P-limited environments. For example, maize (*Zea mays*) phenotypes characterised by higher root AP secretion showed higher tolerance to P-depleted soils (Gaume *et al*., 2001), soil organic P was depleted and P availability to wheat (*Triticum aestivum* L.) was increased when intercropped with AP-secreting chickpea (*Cicer arietium* L.) (Li *et al*., 2003). Li and Tadano (1997) found soil organic P of a nutrient-poor soil to have been depleted by *L. albus*. It is clear that secreted root phosphatase increase plant-available inorganic phosphate by hydrolysing organic forms of soil P. However, the precise contribution of phosphatases to plant P acquisition remains unknown. The confounding influence of microbial activity, as well as the complex dynamics of phosphate interactions with soil constituents, challenge any study attempting to quantify the contribution of secreted AP to plant acquisition of P in field environments (e.g. Adams and Pate 1992; Raghothama, 1999; Lung and Lim, 2006).

When applied in pot and solution culture, organic phosphate salts such as phytic acid (IHP) may be utilised by phosphatase-secreting plants: Lung and Lim (2006) found phytase-secreting tobacco plants supplied with insoluble IHP to have performed better than a P-deficient control, while Adams and Pate (1992) found IHP to be at least equally available to mineral $KH_2PO_4$ as a source of P for growing lupins in sand, but less suitable in P-fixing soils, where the degree of adsorption of IHP significantly affected availability of P to plants. In comparison to non-cluster root-forming species, *L. albus* was more effective at utilising IHP, even in instances where root phosphatase activities were similar. This suggests that availability of IHP to cluster root-forming plants may be further enhanced by a means other than phosphatase secretion, such as carboxylate exudation (Adams and Pate, 1992). Clearly, studies of root AP/phosphate/carboxylate and rhizosphere interactions are complex and require further study.

*Cluster root-secreted AP*

The study of cluster root-secreted AP has focused almost exclusively on white lupin (*L. albus*). Enhanced cluster root secretion of AP can be observed in exudates and extracts of P-deprived plants in comparison to P-sufficient plants. The most pronounced increases, and by far the highest overall AP secretion, is observed in the cluster roots, though the whole root system is active (Adams and Pate, 1992; Gilbert *et al.*, 1999; Wasaki *et al.*, 1999; Wasaki *et al.*, 2003). Secretion of AP can be inhibited by high applications of P (Wasaki *et al.*, 2003). However, roots excised from plants grown under P-sufficient conditions and placed on P-deficient media increased AP secretion within 6 hours, before internal tissue stores of P could be depleted (Wasaki *et al.*, 1999). Thus, the secretion of phosphatase is controlled not only by tissue [P] but apparently also by external [P] (Wasaki *et al*, 1999; 2003).

An isoform of AP unique to cluster roots (LaSAP 2), has been observed in P-deficient *L. albus*, in addition to LaSAP 1 which is secreted throughout the root system in smaller quantities, even in plants supplied with sufficient P (Gilbert *et al.*, 1999). Wasaki *et al.* (2003) proposed that under P-sufficient conditions, LaSAP 1 is synthesized at low levels and is localised at the root epidermis. In response to slight P-deficiency, epidermis-localized LaSAP 1 is rapidly secreted into the rhizosphere. If endogenous P decreases beyond a certain critical threshold, expression of LaSAP 2 is induced, and production and secretion of this enzyme is activated and enhanced.

### *Likely function of AP in cluster roots of Proteaceae*

Cluster roots of *L. albus* and the Proteaceae appear functionally similar e.g. increased cluster root formation in response to P-deficiency, exudation of carboxylates and other inorganic P-solubilising compounds (Lambers *et al.,* 2006). Grierson and Comerford (2000) qualitatively visualised AP secretion by roots of *Banksia sessilis* (Proteaceae), where > 95% of activity was localised around cluster roots, similar to observations of *L. albus* (e.g. Wasaki *et al.*, 1999). Thus, the secretion of AP by Proteaceae is likely to play a similar role in P acquisition to that observed in *L. albus*. However, the secretion of AP by Proteaceae and the contribution of the strategy to P-acquisition remains otherwise uncharacterised.

Cluster roots tend to proliferate at the surface of the soil, preferentially in relatively nutrient-rich patches (Lamont, 1982), though they are also found at greater depths in association with mineral-rich deposits (Pate and Watt, 2002). In *Banksia* species, compound cluster roots proliferate in decomposing leaf litter, forming distinct 'mats' to exploit the nutrient-enriched uppermost soil level (Pate and Watt, 2002). The ratio of organic:inorganic P was found to have been reduced within the cluster mats compared to surrounding surface soils, indicating that *Banksia* promoted the mineralization of organic P deposits (Pate and Watt, 2002). The strategic placement of cluster roots in the uppermost soil layer conforms to the assumed function of secreted AP, given that soil organic matter content is highest at the point of surface deposition (Mitchell *et al*., 1986).

Shane and Lambers (2005) suggest that the contribution of secreted AP and exuded carboxylates to P acquisition is coordinated, as the latter would suppress the soil adsorption and mineral precipitation of any P released by AP. Hayes *et al*., 2000, showed organic anions can facilitate the activity of acid phosphatases. Tang *et al*., 2006, found organic IHP salts associated with cations such as $Al^{3+}$ and $Fe^{2+}$ to be unavailable unless in the presence of carboxylates. Thus, while the topics of cluster root formation and exudation of carboxylates by Proteaceae have been well studied, the role of cluster roots in P-acquisition cannot be fully understood without characterising the role of AP secretion, both in isolation and in combination with other P-solubilising compounds. Phosphorus availability is commonly reported as a limitation to soil productivity (Marschner, 1995), and a broad range of plant adaptations to P-limited soils have been studied extensively from both ecological and agricultural perspectives. (e.g. Raghothama, 1999; Hinsinger, 2001; Lambers *et al.*, 2006; Hawkins *et al.*, 2008). However, the AP-mediated acquisition of P from organic sources remains poorly understood (Gilbert *et al*., 1999; Raghothama, 1999). The secretion of AP by the Proteaceae of highly P-limited Fynbos soils provides an ideal opportunity to extend knowledge in the broader study of plant adaptations to P constraints.

### *Possible application of AP secretion to cutflower production of Proteaceae*

Potentially, a known ability to access organic P sources would also have implications in an agricultural context. A number of species of Proteaceae are cultivated as cutflowers, primarily serving markets in Europe, Japan, and the United States (Leonhardt and Criley, 1999). Phosphorus nutrition is a topic of particular significance to the protea cutflower industry, as relatively low application rates of P have been known to induce symptoms of toxicity in Proteaceae, hindering plant growth and marketability of the cut flower (Shane and Lambers, 2005a; Hawkins *et al*., 2008). Nutrition field trials of commercially grown Proteaceae showed the best-performing plants to have taken up more P than was supplied by fertigation, indicating a possible preference for plant-mediated acquisition of sparingly soluble forms of soil P (Hawkins *et al*., 2008). Thus, poorly soluble forms of organic phosphate could be investigated as a potential source of nutrition for cutflowers.

### *Hypotheses and thesis structure*

The Proteaceae have a remarkable ability to tolerate and adapt to P-limited soils and are particularly notable for their ability to form cluster roots (e.g. Beadle, 1966; Esler *et al.*, 1989; Pate and Watt, 2002; Lambers *et al.*, 2003; Shane *et al*, 2008). The exudation of carboxylates by Proteaceae has been relatively well characterised as an adaptation to P-limited soils, and P acquired by carboxylate solubilisation is assumed to account for a significant proportion of total plant nutrition (Dinkelaker *et al.*, 1997; Roelofs *et al.*, 1999; Lambers *et al.*, 2002, 2003; Shane *et al.*, 2004, 2005). The mechanisms controlling AP secretion by Proteaceae are far less understood, where most knowledge of AP in cluster roots is based on studies of *L. albus* (Fabaceae). This represents a gap in the study of cluster roots, which have been described as the third major root adaptation, after root nodulation and mycorrhizal associations (Skene, 1998). The adaptive significance of AP secretion and the potential contribution of organic P to nutrition of Proteaceae have not been explored even though organic matter frequently represents the majority of soil P in the Fynbos (Mitchell *et al.*, 1984; Witkowski and Mitchell, 1987) and other soils.

This study investigated the secretion of acid phosphatase (AP) by cluster roots of South African Proteaceae and aimed to characterise the role played by AP as part of a suite of plant adaptations to P-limited soils. The central hypotheses of this thesis are that 1) the secretion of AP is an adaptation to P-limited soils; 2) AP secretion is enhanced or suppressed in response to P-form and P-availability; 3) edaphic endemic Proteaceae are adapted to P-forms found in their respective acid, aeolian or calcareous sandy soils and 4) AP-solubilised P makes a similar contribution to P nutrition compared to inorganic P. A broad experimental approach was adopted, where secretion of AP was investigated in wild populations of Proteaceae from dissimilar soils; as well as in plants grown in hydroponics and sand-filled pots and supplied with varying forms and amounts of P. Edaphically specialised species from contrasting soil types were selected for experimentation in order to investigate differences in P-acquisition and preferences for P-supply, Some equivalent experiments were performed on *L. albus* to test generalised assumptions about cluster root function, where a sub-hypothesis was that AP secretion by Proteaceae and *L. albus* are functionally analogous and regulated by similar mechanisms. The study was separated into three experimental chapters, where the secretion of AP by Proteaceae was characterised in Fynbos soils, sand-filled pots supplied with several P substrates, and hydroponic culture.

# Chapter 2 - Acid phosphatase secretion by cluster roots of Proteaceae growing on acid, aeolian or calcareous sands in the Fynbos Biome, South Africa

## Introduction

The Proteaceae of South Africa's fynbos vegetation are characteristically adapted to the low nutrient status of their native soils (Bond and Goldblatt, 1984). Fynbos soils are particularly low in plant-available phosphate (P) (generally 0.8-8.0 mg P $kg^{-1}$ but up to 70 mg P $kg^{-1}$ in coastal areas; Witkowski and Mitchell, 1987), as the majority of soil P occurs in sparingly soluble forms, either as 1) inorganic metal-phosphate complexes or 2) as organic P (Mitchell *et al*., 1984). Soil organic P exists predominantly as stable inositol phosphates, as well as phospholipids, nucleotides, phosphoproteins and phosphonates (Tate, 1984; Tisdale *et al*., 1993). As a large proportion (28-60%) of the poorly-available P in fynbos soils may occur as organic matter (Mitchell *et al*., 1984), organic P sources could potentially be a major source of P for plant nutrition.

Cluster roots occur in Proteaceae and a number of other families as an adaptation to soils that are low in available P (Purnell, 1960; Lamont, 1982; Skene, 1998). Cluster roots increase mobilisation of P by releasing P-solubilising compounds (Lambers *et al*., 2006) such as 1) root-exuded carboxylates, which enhance the solubilisation of P from inorganic complexes (Gardner *et al*., 1982; Lambers *et al*., 2002; Jones *et al*., 2003), and 2) root-secreted acid phosphatase (AP) enzymes into the rhizosphere (Adams and Pate, 1992). Root-secreted phosphatases hydrolyse soil organic matter such as phosphate esters, rendering stable organic P sources available for plant uptake (Tarafdar and Jungk, 1987; Adams and Pate, 1992; Duff, 1994). While AP secretion has been qualitatively visualised in the Australian Proteaceae species, *Banksia sessilis* (Grierson and Comerford, 2000), the regulation of AP secretion by cluster root-producing plants has only been characterised in detail in *Lupinus albus* (e.g. Adams and Pate, 1992; Gilbert *et al*., 1999; Wasaki *et al.,* 2003). Studies of *L. albus* and other AP-secreting plants indicate that AP secretion is regulated and increases under phosphate deficient conditions (Gilbert *et al*., 1999; Wasaki *et al.,* 2003). The secretion of AP by *L. albus* cluster roots is partially regulated by whole plant P-status, where AP activity of P-deprived plants increases markedly in comparison to plants supplied with phosphate (Gilbert *et al.*, 1999). However, rapid increases observed in response to P-free media indicate that regulation of AP activity may be also sensitive to immediate P availability in the rhizosphere (Wasaki *et al.*, 2003), although a mechanism for such ‘P-sensing’ was not proposed.

This study aimed to compare cluster root-secreted acid phosphatase (AP) activities of South African Proteaceae from a range of soil types, varying in pH, phosphate availability, and total content of organic matter and metal cations such as $Fe^{2+}$ and $Ca^{2+}$. Considering the lack of knowledge regarding the contribution of AP activity to plant P nutrition in general and within the Proteaceae in particular, we tested the following hypotheses: 1) AP is secreted by Proteaceae as an adaptation to P-limited soils and 2) is enhanced or suppressed in response to P-form and P-availability on soils with varying P-forms/availability. It was thought that AP activity would be present in all Proteaceae tested and would vary not only between species but also within species growing in different soils, i.e. would be plastic/sensitively regulated. Higher AP activities were expected in species from soils of low P availability. Therefore, higher AP activities were also expected in more acidic soils, as ancient soils of low pH are typically lowest in available P (Witkowski and Mitchell, 1987). It was anticipated that higher AP activity would be observed in soils with high organic matter content, and which therefore contain a higher proportion of soil P in organic forms. The predominant form of metal cation was not expected to directly influence AP activity, except possibly as an indicator of soil type (e.g. $Ca^{2+}$ predominates in the comparatively P-rich limestone soils, whereas $Fe^{2+}$ predominates in acidic sandstone soils which are typically low in available P).

## Materials and Methods

### *Cluster root collection*

Cluster roots from wild populations of Proteaceae were collected during months of winter/spring rainfall (June – September 2007, 2008) from several dissimilar soils (limestone/calcareous and acid sandstone soils, Albertinia, (34.37°S, 21.66°E); Table Mountain acidic sand, Cape Point, (34.22°S,18.41°E); acidic red sandstone/shale clay, Stellenbosch (33.56° S, 18.51° E)**;** aeolian Strandveld sand, Hopefield, (33.02°S, 18.26°E)) and assayed for AP activity. Cluster roots from the generalist species *Protea* *repens* (L.) were collected at each site, allowing a separation of species-specific traits and the influence of soil characteristics. Other species sampled were *Protea obtusifolia* (H. Buek. Ex Meisn.) and *Leucadendron meridianum* (I. Williams) from Canca limestone Fynbos near Albertinia, *Leucospermum praecox* (Rourke) and *Leucadendron salignum* (P.J. Bergius) from Albertinia sand Fynbos (Albertinia), and *Leucadendron foedum* (I. Williams) from Hopefield sand Fynbos (Hopefield) (Mucina *et al.*, 2005). Roots were collected beneath the plant canopy from the uppermost 10 cm of soil. Between 4 and 6 plants were sampled at each site. Cluster roots of the approximate physiological maturity described by Shane *et al.* (2004a) at day 12-13 were selected (Fig. 2.1). Root maturity was assessed visually based on experience of observing cluster roots of Proteaceae in hydroponics. Roots were carefully rinsed in deionized water to remove excess soil, placed in a chilled (4°C) buffer solution (15 mM MES with 500 μM $CaCl_2$, pH 5.5), and maintained on ice in transfer to the laboratory (30 – 120 minutes). In the laboratory, roots were further rinsed free of soil and organic matter and warmed to 25°C in gently agitated buffer solution.

*Soil analysis*

Four soil samples from each site were taken from beneath the plant canopies, where each sample comprised three pooled subsamples (0-30 cm). Oven-dried, sieved (2 mm) soils were analysed for pH, total P, Olsen P (for soils above pH 7.0), Bray-II P (for soils below pH 7.0), Fe, Ca and percentage C. Soil pH was determined by extracting 10 g soil in 25 ml 1 M KCl, shaking at 180 rpm for 60 min, centrifuging at 10 000 rpm for 10 min and measuring pH in the supernatant. Soil was prepared for P analysis by extracting 6.6 g soil in Bray II solution (Bray and Kurtz, 1945) before filtering and analysing using ICP-AES. The Fe and Ca were analyzed in the sample solution using ICP-AES. Percentage C was analyzed according the to the Walkley Black method (Nelson and Sommers, 1982).

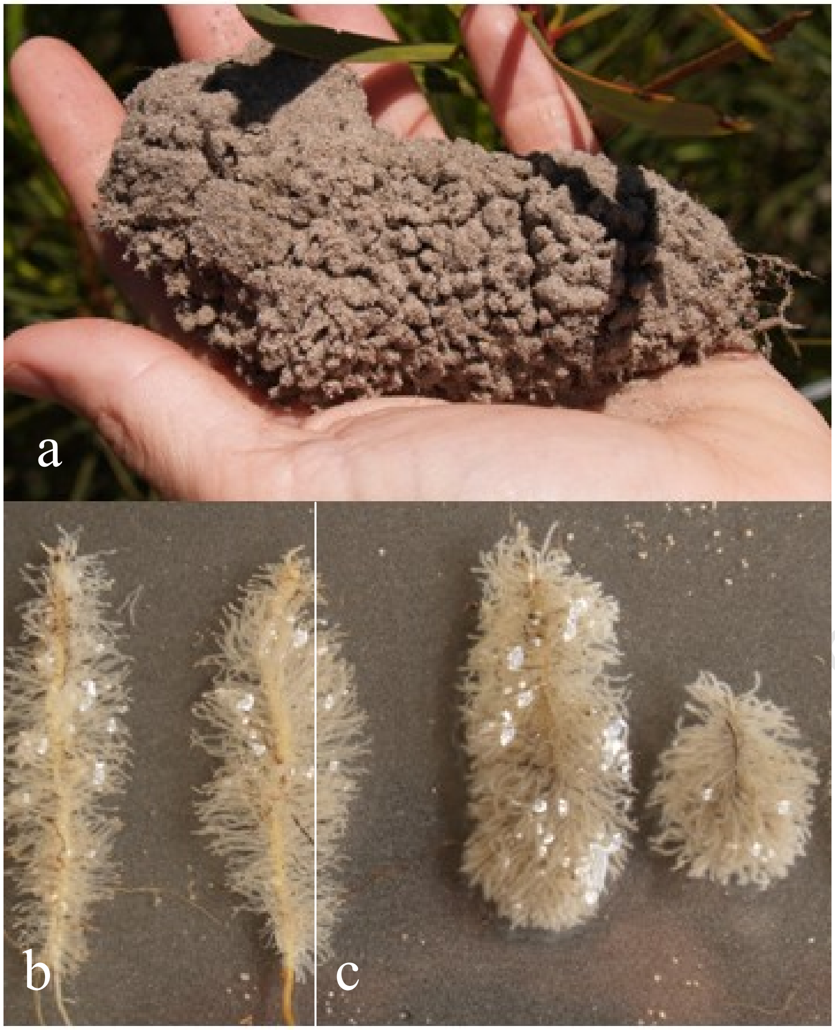

**Fig. 2.1**: ***Protea repens*** cluster root after a) collection from the field, b) washing free of soil (immature stage), c) washing free of soil for AP assay (mature stage)

*Acid phosphatase assay*

Comparisons of plant investment in AP were used to infer the relative contributions of organic matter to P nutrition of Proteaceae in nutrient-poor soils. Acid phosphatase activity was assayed according to the method of Gilbert *et al.* (1999). Cleaned, excised cluster roots were blotted dry, weighed, and placed in 4 ml of 15 mM MES with 500 µM $CaCl_2$ (pH 5.5) with 10 mM *p*-nitrophenyl phosphate (*p*-NPP) at 25°C. Roots were incubated for 30 min, before the reaction was terminated by the addition of 6 ml of 0.25 M NaOH. Root AP activity was based on the amounts of *p*-nitrophenol (*p*-NP) released as measured spectrophotometrically at 412 nm, relative to known *p*-PP standards, and expressed in terms of acid phosphatase activity per unit fresh cluster root mass (µmol *p*-NP $g^{-1}s^{-1}$).

*Statistical analyses*

Statistical analyses were conducted using Statistica 8.0 (Statsoft Inc., USA). The AP data for *P. repens* in different soils types were analysed for statistical significance using the Newman-Keuls multiple range tests after a one-way ANOVA. Correlation matrices were used to correlate AP activities and soil parameters. Acid phosphatase activities of multiple species on the same soil were analysed for significant differences using a nested ANOVA, where soil type and species were treated as factors, followed by a Newman-Keuls multiple range test. All statistics were performed at the $p < 0.05$ level.

## Results

*Soil*

Sandstone soils, Table Mountain sands and the red Stellenbosch clay were highly acidic, low in available P, and $Fe^{2+}$ was the dominant metal cation, whereas the Strandveld and limestone soils were characterised by higher $Ca^{2+}$, alkaline pH, and 2 -4 fold higher P availability (Table 2.1). Organic matter was lowest in Strandveld aeolian sand (0.55% C) and ranged from 1 to 2% C in the other soils.

*Acid phosphatase activity of Protea repens*

Acid phosphatase activity of *P. repens* differed significantly between soils ($p < 0.001$; Fig. 2.2). Trends were similar in 2007 (data not shown) and 2008. Activity was highest in plants from acidic Table Mountain sands and lowest in calcareous sands (Fig. 2.3a). Acid phosphatase activity was negatively correlated with soil pH ($r = -0.593$; -0.688 in 2007 and 2008, respectively) and available P (defined as Olsen P for alkaline soils and Bray-II P for acidic soils) ($r = -0.623$; -0.565 in 2007 and 2008, respectively) (Fig. 2.3a & b). Soil organic matter (%C) and availability of $Ca^{2+}$ and $Fe^{2+}$ had no significant effect on AP activity.

*Acid phosphatase activity of mixed Proteaceae populations on varying soil types*

Where several species were sampled from the same soil, some differences were observed between species (Fig. 2.4). For example, AP activity of *P. repens* in Strandveld and limestone was significantly higher than that of *Ld. foedum* and *P. obtusifolia*, respectively

($p \leq 0.05$) (Fig. 2.4). However, these interspecific differences were smaller than the effect of site (Fig. 2.4, $p = 0.146$ and $p < 0.001$, nested ANOVA for species and sites, respectively). For example, AP activities of plants sampled in limestone soils were significantly lower ($p < 0.001$) than the activities of *Ls. praecox* and *Ld. salignum* in a neighbouring sandstone soil (Figs. 2.4 and 2.5). Soil pH was weakly negatively correlated with AP activity of grouped species ($r = -0.353$, Fig. 5a). Unlike *P. repens*, P-availability was not a significant determinant of AP activity of grouped species (Fig. 2.5b). This was mostly due to the high AP activity of plants in aeolian strandveld sand, despite a relatively high available P (Fig. 2.5b).

**Table 2.1**: Chemical characteristics of soils from study sites. Values are means of four replicates ± standard errors. Letters indicate differences between soils at the $p < 0.05$ level (Newman-Keuls multiple range test after one-way ANOVA).

| **Site** | **Cape Point** | **Stellenbosch** | **Albertinia I** | **Albertinia II** | **Hopefield** |
|---|---|---|---|---|---|
| **Soil type** | Table mountain sand | Red clay/sand | Sand | Calcareous sand | Aeolian sand |
| **Parent material** | Table Mountain Sandstone | Shale/sandstone | Sandstone | Limestone | Aeolian sand |
| **pH (KCl)** | 4.33 ± 0.19 $^{a}$ | 4.63 ± 0.15 $^{a}$ | 5.55 ± 0.15 $^{b}$ | 7.75 ± 0.10 $^{d}$ | 6.45 ± 0.61 $^{c}$ |
| **Bray II P (mg kg$^{-1}$)** | 2.01 ± 0.35 $^{a}$ | 1.90 ± 0.24 $^{ab}$ | 1.90 ± 0.24 $^{a}$ | 4.0 ± 0.98 $^{b}$ | 113.01 ± 34.72 $^{c}$ |
| **Olsen P (mg kg$^{-1}$)** | not determined | not determined | 0.67 ± 0.5 $^{a}$ | 4.98 ± 0.98 $^{c}$ | 8.42 ± 0.76 $^{c}$ |
| **Fe (mg kg$^{-1}$)** | 13.37 ± 4.09 $^{a}$ | 11.06 ± 4.70 $^{a}$ | not determined | not determined | 9.62 ± 1.91 $^{a}$ |
| **Ca (mg kg$^{-1}$)** | 3.28 ± 0.91 $^{a}$ | 2.24 ± 0.41 $^{a}$ | 2.02 ± 0.34 $^{a}$ | 15.46 ± 4.33 $^{ab}$ | 23.51 ± 11.05 $^{b}$ |
| **C (%)** | 1.02 ± 0.37 $^{ab}$ | 1.25 ± 0.30 $^{b}$ | 2.02 ± 0.10 $^{b}$ | 1.49 ± 0.22 $^{b}$ | 0.55 ± 0.06 $^{a}$ |

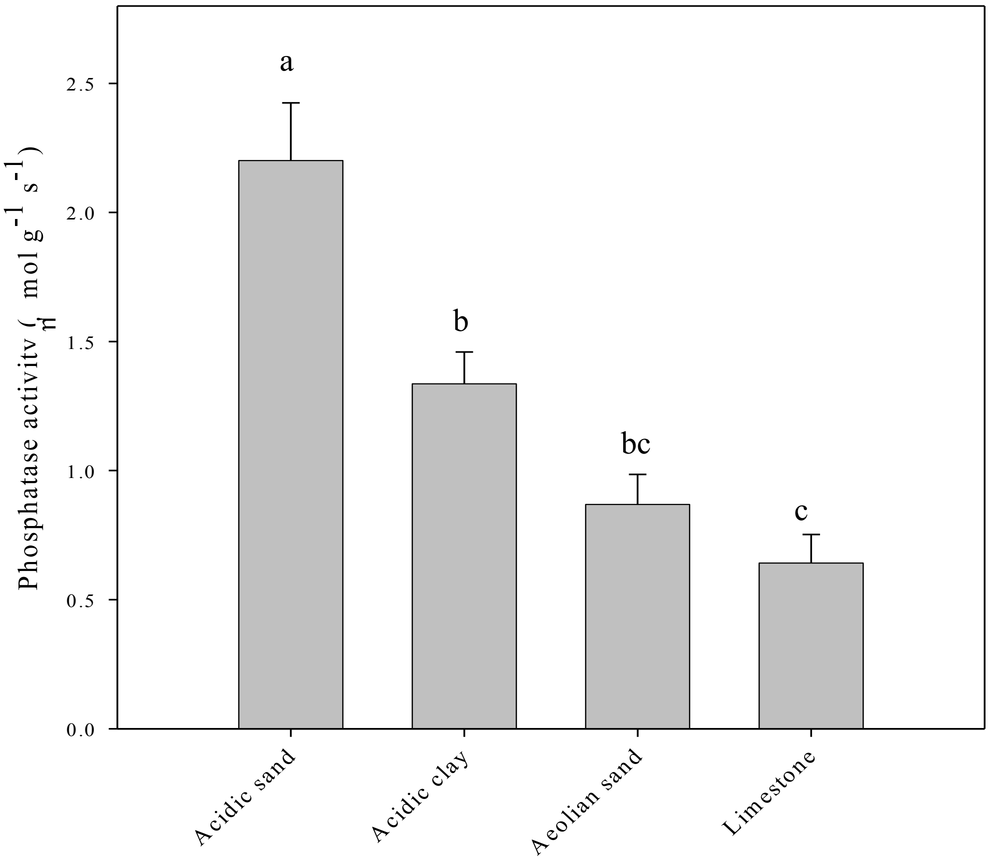

**Fig. 2.2**: Acid phosphatase activity of cluster roots of *Protea repens* harvested from various Fynbos soil types in September, 2008. Values are means ± standard error (n ≥ 4). Letters indicate significant differences at $p < 0.05$ as determined by post-hoc Newman-Keuls multiple range test.

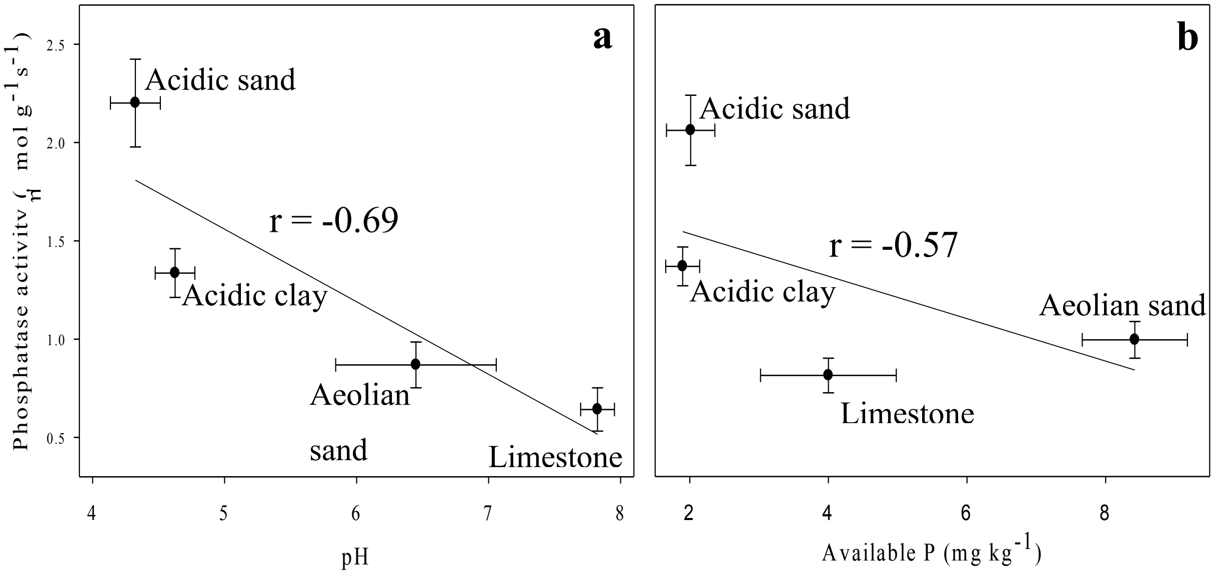

**Fig. 2.3**: Acid phosphatase activity of cluster roots of *Protea repens* harvested from soils varying in a) pH and b) available phosphate. Values are means ± standard error (n ≥ 4).

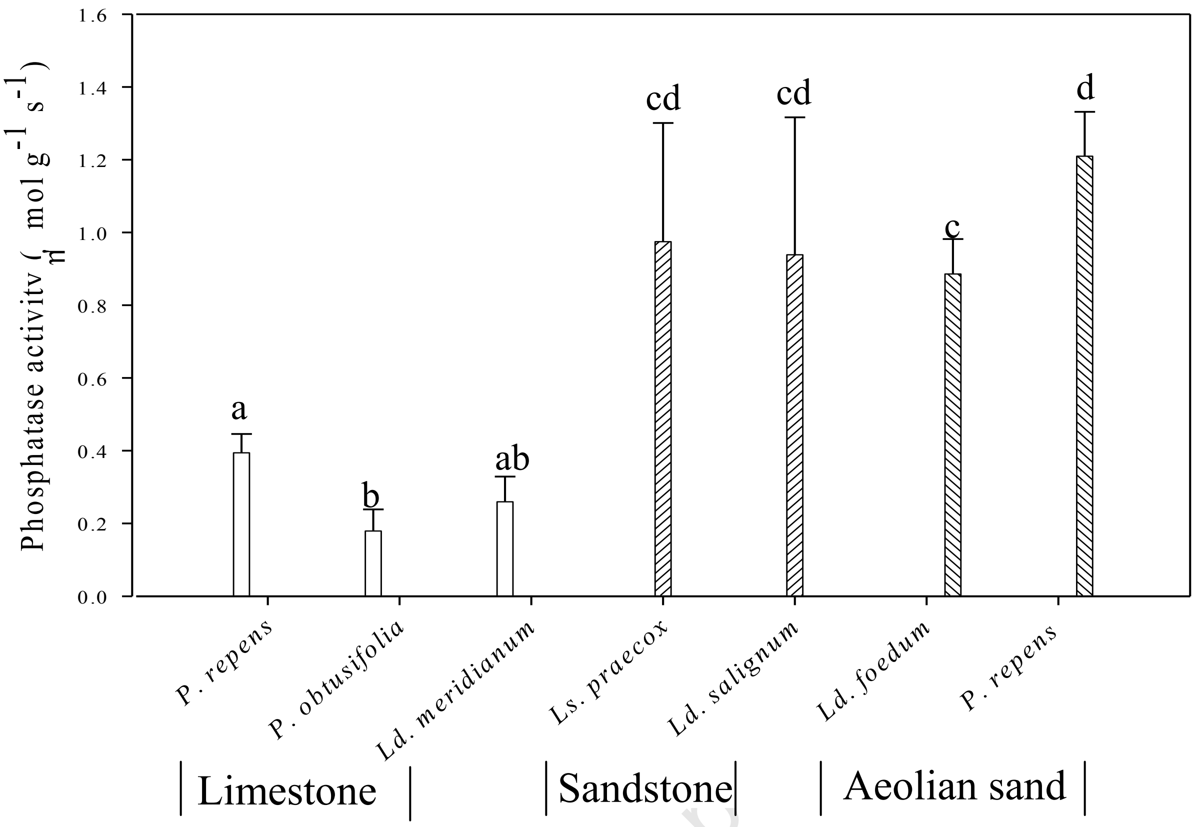

**Fig. 2.4**: Acid phosphatase activity of cluster roots of Proteaceae species harvested from various Fynbos soils in September, 2007. Values are means ± standard error (n ≥ 4). Letters indicate significant differences at $p < 0.05$ as determined by post-hoc Newman-Keuls multiple range test.

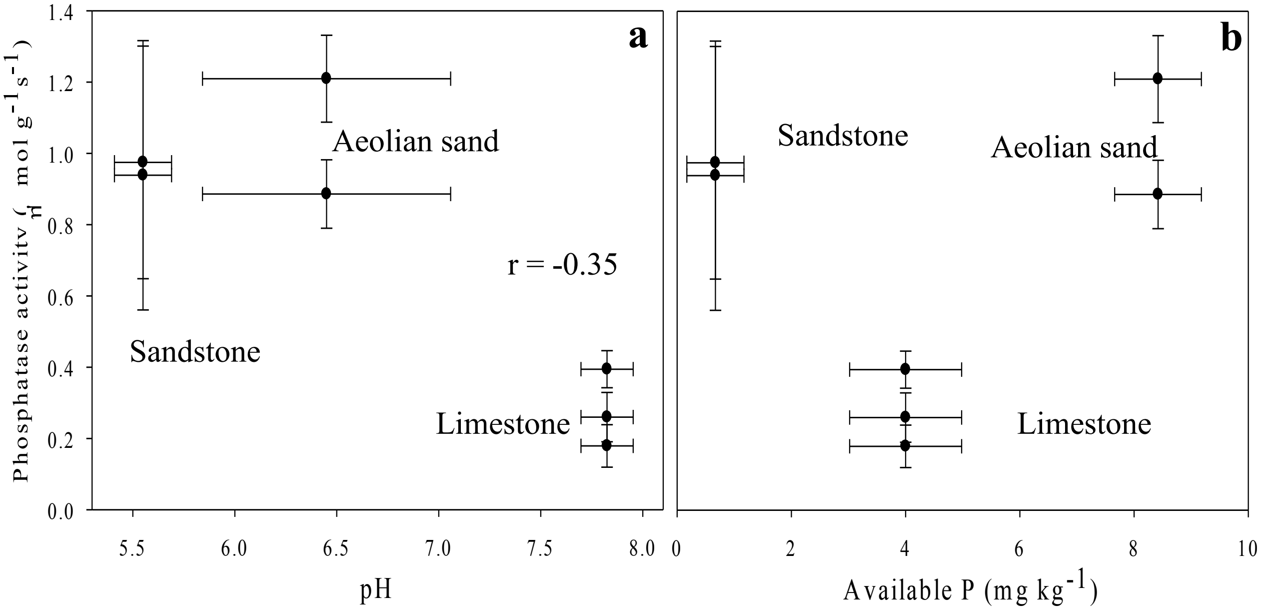

**Fig. 2.5**: Acid phosphatase activities of cluster roots of several Proteaceae species, in soils of varying a) pH and b) available phosphate. No R value is shown for (b) since AP activity was not correlated with available phosphate.

## Discussion

The similarities in AP activity of species within each soil type, and the differences between soils, indicate that phosphatase secretion is not influenced solely by genotype, and is largely a function of plant-available phosphate. This is clearly demonstrated by the differing AP activities of one species, *P. repens*, in various soil types. The significant difference in AP activity ($p < 0.05$) between *P. repens* populations growing on different soil types shows that AP secretion is plastic (at least in *P. repens*), and varies with soil pH and P availability. Other edaphic factors may be involved but those directly linked to P-source, such as percentage organic C, calcium and iron, were not correlated with AP activity.

As expected, AP activity of *P. repens* was highest in soils low in available P. This is consistent with previous studies of AP-secreting plants, where AP secretion increases in P-deficient conditions (Adams and Pate, 1992; Gilbert *et al.*, 1999; Wasaki *et al.*, 1999; Gaume *et al.*, 2001; Playsted *et al.*, 2006). The higher AP activities observed in low pH soils could result from the fact that pH was highly correlated to both total P ($r = 0.95$, $p > 0.001$) and P availability ($r = 0.51$, $p = 0.002$). Therefore, at lower soil pH, P availability is likely to be relatively low and plants are more likely to express P-acquisition traits such as enhanced AP secretion. Since AP catalyses optimally below pH 7.0 (Duff *et al.*, 1994), AP secretion is less likely to be useful as an adaptation to P-deficient conditions in alkaline soils, which may account for the relatively minor investment in AP secretion observed in the alkaline limestone and aeolian soils.

Patterns in data comparing AP activity of a mixture of species of Proteaceae species in various soils was probably compromised by varying species-specific traits. For example, *P. repens* exhibited higher AP activity than neighbouring species in limestone (*P. obtusifolia*) and strandveld (*Ld. foedum*) soils. This may be why AP activity of mixed populations was correlated with soil pH ($p < 0.05$) but not clearly related to P-availability as was the case when *P. repens* was considered in isolation. However, site-specific differences were large relative to interspecific variation, indicating that AP metabolism is primarily influenced by edaphic factors.

The observed AP activities of *P. repens* and *Ld. foedum* populations in the aeolian sand (Fig. 2.4, 2.5) were not as closely correlated to soil pH and P-availability as might have been expected from observation of species in other soils. Plants sampled had a relatively high rate of AP secretion, comparable to that observed in acidic and P-deficient soils, despite occurring in a comparatively P-rich soil of pH 6.45 ± 0.61, i.e. similar to a limestone soil. It is suggested that the high rate of AP secretion in this comparatively P-sufficient soil may be linked to the poor buffering capacity of the sand, as shown by the broad range of observed soil pH (5 – 7.9). Root-induced acidification of the rhizosphere could reduce availability of soil phosphate for plant uptake, thereby increasing AP secretion in response to the resulting decline in P-availability. An alternative explanation for the relatively high AP activity observed in plants from the relatively P-rich aeolian sands is the aridity and low water holding capacity of the site, where the reduced availability of water may reduce the mass flow and diffusion, and therefore the effective availability, of P (Cramer *et al.*, 2009).

The seemingly plastic ability of *P. repens* to secrete AP at higher rates than other Proteaceae in the same soil may be a factor in the widespread distribution range of the species, allowing it to establish on a wide range of soils by accessing organic P forms, irrespective of the dominant form of inorganic P. In contrast, the low AP activity in *Ld. meridianum* and *P. obtusifolia*, the limestone species, indicates that AP-hydrolysed organic P is likely to contribute less to plant nutrition in richer alkaline soils. Possibly, in adapting to these comparatively rich soils, limestone species favoured P-acquisition strategies such as oxalate exudation in order to access P from inorganic calcium phosphate minerals, as observed in *Grevillea crithmifolia* (Shane and Lambers, 2005b) or acidifuge forest species (Strom *et al.*, 1994), and do not require the enhanced ability to secrete AP. Alternatively, limestone-adapted Proteaceae may have root alkaline phosphatase or be capable of enhanced AP secretion under P-deficient conditions but invest preferentially in other P-acquisition strategies.

## Conclusion

The secretion of acid phosphatase from cluster roots of Proteaceae is clearly an adaptation to P-deficient conditions, increasing plant-available phosphate by enzymatic hydrolysis of organic compounds such as phosphate esters. The inverse relationship between soil [P] and AP secretion in *Protea repens* demonstrated that AP activity of a plant population can be plastic, increasing or decreasing depending on soil P availability and presumably, according to internal plant phosphate status. This strategy was of variable importance according to species and soil type, where the highest AP secretion occured in plants adapted to highly nutrient-poor, acidic soils, and the lowest AP activity was observed in richer alkaline soils. The proliferation of dense mats of AP-secreting roots in soil profiles of high organic matter content clearly demonstrates an adaptation intended to enhance nutrient acquisition. While it was evident that AP activity differed both within and between species and is a likely adaptation to low soil [P], the absolute contribution of secreted AP to plant P-acquisition cannot be inferred from these results. Further study of AP secretion as a plant adaptation to phosphate-limitations is required in order to understand the contribution of phosphatase to plant P-acquisition and the dynamics of soil nutrient cycling.

# Chapter 3 - The availability of three forms of sparingly soluble phosphate to Proteaceae from dissimilar edaphic habitats

## Introduction

The soils of the Fynbos in the Cape Floristic Region, South Africa, are generally considered to be particularly low in available phosphate (P) (e.g. Kruger *et al.*, 1983). The majority of Fynbos soil P occurs in stable and sparingly soluble forms, though the composition of sparingly soluble P (SSP) differs widely between soil types (Witkowski and Mitchell, 1987). Soil P content and speciation is largely dependent on parent material, pH and age (Tisdale *et al.*, 1993). In calcareous soils derived from limestone or aeolian sands, inorganic P largely occurs as calcium-bound complexes/mineral salts such as hydroxyapatite. In acidic soils derived from sandstone or granite, inorganic P predominates as sparingly soluble Fe/Al complexes and minerals (Witkowski and Mitchell, 1987). Soil organic matter content is highly variable, but organic SSP may account for up to 60% of total soil P in Fynbos soils (Mitchell *et al.*, 1984), largely occurring as phosphate esters such as phytic acid (inositol hexaphosphate, IHP) (Vincent *et al.*, 1992; Tisdale *et al.*, 1993). The majority of Fynbos soil organic P is deposited by litterfall and deposition of ash from fire (Lamont, 1982; Mitchell *et al.*, 1986; Witkowski and Mitchell, 1987).

Proteaceae form cluster roots as an adaptation to the P-limited soils of the Fynbos and other nutrient-poor biomes, enhancing the acquisition of SSP sources by exuding P-solubilising compounds into the rhizosphere, notably carboxylates and acid phosphatases (AP) (Lambers *et al.*, 2006). Both the speciation of soil P-forms and the efficacy of P-solubilising root exudates are influenced by complex coordination of biological, climatic and chemical factors (Marschner, 1995). Thus, the contribution of root-exuded compounds to P-nutrition of Proteaceae must be highly variable between the heterogenous soils of the Fynbos. It has been previously suggested that edaphic confinement of Fynbos natives such as the Proteaceae may be associated with specific adaptations to native soil nutrition (Mustart and Cowling, 1993; Richards *et al.*, 1997). Species from dissimilar soils are unlikely to favour the same P-acquisition strategies. For example, limestone endemic species invest less in AP secretion than calcifuge species in acidic low-P soils (Chapter 2), and exude a different range of carboxylates (Roelofs *et al.*, 2001). Proteaceae endemic to P-deficient and comparatively P-fertile limestone soils differ in ability to regulate P-uptake, suggesting specific adaptations to native nutrient availability (Shane *et al.*, 2008). While it is likely that edaphically endemic Proteaceae in dissimilar habitats (e.g. calcicole and calcifuge species) acquire P from different sources (e.g. CaP or FeP), it is unclear whether P-acquisition mechanisms/strategies differ between edaphically specialised Proteaceae, and whether preferences for native forms of soil P contribute to edaphic endemism.

Studies comparing edaphically specialised Proteaceae generally select species from adjacent but contrasting habitats whose distribution is considered non-overlapping. For example, the limestone-endemic calcicole species, *Leucadendron meridianum* (I. Williams) and *Proteaceae obtusifolia* (H. Buek. ex Meisn.), have been frequently compared to calcifuge species occurring on adjacent soils, such as *P. compacta* (R. Br.) (Esler *et al.,* 1989; Mustart and Cowling, 1993; Shane *et al.*, 2008). The limestone soils to which *Ld. meridianum* and *P. obtusifolia* are endemic are considered relatively nutrient-rich, where the majority of soil P is associated with Ca and is relatively available (Witkowski and Mitchell, 1987; Mustart and Cowling, 1993), whereas *P. compacta* and *Ld. coniferum* (L. Meisn.) are typical of acidic colluvial sands (Mustart and Cowling, 1993) where P is relatively less available in the form of Fe complexes/minerals or as organic matter (Witkowski and Mitchell, 1987). *Leucadendron foedum* (I. Williams) is endemic to calcareous aeolian sands (Rourke, 1980), which are typically high in total and Ca-associated P and low in organic matter (Witkowski and Mitchell, 1987). *Protea repens* (L.) is considered an edaphically adaptable generalist (Rourke, 1980), and can be found on a wide variety of soil types (Rebelo, 1995), making it of particular interest to the study of edaphic specialisation.

To determine whether Proteaceae from dissimilar soils differed in P-acquisition ability, the six species of edaphically specialised Proteaceae mentioned above were grown in pots of acid-washed sand and supplied with three forms of sparingly soluble P. The SSP forms tested included an organic P, magnesium inositol hexaphosphate (MgIHP), and two inorganic P forms, hydroxyapatite, $Ca_5(PO_4)_3(OH)$, and ferric phosphate, ($FePO_4$), all of which have been found previously to be comparable to soluble $KH_2PO_4$ as a P source for cluster root-producing *Lupinus albus* (Adams and Pate, 1992; Sas *et al.*, 2001). Thus these P sources should be at least partially accessible to cluster-rooted Proteaceae. Determining the availability of MgIHP to Proteaceae was of particular interest, as organic matter frequently accounts for the majority of Fynbos soil P (Witkowski and Mitchell, 1987), yet the potential total contribution of organic P to nutrition of Proteaceae is unknown. This chapter tested hypotheses 2, 3 and 4 of the thesis, namely: AP activity is enhanced or suppressed in response to P-form and P-availability in soils; edaphically endemic Proteaceae are adapted to P-forms found in their respective acid, aeolian or calcareous sandy soils; and organic P forms such as MgIHP make a similar contribution to P nutrition compared to inorganic P. This may partially explain the edaphic specialisation for which the Proteaceae have been noted (e.g. Cowling *et al.*, 1992; Mustart and Cowling, 1993). Quantifying the potential availability of each form of SSP to Proteaceae would contribute to the broader study of cluster roots as an adaptation to P-limited soils, and could be used to inform researchers of the potential importance of each P-source in assessing soil nutrient availability.

## Materials and Methods

### *Plant growth*

Seeds were germinated in a coarse 'Fynbos' soil mix (Master Organics, Ottery, Cape Town), under a day/night temperature of 25/18°C. Once seedlings had accumulated a minimum of 20 mature leaves (10 in the case of the larger-leafed *P. compacta* and *P. obtusifolia*), they were transplanted to 10 L pots containing equal parts of 2 mm and 0.5 mm grain-size acid-washed sand, thoroughly mixed in a cement mixer with finely ground magnesium inositol hexaphosphate (MgIHP), hydroxyapatite, $Ca_5(PO_4)_3(OH)$ (CaP), or ferric phosphate, $FePO_4$ (FeP) fertilisers at a rate of 10 $\mu g\ P\ g^{-1}$ (n = 15). Pots were transferred to a glasshouse and arranged in a randomised complete block design. Pots were irrigated to field capacity with P-free nutrient solution (see Appendix) solution three to five times per week, the frequency varying according to seasonal conditions.

### *Plant measurement and harvest*

Plant height and leaf number data were recorded every 4 weeks. After 9 to 12 months growth, plants were harvested and separated into stem, leaves, roots and cluster roots. After measuring total leaf area, final stem length and leaf number, all plant parts were oven-dried for 3 d at 70°C and their mass determined. Stem length was used to calculate relative stem elongation rate.

### *Acid phosphatase assay*

Acid phosphatase activity was assayed according to the method of Gilbert *et al*. (1999). The rinsed cluster roots were gently blotted dry, weighed, and placed in 4 ml of 15 mM MES with 500 µM $CaCl_2$ (pH 5.5) with 10 mM *p*-nitrophenyl phosphate (*p*-NPP) at 25°C. Roots were incubated for 30 min, before the reaction was terminated by the addition of 6ml of 0.25M NaOH. Root AP activity was calculated from the amount of *p*-nitrophenol (*p*-NP) released as measured spectrophotometrically at 412 nm, relative to known *p*-NP standards, and expressed as acid phosphatase activity per unit dried cluster root mass (µmol *p*-NP $g^{-1}$ $s^{-1}$).

### *Determination of leaf and sand phosphate concentration*

Approximately 0.1g of milled oven-dried leaf tissue was digested using the triacid method of Piper (1950). Phosphate content of the digest solution was determined colorimetrically using the molybdenum blue method (Holman, 1943). Availability of P from samples of SSP-treated sand was determined by extraction with 1N $NH_4F$ (Bray II P) (Benton Jones, 2001), followed by colorimetric determination of extractant P content using the molybdenum blue method (Holman, 1943).

### *Statistical analyses*

Statistical analyses were conducted using Statistica 8.0 (Statsoft Inc., USA). Differences in plant growth between P-treatments were tested for statistical significance (critical $p < 0.05$) using the Newman-Keuls multiple range tests after a one-way ANOVA. Pearson correlation coefficients were determined for cluster root mass, percentage cluster root/total root mass, height, shoot mass and biomass. Inclusion of Pearson correlation coefficients was contingent on the false discovery rate criteria (Benjamini and Hochberg, 1995). Thus correlation coefficients were only included when $\alpha_i < 0.05$, where $\alpha_i = p_i\, n/i$ and $i$ = the rank order of the probability of the individual Pearson correlation coefficient ($p_i$) in $n$ total correlation comparisons.

## Results

### *Plant growth*

All plants appeared healthy and free of pests and diseases throughout the growing period (Fig. 3.1). The only plants which exhibited clear visual symptoms of P-deficiency were *P. obtusifolia* seedlings supplied with MgIHP and FeP (Fig. 3.2). In general, species from alkaline soils (aeolian and limestone parent materials) showed a preference for CaP as a P-source while species originating from acidic soils (sandstone and granitic parent materials) were less sensitive to the P-source (Fig. 3.3). Growth of the two calcifuge species, *P. compacta* and *Ld. coniferum*, did not vary between P treatments (Fig. 3.3, Table 3.1). In *P. compacta*, cluster root mass was not correlated with plant growth, whereas in *Ld. coniferum* it was correlated with biomass ($r = 0.45$, $p = 0.008$) (Fig. 3.4, Table 3.2 and 3.3).

**Fig. 3.1**: *Protea compacta* seedlings in acid-washed sand, supplied with sparingly soluble phosphate sources (CaP, FeP or MgIHP). Plants were largely healthy and free of pests and disease.

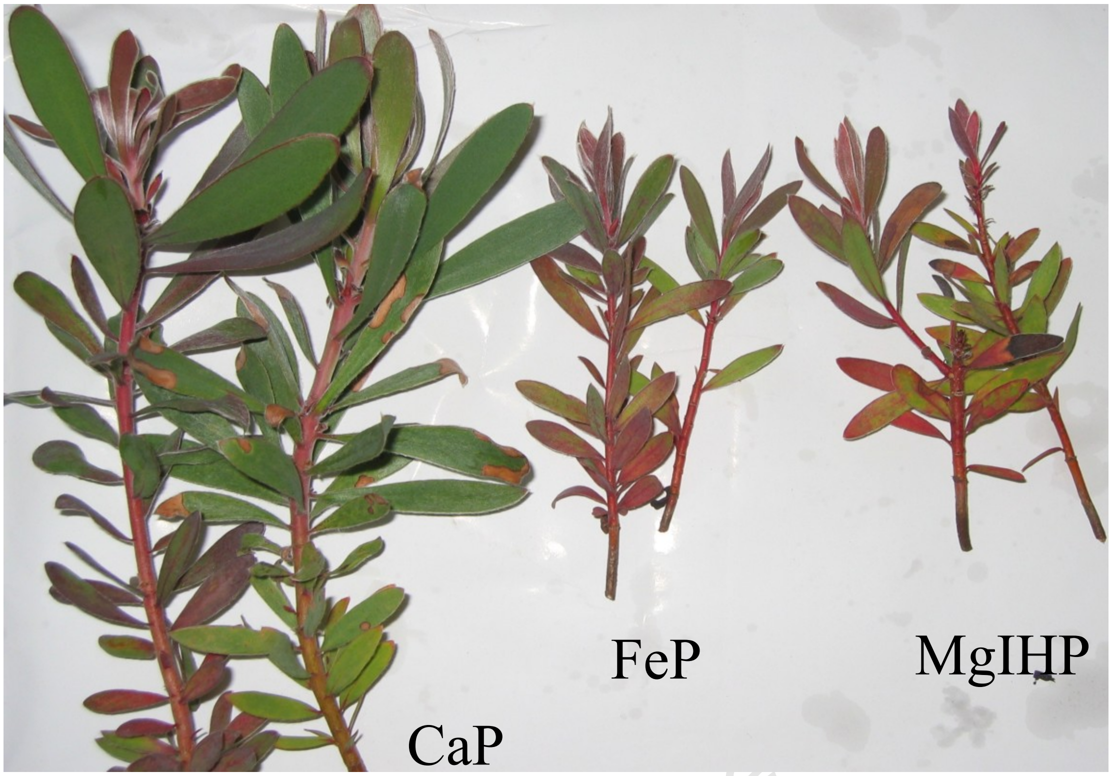

**Fig. 3.2**: Stems of *Protea obtusifolia* seedlings supplied with CaP, FeP and MgIHP. Plants supplied with the latter two treatments showed visible symptoms of phosphate deficiency e.g. reduced shoot growth, red/purple leaves.

Seedlings of the generalist species *P. repens* supplied with CaP grew significantly taller and accumulated more biomass (Fig. 3.3, Table 3.1), with a greater mass of cluster roots than those supplied with FeP or MgIHP (Fig. 3.4, Table 3.1). Although leaf number and total leaf area did not differ between treatments, shoot mass was greatest in CaP-supplied plants (Table 3.1). Cluster root mass of *P. repens* was significantly correlated with plant height, shoot mass and biomass (Table 3.2). Seedlings of the Strandveld species, *Ld. foedum*, grew tallest and accumulated the most biomass when supplied with CaP, while FeP-supplied plants grew the least (Fig. 3.3, Table 3.1). The relative investment in cluster roots (i.e. % cluster root mass/total root mass) by the larger CaP plants was significantly less than in MgIHP and FeP-supplied plants (Fig. 3.4, Table 3.1). Uniquely among the species sampled, investment in cluster roots (% cluster root/total root mass) by *Ld. foedum* was negatively correlated with stem elongation, shoot mass and biomass whereas total cluster root mass was not correlated with growth of *Ld. foedum* (Table 3.2).

The two limestone or calcicole species, *Ld. meridianum* and *P. obtusifolia*, differed in their responses to P treatment. *Leucadendron meridianum* seedling growth showed very little variation with P-supply (Fig. 3.3, Table 3.1). The only significant differences were in total cluster root mass and the ratio of cluster root/normal root, where more biomass was allocated to cluster root development in FeP and MgIHP-supplied plants than those supplied with CaP (Fig. 3.4, Table 3.1). Cluster root mass was weakly correlated with stem elongation and biomass (Table 3.2). Differences in growth of *P. obtusifolia* were readily discernable between treatments (Fig. 3.2). Seedlings supplied with MgIHP and FeP were significantly smaller in all measured dimensions than those supplied with CaP (Fig. 3.3, Table 3.1). Cluster root mass was significantly higher in CaP-supplied plants (Fig. 3.4, Table 3.1), and was strongly correlated with SER, shoot mass and biomass (Table 3.2).

*Substrate phosphate availability and leaf phosphate concentration*

The initial Bray II-extractable P was highest in sands treated with CaP, where approximately 27% of total P was extracted *by* $NH_4F$, followed by FeP (17%) and MgIHP (10%). Therefore, initial P-availability was estimated to be approximately 2.7, 1.7 and 1 $\mu g\ P\ g^{-1}$. In all species except *Ld. coniferum* and *P. obtusifolia*, leaf % P was highest in CaP-supplied plants, but did not differ between MgIHP and FeP (Fig. 3.4).

*Phosphatase activity*

During the plant harvest, viable cluster roots of the approximate maturity described by Shane *et al*. (2004a) were found on all plants except *P. compacta* and *Ld. coniferum*. Significant differences in cluster root acid phosphatase (AP) activities were observed between treatments in all remaining species except *P. obtusifolia*, the activity of which was almost negligible (Fig. 3.4). Roots collected from CaP-supplied *P. repens, Ld. foedum* and *Ld. meridianum* secreted less AP than those supplied with FeP or MgIHP. In the latter two species, AP secretion did not differ between FeP and MgIHP treatments, whereas in *P. repens* the activity of MgIHP-supplied plants was greater than FeP and CaP (Fig. 3.4).

**Table 3.1**: Differences in growth of four species of Proteaceae supplied with three forms of phosphate (Ca = calcium phosphate, Fe = iron phosphate and IH = magnesium phytate (inositol phosphate)). *Protea compacta* and *Leucadendron coniferum* are not shown as these plants did not differ between treatments. Significant differences were determined by Newman-Keuls multiple range tests after a one-way ANOVA at the $p < 0.05$ level.

| | Strandveld | Generalist | Limestone | |
|---|---|---|---|---|
| | *Ld. foedum* | *P. repens* | *Ld. meridianum* | *P. obtusifolia* |
| Stem elongation rate | Ca > Fe, IH | Ca > Fe, IH | Ca > Fe, IH | Ca > Fe, IH |
| % Leaf number increase | Ca > Fe, IH | | | Ca > Fe, IH |
| Total leaf area | Ca > IH > Fe | | | Ca > Fe, IH |
| Shoot mass | Ca > Fe, IH | Ca > Fe, IH | | Ca > Fe, IH |
| Root mass | Ca > IH > Fe | | | Ca > Fe, IH |
| Biomass | Ca > IH > Fe | Ca > Fe, IH | | Ca > Fe, IH |
| Cluster root mass | IH > Fe, Ca | Ca > Fe > IH | Fe ≥ IH ≥ Ca | Ca > Fe, IH |
| % Cluster root/total root mass | Fe, IH > Ca | | Fe ≥ IH ≥ Ca | |

**Table 3.2**: Pearsons's correlation coefficients for plant growth dimensions (height, shoot mass, biomass) and cluster root mass (cluster root/total root mass in the case of *Leucadendron foedom*) by five species of Proteaceae supplied with three forms of phosphate ($p < 0.05$). *Protea compacta* cluster root mass was not correlated with plant growth.

| | | Stem elongation rate | Shoot mass | Biomass |
|---|---|---|---|---|
| *Ld. coniferum* | Cluster root mass | - | - | r = 0.45<br>p = 0.008 |
| *P. repens* | Cluster root mass | r = 0.34<br>p = 0.04 | r = 0.37<br>p = 0.02 | r = 0.49<br>p = 0.002 |
| *Ld. meridianum* | Cluster root mass | r = 0.34<br>p = 0.04 | - | r = 0.37<br>p = 0.03 |
| *P. obtusifolia* | Cluster root mass | r = 0.55<br>p <0.001 | r = 0.71<br>p <0.001 | r = 0.77<br>p <0.001 |
| *Ld. foedum* | Cluster root/total root mass | r = - 0.41<br>p = 0.01 | r = - 0.55<br>p <0.001 | r = - 0.56<br>p <0.001 |

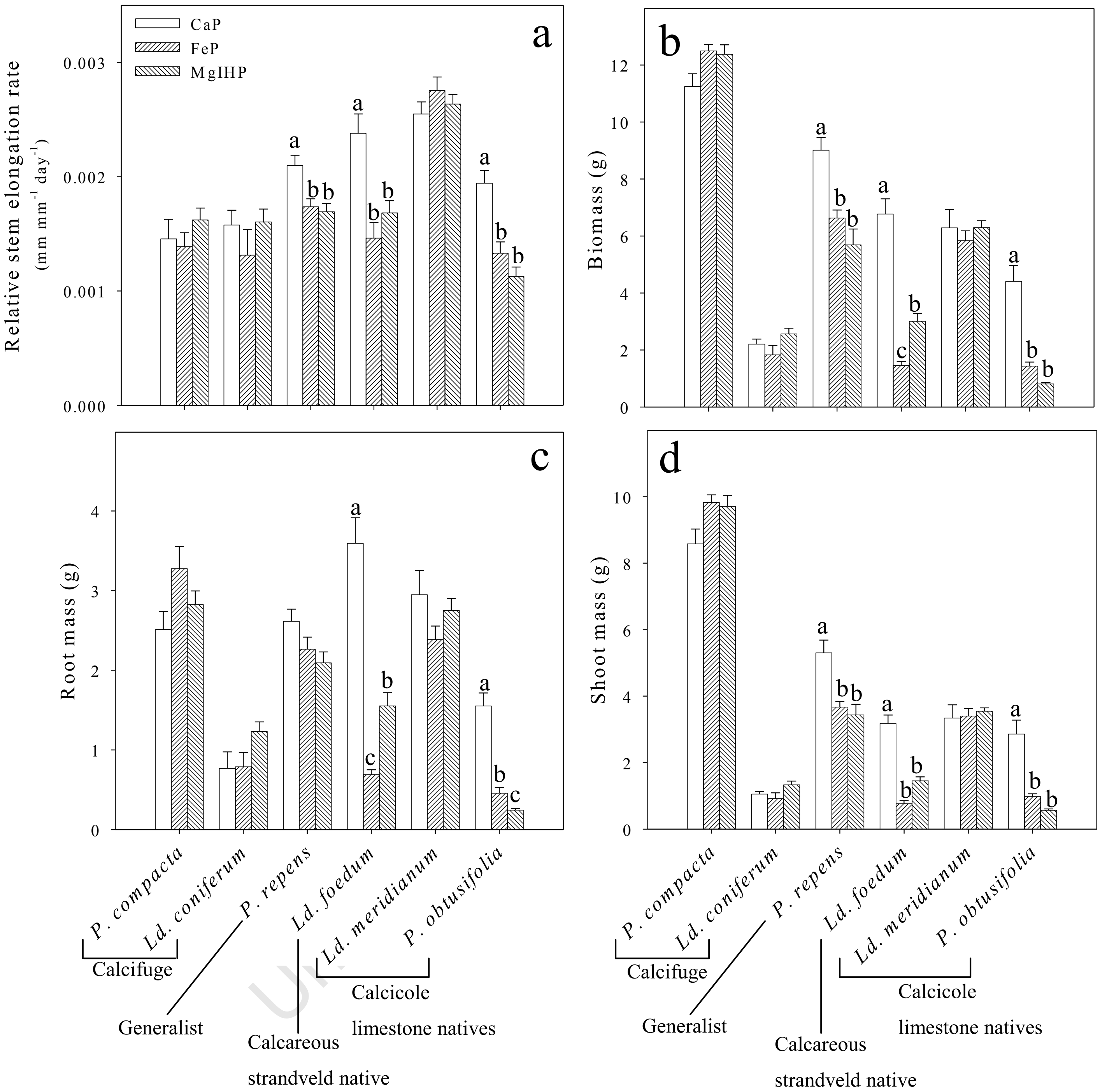

**Fig. 3.3**: Differences in a) stem elongation rate, b) biomass, c) root mass, d) shoot mass of six species of Proteaceae supplied with three forms of phosphate, CaP = calcium phosphate, FeP = iron phosphate and MgIHP = magnesium phytate. Values are means ± standard error ($11 \leq n \leq 15$). Letters indicate significant differences within a species, determined by Newman-Keuls multiple range tests after a one-way ANOVA at the $p < 0.05$ level.

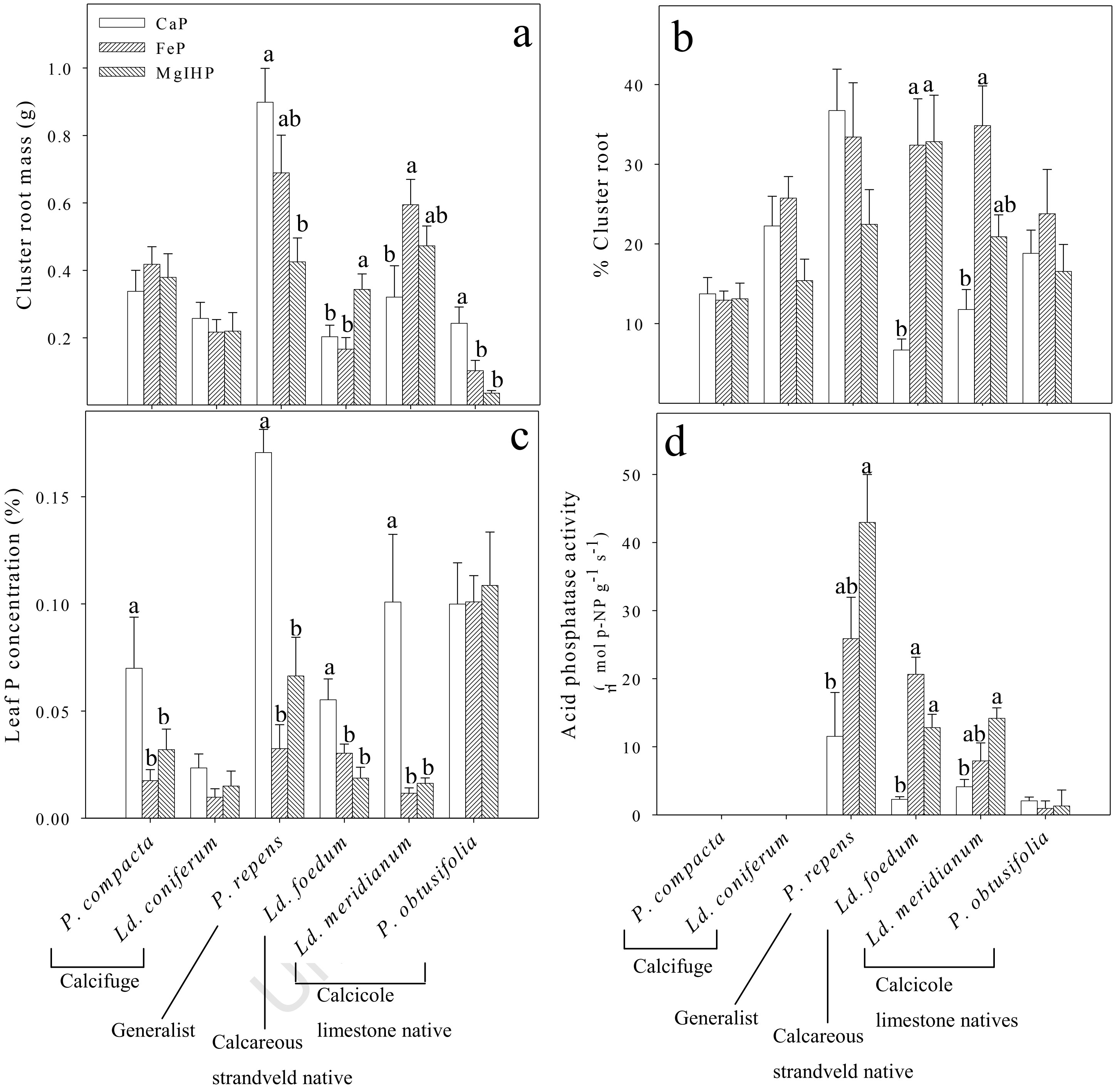

**Fig. 3.4**: Differences in a) cluster root mass, b) % cluster root/normal root mass, c) percentage leaf P concentration, d) root acid phosphatase activity of six species of Proteaceae supplied with three forms of phosphate, CaP = calcium phosphate, FeP = iron phosphate and MgIHP = magnesium phytate. Values are means ± standard error (11 ≤ n ≤ 15). Significant differences at the $p < 0.05$ level are indicated by different letters above the bars for each species after one-way ANOVA.

## Discussion

Most species exhibited some ability to access phosphate from all three sources. In all species, CaP-supplied plants accumulated similar or greater biomass than those supplied with FeP or MgIHP. This could be expected, as Bray II-extractable P was highest in sand supplied with CaP (27 % availability), followed by FeP and MgIHP (17%, 10% respectively). However, the ability to utilise the latter two phosphate sources varied between species from dissimilar soils, and was least in those species from comparatively P-rich calcareous soils. This supported hypothesis 3, namely that edaphically endemic Proteaceae are adapted to P-forms found in their respective acid, aeolian or calcareous sandy soils, particularly in the case of limestone endemics, where P occurs primarily as CaP.

Plant growth of the two calcifuge species, *P. compacta* and *Ld. coniferum*, did not differ between P treatments: both species appeared able to access P equally from organic and inorganic sources. These species are found in highly acidic and nutrient-poor soils (e.g. Esler *et al.*, 1989), where the majority of P occurs as poorly available organic matter and inorganic iron complexes/minerals (Witkowski and Mitchell, 1987). Therefore it could be predicted that these species would have an enhanced ability to utilise poorly available P.

The generalist species, *P. repens*, could be expected to be similarly able to utilise diverse SSP sources, as it occurs on a range of soil types of varying P speciation and availability. Indeed, while seedlings of *P. repens* grew taller and heavier when supplied with CaP, growth of this species was less compromised by P supply from FeP and MgIHP than was the case for the calcicole species. *Protea repens* differed from other species sampled in that the largest plants (i.e. those supplied with CaP) accumulated the most cluster root mass. In these plants cluster root mass was proportional to plant size. This could be a result of the larger plants having greater nutritional demand for P.

Although all calcicole species displayed a preference for CaP, the ability to utilise FeP and MgIHP varied between the three calcicole species studied. A preference for CaP is consistent with the calcareous aeolian sand and limestone-based soils in which these species occur, where phosphate predominates in Ca-bound forms, Fe content is low and organic matter is variable (Witkowski and Mitchell, 1987; also see previous chapter).

Both the limestone-endemic *Ld. meridianum* and calcareous Strandveld-native *Ld. foedum* produced more cluster roots and secreted more AP when supplied with FeP or MgIHP than when supplied with CaP, suggesting that the former P-sources were less available to both species. However, whereas overall seedling growth of *Ld. meridianum* was comparable between treatments, growth of *Ld. foedum* was reduced in FeP/MgIHP-supplied plants relative to those supplied with CaP. This demonstrated that *Ld. meridianum* utilised P from all three sources, although when supplied with the less available P-forms an increased investment in cluster roots was required in order to maintain growth rates comparable to that of plants supplied with CaP. In comparison, the poor growth of *Ld. foedum* supplied with FeP or MgIHP, despite an increased formation of cluster roots, suggests that the nutrient acquisition mechanisms of this species are less effective or narrower in specificity than those of sampled calcifuge species or the calcicole, *Ld. meridianum*. This would reduce the capacity of *Ld. foedum* seedlings to establish off the soils to which it is native.

In comparison to the other calcicole species, the limestone-endemic *P. obtusifolia* was entirely unable to utilise FeP or MgIHP as P-sources, where seedlings displayed typical symptoms of P-deficiency in Proteaceae, such as smaller leaves, lower biomass accumulation, and purple petioles/leaves (Van Staden, 1967). The near absence of cluster roots and negligible AP secretion of *P. obtusifolia* supplied with FeP/MgIHP contrasted with observations of other species, where plants supplied with less available SSP typically increased formation of cluster roots. This indicates a degree of phosphate deficiency not seen in other sampled species, where *P. obtusifolia* supplied with FeP or MgIHP presumably could not accumulate sufficient endogenous resources to develop cluster roots for P acquisition.

It is possible that the superior growth of *P. obtusifolia* supplied with CaP is due to specific adaptations to native soil nutrition (as suggested by Mustart and Cowling, 1993; Richards *et al.*, 1997) e.g. an enhanced ability to acquire CaP, or an inability to utilise SSP other than those bound by calcium. This explanation assumes a mechanism similar to that suggested by Strom *et al.* (1994), where European calcicole and calcifuge species differed in composition of exuded carboxylates, reflecting adaptations to native soil nutrition but restricting the ability to utilise non-native SSP. However, as *P. obtusifolia* produces smaller and less nutritious seeds (0.1 mg P/seed) than Proteaceae from highly nutrient poor soils, e.g. *P. compacta* (0.8 mg P/seed) (Esler *et al.*, 1989), young seedlings have a comparatively reduced capacity to establish and develop without supplementary P. It seems likely that endogenous P reserves in the seeds of *P. obtusifolia* are insufficient to support the development of functional and effective cluster roots, and a certain critical amount of supplementary P is required in order for seedlings to establish, which in this case may have been provided only by the most readily available of SSP tested, CaP. Therefore, while the poor growth of *P. obtusifolia* supplied with FeP and MgIHP was due to an inability to utilise either as a P source, it is unclear whether the species lacked the physiological ability to utilise these less soluble sources, or whether all three SSP are broadly available via cluster root-mediated mechanisms, provided that seedlings first have sufficient resources to develop cluster roots. Either possibility would explain the restricted geographic range of *P. obtusifolia* to limestone soils (Mustart and Cowling, 1993).

*Protea obtusifolia* and *Ld. foedum* showed a reduced ability to utilise FeP or MgIHP as a P-source, which would likely prevent seedling establishment on nutrient-poor acidic soils. Conversely, the ability to access multiple sources of phosphate would be a distinct adaptive advantage to low-P soils. For example, the widespread distribution of *P. repens* over varying soils must be partly attributable to a ready ability to acquire P from a diverse range of poorly available sources. The ability to acquire P from diverse sources can be only partly responsible for geographic distribution since edaphically restricted calcicole (*Ld. meridianum*) and calcifuge species (*P. compacta* and *Ld. coniferum*) were seemingly able to utilise native and non-native P-sources in this experiment yet are typically unable to establish off their native soils. In a reciprocal seed-sowing experiment using calcifuge and calcicole species, Mustart and Cowling (1993) found that in both P-poor colluvial sands and comparatively P-rich limestone soils, seedlings of native species outgrew non-native species, though all species germinated readily on both substrates. It has previously been reported that calcicole Proteaceae adapted to fertile limestone soils have an inherently faster growth rate than those from P-poor acidic soils (Shane *et al.*, 2008). It is suggested that, if transplanted into limestone soils of relatively high P-availability, neither calcicole nor calcifuge species will be limited by P-availability, but populations of non-native calcifuge species would be outcompeted by the faster-growing calcicole species. If transplanted into acidic P-poor soils, the faster growth of calcicole species would be limited by P-availability and outcompeted by native calcifuge species with an enhanced ability to utilise poorly available native P. Therefore, a species' edaphic distribution may be partially restricted by specific adaptations to native soil P dynamics (e.g. predominant P-form, P-availability), but is likely subject to the influence of other soil variables such as pH, water holding capacity, and availability of other macronutrients (e.g. Fe; Strom *et al.*, 1994).

In general, AP secretion was higher in those plants supplied with the least available forms of P, supporting hypothesis 2, namely that AP activity is enhanced or suppressed in response to P-form and P-availability.

## Conclusion

MgIHP was found to be comparable to inorganic FeP (and in some cases, CaP) as a sole source of P for Proteaceae in sand culture. This is consistent with previous studies of AP-secreting plants (e.g. Adams and Pate, 1992; Gilbert *et al.*, 1999), where P-availability was increased by enzymatic hydrolysis of otherwise unavailable organic sources. It is likely that a mix of organic and inorganic SSP would be more effective than an equivalent amount supplied by a single source, as AP was secreted to some degree by all plants, irrespective of the presence of MgIHP. The suppressed AP secretion of P-sufficient, CaP-supplied plants (e.g. *P. repens*, *Ld. meridianum*) shows that enzymatic hydrolysis of organic SSP is unlikely to be the primary phosphate acquisition mechanism of P-sufficient Proteaceae, but is a strategy enhanced in response to P-limited conditions. This is in agreement with the findings of the Chapter 2, where AP secretion by Proteaceae was highest in those plants growing in acidic soils of very low P-availability. It is likely that the contributions of organic matter to plant-acquired P are of greater significance to those Proteaceae growing in poorer soils.

# Chapter 4 - The effect of rhizosphere phosphate availability and supply form on acid phosphatase secretion by hydroponically-grown Proteaceae

## Introduction

Previous experiments have shown a generally inverse relationship between availability of phosphate (P) and the secretion of acid phosphatase (AP) by Proteaceae, where AP secretion by Fynbos Proteaceae was negatively correlated with soil P-availability (Chapter 2), and AP of pot-grown Proteaceae was higher in plants supplied with poorly available P-substrates (iron phosphate, magnesium inositol phosphate) than those supplied with relatively available calcium phosphate (Chapter 3). These results conform with the suggestion that root secretion of AP by Proteaceae and species from other genera is an adaptive response to P-limited environments (e.g. Reid and Bieleski, 1970; Goldstein *et al.*, 1988; Gilbert *et al.*, 1999; Gaume *et al.*, 2001; Wasaki *et al.*, 2003; Playsted *et al.*, 2006). However, the confounding influence of microbial AP, the complex dynamics of phosphate interactions with soil constituents, and the coordinated nature of P-acquisition mechanisms, challenge any study attempting to characterise the regulation of AP secretion or quantify the contribution of secreted AP to plant nutrient acquisition, (e.g. Adams and Pate 1992; Raghothama, 1999; Lung and Lim, 2006; Lambers *et al.*, 2006).

Understanding of the regulation of AP is mostly based on observations of hydroponically cultured *Lupinus albus* (Fabaceae) (Gilbert *et al.*, 1999; Miller *et al.*, 1999; Wasaki *et al*, 2003), a system in which nutrient availability can be controlled and undamaged roots can be excised for assaying of AP. In P-deficient conditions, the entire root system of *L. albus* secretes AP, although cluster roots secrete significantly greater quantities than normal root segments (Wasaki *et al.*, 1999; 2003; Gilbert *et al.*, 1999). Gilbert *et al.* (1999) identified two isoforms of AP in *L. albus*: isoform 1 (LaSAP 1), which was secreted by all portions of the root system, even in those plants supplied with moderate P; and isoform 2 (LaSAP 2), which was unique to the cluster roots of P-deficient plants. The existence of an isoform unique to P-deficiency suggests a specialised role for AP as part of a coordinated adaptive response to P-limited environments. Isoforms of AP specific to

P-deficiency were also reported in studies of P-deficient *Spirodela oligorrhiza* (Reid and Bieleski, 1970) and *Lycopersicon esculentum* (Goldstein *et al.*, 1988), in addition to at least one form of AP which was expressed when P availability was not limited. Wasaki *et al.* (2003) proposed the following scheme to describe the relationship between plant P-status and secretion of AP by *L. albus*:
i) Under P-sufficient conditions, LaSAP 1 is synthesized at low levels and accumulates at the epidermis of normal and cluster roots; ii) When external P supply is reduced, epidermis-localised LaSAP 1 is almost immediately secreted into the rhizosphere by cluster roots; iii) When plant cells experience a critical degree of internal P-deficiency, LaSAP 2 is synthesised in cluster roots and secreted freely into the rhizosphere. Total root AP activity is greatest at this phase. The respective P-hydrolysing efficiency and substrate specificities of the two isoforms of AP were not reported. However, the fact that LaSAP 1 is accumulated at the epidermis by the entire root system even under relatively P-sufficient conditions, whereas LaSAP 2 is specific to cluster roots of P-deficient plants, suggests that the two enzymes are functionally distinct.

Based on the exceptionally large quantities of AP secreted by cluster roots, and the apparent complexity of AP regulation, it is thought that the strategy of AP secretion is an important part of a coordinated adaptive response to P-limited soils, acting in coordination with other P-mobilising root exudates (e.g. Shane *et al.*, 2003; Lambers *et al.*, 2006). The function and regulation of AP secretion by Proteaceae has so far been shown to be analogous to *L. albus*, increasing in response to P-deficient conditions (Chapter 2, 3). However, it was unclear whether cluster root AP secretion was regulated only by endogenous plant P-status, or also by root response to immediate rhizosphere P-availability, as observed in *L. albus* (Wasaki *et al.*, 1999). It is not known whether multiple isoforms of AP are synthesised by Proteaceae, analogous to those observed in *L. albus*, and assay methods used previously (Chapter 2, 3) did not distinguish between epidermis-bound and freely secreted AP activity (analogous to LaSAP 1 and 2, respectively, in *L. albus*). In order to understand and model the role of AP secretion by Proteaceae as part of a coordinated adaptive response to P-limited soils, it must be established to what degree secreted AP is mobile in soil solution, i.e. whether AP is bound to the root epidermis or released into the rhizosphere. In addition, the influence of total plant P-status on AP synthesis and secretion must be distinguished from root response to immediate rhizosphere P-availability. Hydroponic culture of Proteaceae provided the ideal system in which to test these ideas.

It was hypothesised that Proteaceae would increase secretion of AP a) as a rapid root-mediated response to changes in rhizosphere P-availability, and b) as a gradual plant-mediated response to low endogenous P content. It was further hypothesised that AP secreted by Proteaceae comprised both root-bound and solution-mobile components, comparable to *L. albus*. To test these hypotheses, the effect on AP secretion of up to 72 hours of exposure to a range of concentrations of inorganic and organic P was measured in hydroponically-grown *Leucadendron foedum* (I. Williams), *Ld. coniferum* (L. Meisn.) (Proteaceae), and *L. albus* (Fabaceae). In addition, the influence on AP activity of excised cluster roots of short-term (30 min) exposure to varying P-supply forms and availabilities was investigated. α-Naphthyl phosphate (NP) was supplied as a P-substrate to *L. albus* and *Ld. foedum* as a stain for AP activity, providing a measure of the changes in AP secretion with root maturity and a practical means of comparing the function of both isoforms of AP over the lifespan of a root.

## Materials and Methods

### *Growth and preparation of plants*

Seedlings of *Ld. coniferum* and *Ld. foedum* were washed free of soil and transferred to hydroponic tanks, where they were maintained at a minimum/maximum temperature of 17/25ºC and supplied with a nutrient solution (see Appendix) with the addition of 15 mM MES buffer (pH 6.5). After approximately 3 months, P was withdrawn from nutrient solutions. Plants were maintained in P-free solution until sufficient cluster roots had formed for experimentation. Seedlings of *L. albus* were similarly grown in P-free hydroponic solution with MES for 21 d until cluster roots ranging in age from emergent to senescent could be observed occurring sequentially on lateral roots. Experiments were conducted separately over several months whenever sufficient cluster roots were available for experimentation due to the inherent difficulties of scheduling cluster root development in perennial Proteaceae. Therefore, although efforts were made to treat plants uniformly, plant age and P-status of Proteaceae could not be controlled as precisely as for the fast-growing annual *L. albus*.

### *Hydroponic phosphate treatments*

*Leucadendron coniferum* was used to compare the effects of inorganic ($KH_2PO_4$) and organic (sodium inositol phosphate, NaIHP) P on AP secretion and to investigate the rapidity of plant response to declining P-availability. Sixteen *Ld. coniferum* seedlings were randomly assigned to one of four hydroponic tanks, designated '$PO_4$', 'IHP', 'P-limited' and 'control'. Phosphate was supplied as $KH_2PO_4$ at a concentration of 10 µM over 4 d to '$PO_4$', 'IHP' and 'P-limited' plants, while P was entirely withheld from 'control' plants. After 4 d of preparatory exposure to P, 10 µM $KH_2PO_4$ and NaIHP was supplied to $PO_4$ and IHP plants, respectively, while P was withheld from P-limited as well as control plants. After commencement of treatments, cluster roots were excised and assayed for soluble and total AP activity (see below for assay method) at intervals approximating 0.5, 8, 24, 48 and 72 hours ($n = 4$) (roots from each treatment were not assayed simultaneously, as processing larger numbers of roots would lead to lengthy delays between excision and assaying. Nutrient solutions were renewed every 24 hours.

*Ld. foedum* was used to investigate the rapidity of response to increased P-availability, and was treated similarly to *Ld. coniferum*, except that plants were not supplied with P before treatment application and that $PO_4$ and IHP plants were treated with a higher concentration of P (50 µM). Cluster roots were excised and assayed for total and soluble AP activity at $t = 0.5$, 24 and 48 h ($n = 4$). Phosphate-deprived *L. albus* was treated similarly to *Ld. foedum* and supplied with 5, 30 and 90 µM $KH_2PO_4$ and NaIHP and assayed for total and soluble AP activity over 24 h. In a separate experiment, *L. albus* was supplied with 100 µM α–naphtyl phosphate ($C_{10}H_{10}NaO_5P$) as both an alternative organic P substrate and bio-indicator of APase activity. After 24 hours, roots were excised and examined for purple staining. Portions of root were examined and photographed under magnification with a Stereoscopic Zoom microscope (Nikon).

### *Incubation of excised roots in phosphate substrate*

Excised cluster roots were harvested from seedlings of *Ld. foedum* grown hydroponically in P-free solution as described above. Harvested cluster roots were immediately collected into 25°C 15 mM MES containing 500 µM $CaCl_2$ buffered at pH 5.5 and transferred to 4 ml of buffered incubation solution containing P-substrate. To test the effect of increasing rhizosphere P-availability, excised cluster roots of P-deprived *Ld. foedum* were incubated with 0, 0.1, 1, 5, 10, 20, 50 and 100 µM $KH_2PO_4$ (n = 4). In a subsequent experiment, the influence of inorganic and organic substrates was compared by assaying total and soluble AP before (n = 8) and after (n = 4) incubation with 50 µM $KH_2PO_4$ (inorganic), NaIHP (organic) or a P-free buffer solution. Roots were shaken in the buffer/substrate solution at 100 rpm for 30 min, after which they were removed and washed in distilled water. Roots were then assayed for soluble root-secreted and total cluster root AP activity.

*Total and soluble acid phosphatase assay*

Total root acid phosphatase activity was assayed by the method of Gilbert *et al.* (1999). Excised cluster roots were gently blotted dry and placed in 4 ml of 15mM MES containing 500 µM $CaCl_2$, buffered at pH 5.5, in the presence of 10 mM *p*-nitrophenyl phosphate (*n*-NPP) at 25°C. Roots and substrate were shaken at 100 rpm for 30 min, before terminating the reaction with addition of 4 ml of 1.0M NaOH. Roots were oven-dried at 70°C for 2 d and their dry mass recorded. Root AP activity was calculated by measuring the release of *p*-nitrophenol (*p*-NP) spectrophotometrically at 412 nm, relative to known *p*-NP standards, and expressed in terms of cluster root dry mass (µmol *p*-NP $g^{-1}s^{-1}$). Soluble root acid phosphatase activity was determined by placing excised roots in 4 ml of 15 mM MES containing 500 µM $CaCl_2$, buffered at pH 5.5, and shaken at 100 rpm for 30 min. After this period of soluble enzyme collection, 0.5 ml aliquots of collected exudates were added to 0.5 ml of 20 mM *p*-nitrophenyl phosphate and shaken for an additional 30 min. The reaction was terminated by addition of 1ml of 1.0 M NaOH and soluble AP calculated after determining root dry mass and measuring released *p*-NP.

*Statistical analyses*

Statistical analyses were carried out in Statistica 8.0 (Statsoft, 2008). Acid phosphatase activities were analysed for statistical significance ($p < 0.05$) using one-way ANOVA, assessing either treatment or time as a factor.

## Results

### *The effect of P-availability and supply form on AP secretion by P-sufficient Leucadendron coniferum and P-deficient Leucadendron. foedum and Lupinus albus in hydroponics*

Secretion of AP by *Ld. coniferum* differed between treatments. The highest activity was consistently observed in 'control' plants, those deprived of preparatory P (Table 4.1, Fig. 4.1a). Secretion of AP by those plants supplied with preparatory P (IHP, $PO_4$, P-limited) differed at 48 h, where 'P-limited' plants secreted significantly more AP than 'IHP' and '$PO_4$' plants, which did not differ from each other (Table 4.1, Fig. 4.1a). A temporally similar response to changes in P-availability was observed in P-deficient *Ld. foedum*, where total secretion of AP by plants supplied with P declined significantly relative to control plants within 48 hours (Table 4.1, Fig. 4.1b). Although AP of '$PO_4$' plants did not differ significantly from initial activity, AP of IHP-treated plants declined significantly from 0 to 48 h (Table 4.1, Fig. 4.1b). Relatively minor soluble AP activity was detected in the exudates of some roots from each treatment, but inconsistently and showing no general trend between treatments. Soluble AP activity was always less than 1% of total AP activity (data not shown). Total secretion of AP by *L. albus* supplied with IHP declined significantly from 12 to 24 h relative to control and $KH_2PO_4$– treated plants, which did not differ from each other (Fig. 4.2). No differences were observed between different P-concentrations. Some minor soluble activity was observed at approximately 10% of the magnitude of total AP and did not differ between treatments (data not shown).

**Table 4.1**: The relative magnitude of acid phosphatase secretion by cluster roots of two hydroponically grown Proteaceae in response to different phosphate treatments. Significant differences were determined by Newman-Keuls multiple range test after one-way ANOVA of log-transformed data, where *, **, *** signifies $p < 0.05, 0.01, 0.001$ respectively. '≈' signifies no differences between treatments.

| | *Relative magnitude of AP secretion in response to P-treatment* | |
|---|---|---|
| Time | *Ld. coniferum* | *Ld. foedum* |
| 0.5 | Control > P-limited, IHP, $PO_4$ ** | ≈ |
| 8 | Control > P-limited, IHP, $PO_4$ *** | |
| 24 | | ≈ |
| 48 | Control > P-limited > IHP, $PO_4$ *** | Control > IHP, $PO_4$ ** |
| 72 | Control > P-limited, IHP, $PO_4$ *** | |

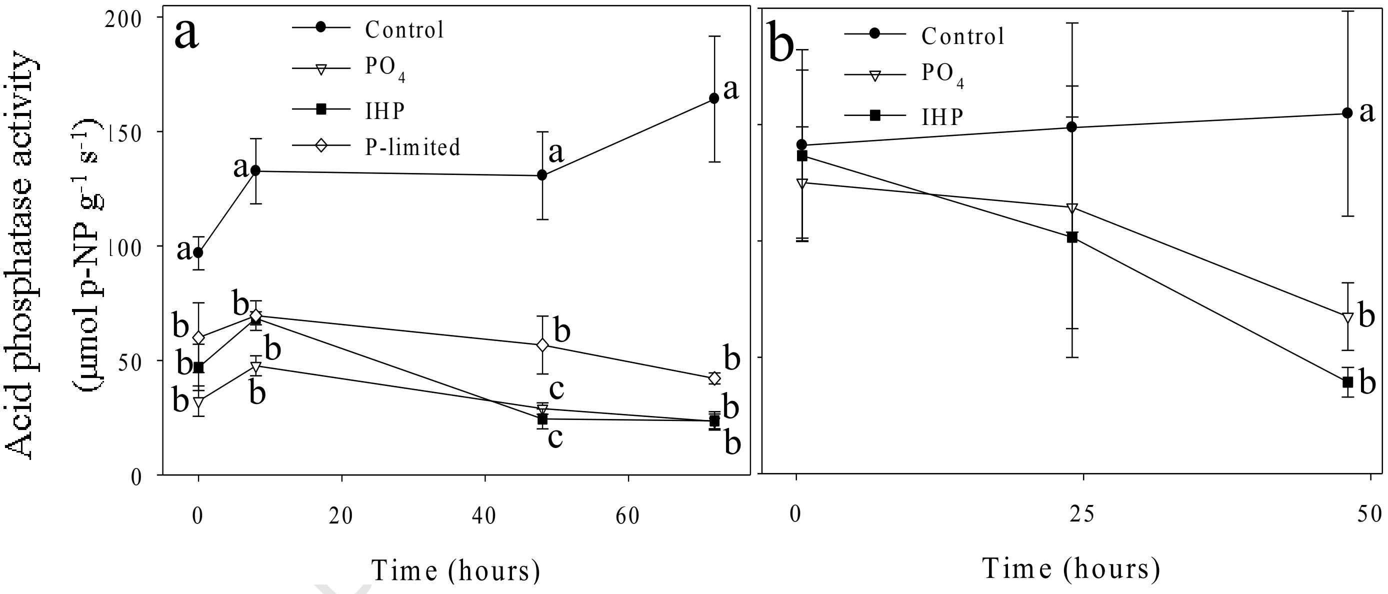

**Fig. 4.1**: Acid phosphatase activity of cluster roots of **a)** P-sufficient *Leucadendron coniferum* and **b)** P-deficient *Ld. foedum,* in response to application of 10 µM and 50 µM of P, respectively, supplied as NaIHP (IHP) or $KH_2PO_4$ ($PO_4$). P-deficient plants were supplied with and then deprived of P at t = 0 h, while control plants continued to be deprived of P. Values are means ± standard error ($n \geq 4$). Letters indicate significant differences at $p < 0.05$ as determined by post-hoc Newman-Keuls multiple range test of log-transformed data.

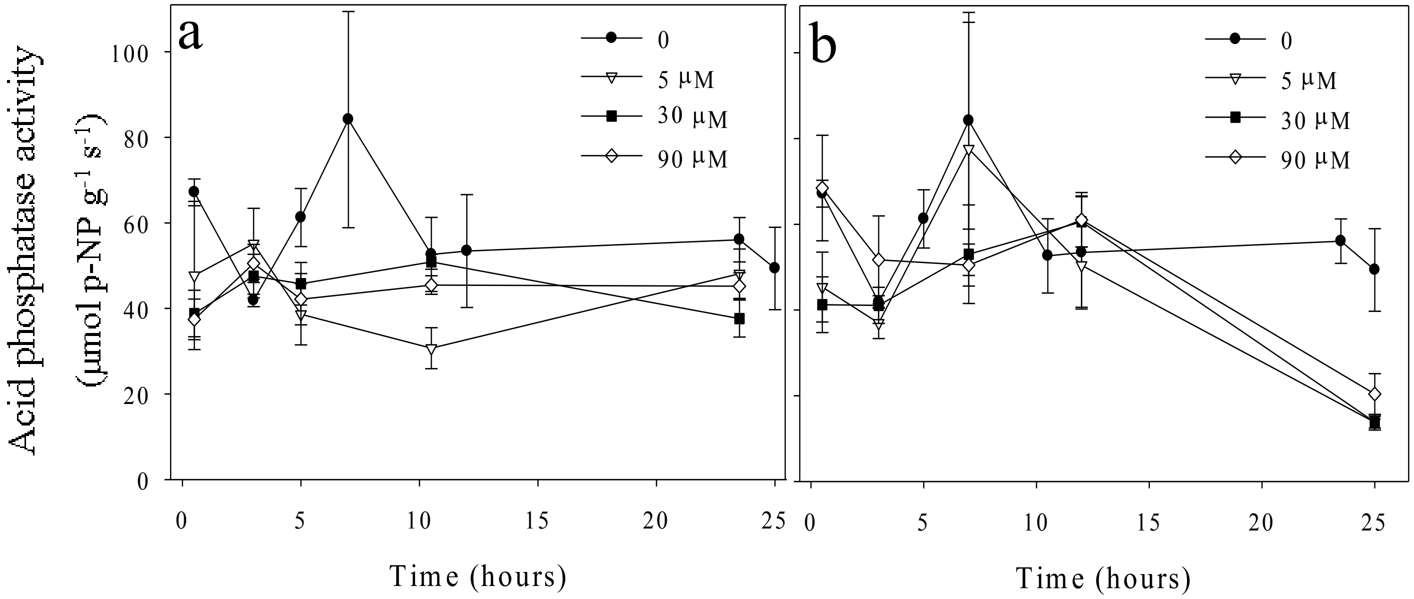

**Fig. 4.2**: Total acid phosphatase activity of cluster roots of *Lupinus albus* supplied with 5, 30 and 90 of μM **a)** $KH_2PO_4$ or **b)** NaIHP, relative to an untreated control. Values are means ± standard error ($n \geq 4$). Treatments did not differ except at t = 25 ($p < 0.01$), as determined by post-hoc Newman-Keuls multiple range test.

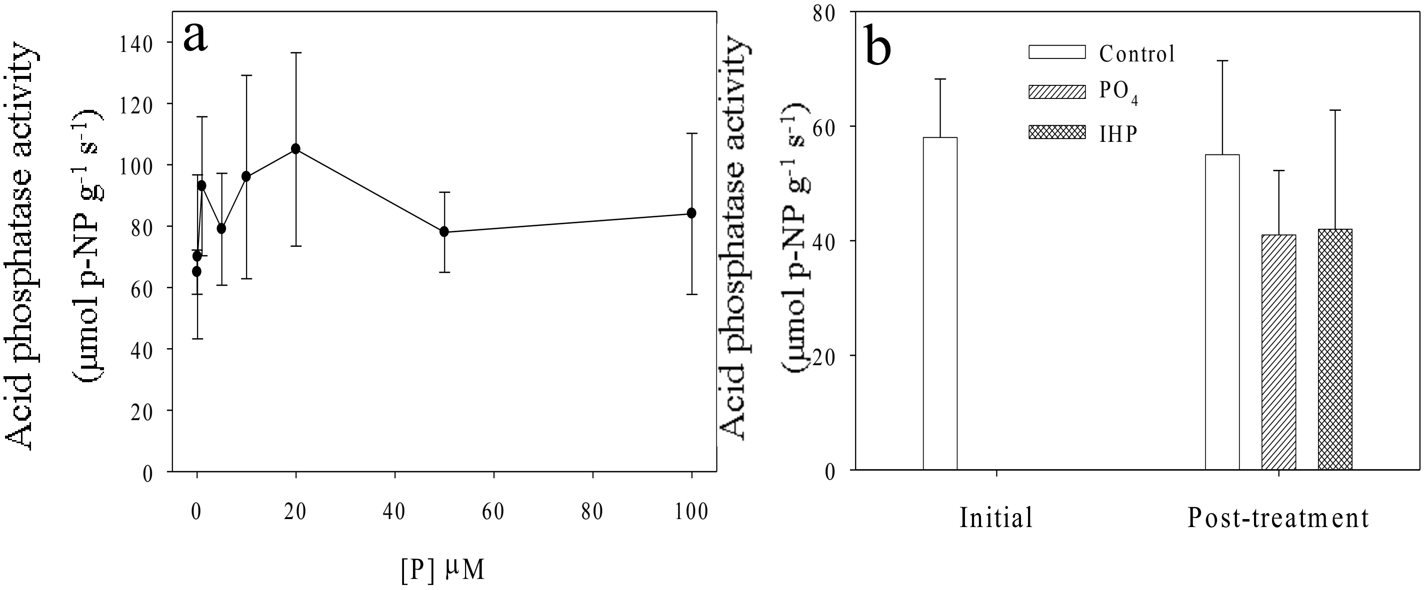

**Fig. 4.3**: Acid phosphatase activity of excised cluster roots of P-deficient *Leucadendron foedum* after 30 min of incubation with **a**) increasing concentrations of $KH_2PO_4$ or **b**) P-free buffer solution, 50 μM $KH_2PO_4$ or NaIHP (Control, $PO_4$, or NaIHP, respectively). Values are means ± standard error ($n \geq 4$). No differences were noted between treatments in either experiment.

### *The effect of P-availability and supply form on total and soluble AP of excised cluster roots of Ld. foedum*

Total AP of excised cluster roots was unaffected by 30 min of exposure to P substrate, irrespective of concentration or supply form (Fig. 4.3a, b). Small amounts of soluble AP activity were observed in the exudates of some roots, but this was very inconsistent (data not shown).

### *Staining of L. albus root tissue by α-napthyl-phosphate*

Purple staining was observed on the surface of young root tissue of *L. albus*, particularly on the epidermis of tertiary rootlets of emergent or young cluster roots (Fig. 4.4 a – b; 4.5 a - f). No staining was observed on mature or senescent roots of *L. albus* (Fig. 4.4 c), and could not be induced in *Ld. foedum* in roots of any age (Fig. 4.4 d - f). Purple staining was also observed within the stele of cluster rootlets (Fig. 4.4 a, b, f) and parent lateral roots (Fig. 4.4 c, e). Figure 4.4 e depicts a lateral root with two clearly stained xylem vessels within the diarch stele. Cortical tissue was unstained (Fig. 4.2 c - f).

**Fig. 4.4**: Cluster roots of hydroponically grown plants of varying stages of maturity after 24 h of exposure to α-napthyl-phosphate (NP). Purple staining indicates localised hydrolysis of NP by acid phosphatase (AP). Panel a - c) *Lupinus albus*; d - e) *Lecudadendron foedum*.

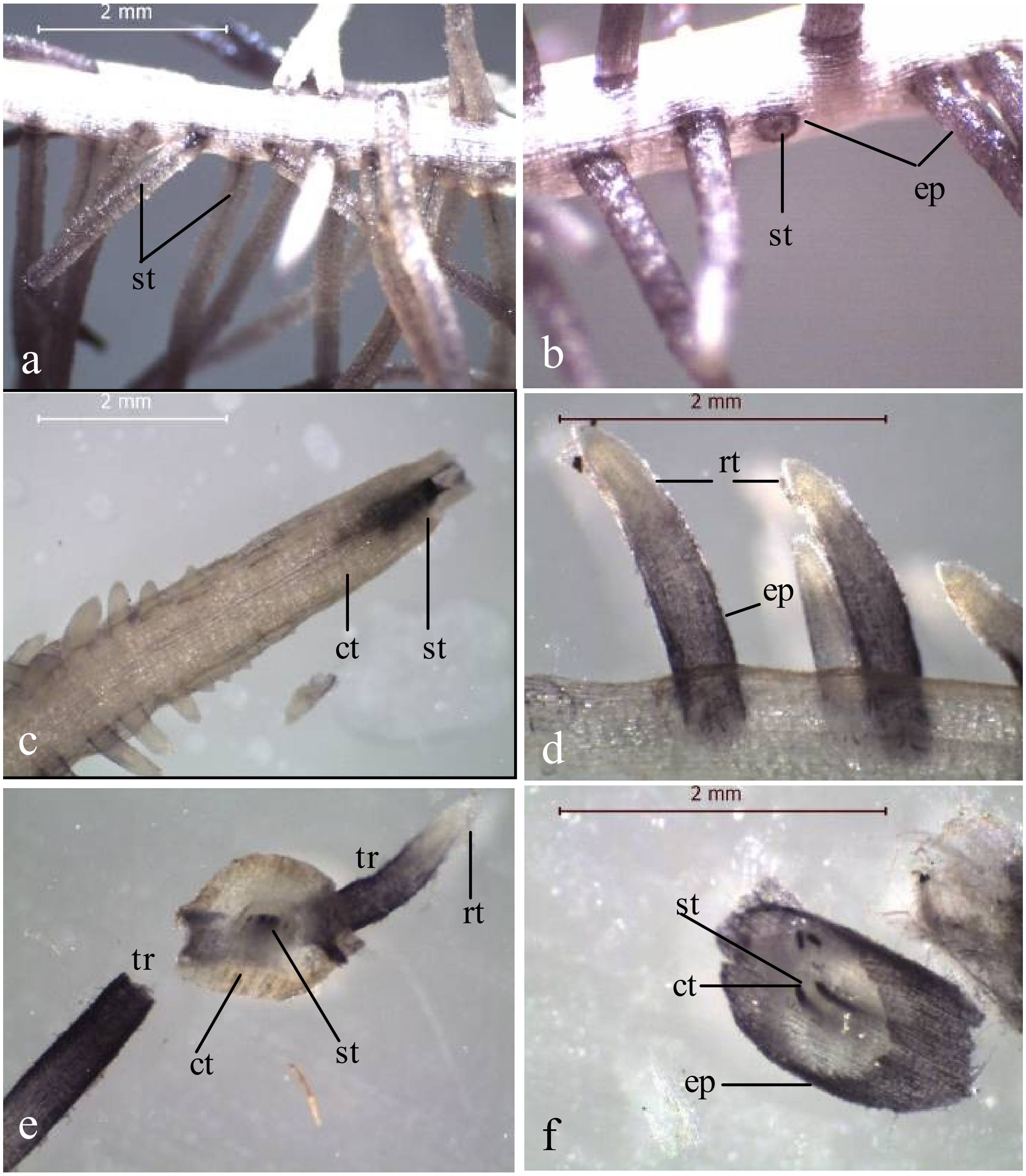

**Fig. 4.5**: Immature cluster roots of hydroponically grown *Lupinus albus* after 24 h of exposure to α-napthyl-phosphate (NP). Purple staining indicates localised hydrolysis of NP by acid phosphatase (AP). Panels a – b) whole roots stained at the epidermis (ep) and in the stele (st) but unstained within cortical cells (ct); c) transverse section of lateral root tip with localised staining in the stele; d) emergent tertiary roots with AP activity absent from root tips (rt); e) cross-section of lateral root with epidermal AP activity of emerging tertiary roots that can be traced continuously to the stele of the lateral root; f) cross-section of lateral root with AP activity visible in the epidermis and in the diarch stele.

# Discussion

In general, results were consistent with the hypothesised role of AP secretion as an adaptive response to P limitations. AP activity of *Ld. coniferum* was observed at two distinct levels, where control plants deprived of P secreted significantly more than those which had been supplied with low levels of preparatory P, demonstrating a down-regulation of AP secretion within 4 d of P supply. In the case of those plants which had been briefly supplied with and then deprived of P, AP activity increased significantly within 48 h relative to IHP and $PO_4$–supplied plants. However, despite the divergence of their respective AP activities after 48 h, none of the P-treated plants (P-limited, IHP, $PO_4$) had significantly increased or decreased AP relative to initial activity. Thus, it is difficult to determine whether differences between treatments should be attributed to a) increased AP synthesis by control and P-deficient plants, b) decreased AP synthesis by those plants continuously supplied with P ($PO_4$, IHP), or both. This could have been elucidated by more frequent sampling of AP activity with parallel monitoring of endogenous plant P.

To enhance the magnitude of plant response to changes in P-availability, P-deprived *Ld. foedum* was supplied with P at a higher rate than was previously supplied to *Ld. coniferum*. The AP activity of *Ld. foedum* decreased within 48 h of application of either P-form, similar to the response time of P-sufficient *Ld. coniferum* to P-withdrawal (i.e. in 'P-limited' plants). Although the initial and final AP activities of IHP and $PO_4$ plants were similar, only IHP-treated plants showed a significant decline in AP relative to initial activity. This may indicate that responses to organic and inorganic P are distinct from each other, as observed in *L. albus* (see below). The similar timing and magnitude of response of P-sufficient and P-deficient Proteaceae to reversals in P-availability suggests that down- and up-regulation of AP is primarily controlled by the same mechanism. While Wasaki *et al.* (1999) reported an increase in AP-secretion by *L. albus* within 12 h of exposure to P-deficient media, this rapidity of response could not be replicated in this study. The relatively gradual response to rhizosphere P-availability (i.e. d, rather than h) probably indicates that secretion of AP by Proteaceae is primarily regulated by endogenous P-status rather than rhizosphere P-availability, similar to *L. albus* (Gilbert *et al.,* 1999; Wasaki *et al.*, 2003; Miller *et al.* 2001). Observations of excised cluster roots where short-term applications of P did not measurably influence AP support this hypothesis (these observations were subsequently replicated in *Protea repens* (data not shown). Although soluble AP activity was detected in the exudates of some Proteaceae roots, the activity was relatively small and inconsistent in all treatments, whether applied in hydroponics or to excised roots. The observed soluble AP was thus attributed to AP leakage from damage to roots, rather than the induction of a freely migrating isoform of AP, as was reported for *L. albus* (Gilbert *et al.*, 1999, Miller *et al.*, 2001). The AP activity of cluster roots of both P-supplied and P-deprived Proteaceae was largely epidermally-bound. It may be noteworthy that the observed maximum and minimum AP activities were comparable between *Ld. foedum* and *Ld. coniferum*. It would be interesting to compare these data with a broader range of species, particularly those adapted to high-P soils (e.g. *Leucadendron meridianum*, *Protea obtusifolia*), to determine whether the capacity for AP secretion or the control thereof differs between edaphically specialised Proteaceae. Unfortunately, the two latter species did not grow well in hydroponics and could not be induced to develop sufficient cluster roots for experimentation

In the case of *L. albus*, 24 h was insufficient time for P-deficient *L. albus* to appreciably decrease AP secretion when supplied with $KH_2PO_4$, but a significant decline was observed in plants supplied with NaIHP at all three levels of application from 12 to 24 h. A similar but less marked response to organic P was observed in *Ld. foedum*. This decline is unlikely to be simply regulated by endogenous P-status or rate of P-uptake, since it was not observed in plants supplied with readily available inorganic $KH_2PO_4$, which did not differ from AP activity of P-deficient control plants. It appeared that AP secretion by *L. albus* is in some way sensitive to P-supply form, where addition of organic P induced a rapid response distinct from the gradual decline expected in response to increased P-availability. The rapidity of the observed decline of AP activity, and the relative stability of plants supplied with inorganic P, suggests that synthesis of AP was not downregulated in response to P-sufficiency, but may have been downregulated in order to synthesise another isoform preferentially, as previously reported in response to P withdrawal (Gilbert *et al.*, 1999; Wasaki *et al.*, 2003). Alternatively, the decline in activity observed after 24 h may signify the depletion of epidermal AP after a rapid release into the rhizosphere in response to organic P. As far as can be determined, no mechanism is known by which plants can ‘distinguish’ organic from inorganic P sources and adapt their metabolism accordingly.

The staining of *L. albus* roots with NP supplied as a hydroponic substrate represents a novel means of observing changes in AP activity with root age. The staining on younger roots and the absence of stain accumulation on mature roots was interpreted as an illustration of the change from epidermally-bound to freely-secreted AP, as previously observed in P-deficient *L. albus*. This experiment provides an interesting demonstration of the potentially distinct adaptive functions of the two stages. Acid phosphatases confined to the epidermis of young roots would allow hydrolysis and uptake of small quantities of P from root-intercepted organic matter. Freely-soluble AP would potentially allow a mature root to 'mine' a larger volume of soil than by interception alone, comparable to the function of root-exuded carboxylates (e.g. Lambers *et al.*, 2003). The majority of P hydrolysed by soluble AP would be spatially separated from the root surface. Therefore a greater proportion of P hydrolysed by this mechanism is likely to be reabsorbed by soil constituents or scavenged by microfauna in comparison to root-bound AP (Adams and Pate, 1992). However, the effect may be lessened by the coordinated influence of other P-mobilising root exudates, whose physiological peak occurs at a similar stage of maturity (Neumann *et al.*, 2001; Shane *et al.*, 2003b; Li *et al.*, 2005) and enhance the efficacy of AP in the rhizosphere (Hayes *et al.*, 2000).

The staining within the xylem vessels of cluster rootlets and parent lateral roots, plus the absence of staining within the cortical tissue, represents novel observations of the uptake and transport of intact organic P, where NP is transported intact into xylem before being hydrolysed by endogenous phosphatase, presumably LASAP-1 which is present in xylem throughout the plants (Miller *et al.*, 2001). The mechanism by which NP enters the root is unknown. The stain observed in the stele of a young lateral root tip (4.5 c) preceded formation of cluster roots, demonstrating that a) the mechanism by which organic NP is transported is common to cluster and normal roots; b) synthesis of AP in the vascular tissue precedes epidermal secretion of AP. Root tips were generally unstained at the epidermis, further indicating that AP is not secreted by new/developing root tissue (Fig. 4.5 c, d). Results obtained confirmed the suitability of NP as an indicator for root-bound AP activity. However, the method is unsuitable for staining physiologically mature roots where AP activity is not membrane-bound. The absence of staining on roots of all ages in *Ld. foedum* may suggest that the AP of this species is ineffective at hydrolysing NP. This further demonstrates that while *L. albus* is a useful model plant for studies of cluster root physiology, conclusions are not necessarily broadly applicable to other genera.

## Conclusion

The observation of root uptake and endogenous hydrolysis of organic P by *L. albus* has broad implications for root physiology in general and plant P-acquisition strategies in particular. The accumulation of organic P observed in roots may provide a clue to the mechanism by which *L. albus* appeared to distinguish between inorganic and organic P in hydroponics; possibly endogenous stores of organic P have a regulatory role in AP metabolism. The topic warrants further study to determine a) whether other phosphate esters are similarly available for root uptake; b) whether the phenomenon is peculiar to *L. albus*; c) the mechanism by which NP enters the root and is transported to xylem; d) whether plant uptake of organic P can be observed in plants grown in soil.

## Chapter 5 – Synthesis and conclusions

The Proteaceae are native to some of the world's most impoverished soils (Bond and Goldblatt, 1984) and display a broad suite of adaptations to limited P-availability, of which the family-wide ability to form cluster roots is a particularly prominent example (Purnell, 1960; Lamont, 1982; Shane and Lambers, 2005). One of the most striking features of cluster roots is the exudation of large quantities of P-solubilising compounds, including carboxylates, mucilage, phenolics and acid phosphatases (AP) (Shane and Lambers, 2005). However, while the regulation of cluster root exudation of carboxylates and their potential contribution to plant nutrition has been relatively well studied (e.g. Gardener *et al.*, 1982; Johnson *et al.*, 1996; Dinkelaker *et al.*, 1997; Roelofs *et al.*, 2001; Jones, 2003; Lambers, 2003; Shane *et al.*, 2004a; Pearse *et al.*, 2007), little is known about the secretion of AP and acquisition of organic P sources by cluster roots, except for studies of *Lupinus albus* (Fabaceae) (Adams and Pate, 1992; Gilbert *et al.*, 1999; Miller *et al.*, 1999; Wasaki *et al.*, 1999; 2003). This represents a gap in knowledge of both the broad study of plant/soil nutrient dynamics and the P-acquisition of Proteaceae. This study aimed to characterise the secretion of AP by cluster roots of Proteaceae as part of a sophisticated suite of plant adaptations to P-limited soil. Assumptions of AP metabolism based on previous studies of *L. albus* (Gilbert *et al.*, 1999; Miller *et al.*, 2001; Wasaki *et al.*, 1999; 2003) were used to inform our experimental approach. The overarching hypotheses of this study were that 1) AP is secreted by Proteaceae as an adaptation to P-limited soils; 2) AP is enhanced or suppressed in response to P-form and P-availability on soils with varying P-forms/availability; 3) edaphic endemic Proteaceae are adapted to P-forms found in their respective acid, aeolian or calcareous sandy soils and 4) AP-solubilised P makes a similar contribution to P nutrition compared to inorganic P.

Acid phosphatase was secreted by all species sampled, and in general results support the hypothesised function of secreted AP, where AP activity of Proteaceae in Fynbos soils (Chapter 2), sand-filled pots (Chapter 3) and hydroponic culture (Chapter 4) was inversely proportional to P-availability. Total soil P-content, P-supply form and availability of other nutrients did not influence AP secretion (Chapter 2, 3), which appears to be regulated primarily by endogenous P-status as an effect of P-availability. The application or removal of P induced changes in AP within 48 h (Chapter 4), demonstrating a regulatory mechanism sensitive to relatively short-term fluxes in P availability. It was demonstrated that Proteaceae were able to utilise P from poorly available organic sources, where i) MgIHP-supplied plants in sand-filled pots sustained similar growth to plants supplied with poorly available inorganic P minerals (Chapter 3), as has been previously observed in *L. albus* (Adams and Pate, 1992); ii) hydroponically-grown plants supplied with organic P decreased AP similarly to plants supplied with readily-available inorganic P, demonstrating that uptake of P from the two substrates was comparable (Chapter 4).

Secretion of AP is generally thought to be symptomatic of P-stress, and has been demonstrated experimentally by comparing P-deprived plants with those that have been supplied with up to 1.1 mM P, where the latter was sometimes sufficient to suppress AP secretion (Goldstein *et al.*, 1988; Gilbert *et al.*, 1999; Wasaki *et al.*, 1999). However, this study demonstrated that the notion of a 'P-sufficient' plant can be misleading and is unlikely to occur in wild populations of Proteaceae, where Bray II P-availability of Fynbos soils sampled was consistently lower than 10µg P $g^{-1}$ soil (± 80 µM) (Chapter 2), far below the concentration of P commonly applied to determine the AP secretion of 'P-sufficient' plants. Application of 10 µM P was sufficient to induce a change in AP activity (Chapter 4) and probably reflects more realistically the range of P-availabilities that should be used for assessing the regulation of P-acquisition. All Proteaceae, even those growing in comparatively rich soils (e.g. *P. repens* and *Ld. foedum* in Aeolian strandveld sand (Chapter 2)) and those supplied with relatively available P-forms (Chapter 3), secreted relatively large quantities of AP. Therefore, AP secretion should not be depicted solely as a symptom of P-stress, but as a widely-deployed P-acquisition strategy of healthy Proteaceae in a diverse range of Fynbos soils.

## Differences in AP secretion between Proteaceae from dissimilar soils

There are functional differences in P acquisition and uptake between Proteaceae from the typically acidic, nutrient leached Fynbos and Kwongan and those species endemic to relatively P-rich limestone soil (e.g. *Grevillea crithmifolia*, *Ld. meridianum*, *P. obtusifolia*) e.g. the sensitivity of cluster root initiation to P-availability, the ability to down-regulate P uptake and susceptibility to P toxicity, seemingly reflecting adaptations to native soil P-availability (Shane *et al.*, 2004b; Shane and Lambers, 2005; Shane *et al.*, 2008). In this study, it was hypothesised that the relative abilities to secrete AP and utilise organic P would differ between Proteaceae from soils of contrasting P availability. Secretion of AP was lowest in species growing in relatively P-rich limestone soil (*P. repens*, limestone-endemic *P. obtusifolia* and *Ld. meridianum*) (Chapter 2). When supplied with inorganic hydroxyapatite (CaP) and ferrite (FeP) and an organic phytic acid salt (MgIHP), *P. obtusifolia* differed from other species sampled in that it only grew vigorously when supplied with CaP, the most soluble of the three substrates. Plants supplied with MgIHP or FeP grew little, formed few cluster roots and secreted minimal AP. However, *Ld. meridianum* was able to utilise all substrates equally, and secreted AP at similar levels to other species sampled (*P. repens*, *Ld. foedum*) (Chapter 3). The inability of *P. obtusifolia* to utilise FeP and MgIHP was attributed to the species' relatively small and nutrient-poor seed (Esler *et al.*, 1989), where seed P-reserves alone were insufficient for a seedling to develop functioning cluster roots. Without the influence of root-exuded P-solubilising compounds, only CaP-amended sand provided sufficient soluble P for seedlings to establish P-acquisition structures. The secretion of relatively large quantities of AP by *P. obtusifolia* supplied with CaP demonstrate that the species is capable of hydrolysing organic P, provided seedlings have sufficient resources to form cluster roots. Thus, although AP secretion is likely to be higher in species in more P-limited soils, it seems unlikely that Proteaceae endemic to dissimilar soils differ fundamentally in their capacity to secrete AP or utilise organic P. All species are probably capable of sensitive regulation of AP synthesis according to P demand.

## The potential contribution of AP-hydrolysed organic P to nutrition of Proteaceae

While this study has characterised the secretion of AP by Proteaceae, no attempt was made to quantify the potential contribution of AP-hydrolysed organic P to plant nutrition due to the potential influence of microbial phosphatases, the necessarily destructive sampling methods employed, and the complexities of soil P dynamics. However, data from this and other studies may be used to estimate the size of the organic P pool intercepted by roots and the quantity of AP secreted into the rhizosphere by stands of mature Proteaceae.

The quantities of AP secreted by sampled Proteaceae in Chapter 2, when expressed relative to fresh root weight, were not remarkable in comparison with data from previous studies of AP-secreting plants in soil, where the highest AP activity in literature reviewed was observed in ryegrass, *Lolium multiflorum* (Starnes *et al.*, 2008). Where relative AP activity of *L. multiflorum* is 1, the relative magnitude of AP of previously studied species is 0.28 (*Zea mays*, Gaume *et al.*, 2001), 0.15 (*P. repens*, Chapter 2), 0.003 (*Spriodella oligorrhiza*, Playsted *et al.*, 2006). However, these values do not reflect the potential cumulative total of AP secreted by a stand of Proteaceae, where cluster roots comprised one-third of total root biomass in *Ld. laureolum* (Lamont, 1984), and are commonly observed as dense mats of compound cluster roots that entirely dominate the upper 5mm of the soil profile (Lamont, 1982; Skene, 1998; Pate and Watt, 2002). Phosphatase secreted by Proteaceae is likely to be more effective than an equivalent amount secreted by normal roots of other plants, where enzyme activity in the rhizosphere of cluster roots would be highly concentrated between densely-packed rootlets, minimising the loss of released P to soil readsorption and microbial predation.

Although the total contribution of AP-hydrolysed organic P to Proteaceae was not determined, estimates of total quantity of AP secreted into the rhizosphere and the proportion of organic P intercepted by roots can be made using data from previous studies. In a stand of *Ld. laureolum*, Lamont (1984) found a cluster root density of 200 mg cluster roots (dry weight) per litre soil, where specific cluster root volume was calculated as 39ml $g^{-1}$. Assuming a soil bulk density of 1.3 g $ml^{-1}$ and a fresh:dry weight ratio of 9.5 (obtained from cluster roots of *Ld. coniferum*; Power, unpublished)), this approximates to 1.5 g of fresh cluster roots per kg soil, exploring a soil volume of 10.4 ml per litre of soil. Organic matter content (measured as % C) of fynbos soils may vary from negligible in some sands to 15% in some limestone-derived soils, however 2 to 3% C is typical for acidic nutrient-poor colluvial sands and sandstone-based soils (Mitchell *et al.*, 1984, 1986; Witkowski and Mitchell, 1987; Richards *et al.*, 1997). Assuming a P content of 2.5 % (w/w) for soil organic matter (Tisdale *et al.*, 1993; Mitchell *et al.*, 1986), cluster roots directly explore a soil volume containing 3.27 μM organic P $kg^{-1}$ soil. Using the maximum observed AP secretion by *P. repens* of 2.5 μM p-NP $s^{-1}$ $g^{-1}$ (Chapter 2), total AP activity of fresh cluster roots per kg of soil is equivalent to the amount of AP required to hydrolyse 3.65 μM p-NP $s^{-1}$, or 0.3 M p-NP $day^{-1}$, *in vivo.*

While these estimates demonstrate an example of the potential size of the organic P pool available for AP-mediated hydrolysis, calculations must be interpreted cautiously, as soil and cluster root variables quoted may differ by an order of magnitude between soils. Very conservative estimates of cluster root mass and volume have been used, where in some cases the uppermost surfaces of soil beneath leaf litter may be entirely dominated by dense, continuous 'mats' of cluster roots (Lamont, 1982; Pate and Watt, 2002). There is great variation in the diffusivity of AP (Marinari *et al.*, 2008) and the mobility of organic P (Adams and Pate, 1992, Condron *et al.*, 2005) between soil types, and thus the proportion of soil organic P explored by cluster roots and secreted AP will vary greatly. Nurruzaman *et al.* (2006) reported elevated AP activity and significant depletion of organic P up to 4mm from the rhizosphere of *L. albus*, but this value could be much higher in sandy soils or almost negligible in clay-rich soils (Adams and Pate, 1992). The efficacy of secreted AP would be highly variable between soils and dependent on a complex interaction of biotic and abiotic factors, including availability and predominant forms of organic P, soil pH, clay and mineral content of soils, mobility of AP in soil, substrate specificity of AP, temporal and spatial distribution of secreted AP, presence of other P-solubilising compounds (e.g. carboxylates, see below), and competition with soil microfauna and other plants for hydrolysed P (e.g. Raghothama, 1999; Hayes *et al.*, 2000; Richardson *et al.*, 2000; Hens *et al.*, 2003; Lung *et al.*, 2005; Shane *et al.*, 2005; Marinari *et al.*, 2008).

The potential contribution of roots directly exploring the leaf litter layer above the soil profile has not been considered in any detail, although the annual deposition of 80 g litter $m^{-2}$ $year^{-1}$ observed under a stand of *Leucospermum parile* represents a major input of P to Proteaceae (2.5 g P $m^{-2}$ $year^{-1}$) (Mitchell *et al.*, 1986). Cluster roots frequently colonise this litter layer (Lamont, 1982; Pate and Watt, 2002), and Proteaceae may contribute significantly to the remineralisation of organic P during this stage of the soil P cycle. The proportional contribution of organic P to plant nutrition may increase with stand age as leaf and culm litter, and thus organic P, accumulates after fires (leaf litter layer under a stand of Proteaceae increased from 120 to 660 g $m^{-2}$ from 4 to 9 years after fire) (Mitchell *et al.*, 1986).

## The coordinated influence of acid phosphatases and other root exudates

The influence of secreted AP on plant acquisition of P is typically investigated and discussed in isolation, independent of other P-mobilising compounds (e.g. carboxylates, phenolics, mucilage). However, several researchers have proposed that the coordinated influence of these compounds is greater than the sum of their parts. Adams and Pate (1992) noted that high AP secretion did not necessarily imply an enhanced ability to utilise organic P substrates, suggesting that availability of organic P was enhanced most by those plants which exported large quantities of both AP and carboxylates. Hayes *et al.* (2000b) demonstrated the enhanced hydrolysis of several organic P substrates by AP or phytase in the presence of organic anions, and in certain instances, hydrolysis of substrates was negligible in the presence of AP alone. Similarly, Tang *et al.* (2006) found IHP salts of $Ca^{2+}$ and $Mg^{2+}$ to be available to enzymatic AP-hydrolysis, but IHP associated with cations such as $Al^{3+}$, $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ was unavailable except in the presence of carboxylates, of which citrate had the greatest effect (citrate>oxalate>malate) on adsorbed IHP availability. Hens *et al.* (2003) showed that accumulation of citrate was effective in the solubilisation of stable organic P forms such as IHP, leaving increasingly unstable organic substrates for solubilisation by enzymatic hydrolysis. With particular reference to cluster roots, Helmke *et al.* (2000) and Shane and Lambers (2005) suggested that secreted AP is probably an important adjunct to carboxylates, where any inorganic P released by AP-hydrolysis is more likely to be absorbed by plants in the presence of citrate, which would suppress the readsorption or precipitation of P, while conversely the influence of AP would enhance the efficacy of carboxylates by minimising the conversion of released P into organic forms. Root-exuded phenolics and mucilage, although not extensively studied, are generally thought to assist in P-acquisition by suppressing mineralization/readsorption of solubilised P in the rhizosphere (Shane and Lambers, 2005) and would also probably enhance the efficacy of AP. Therefore, while practical constraints generally dictate that the assorted P-acquisition mechanisms of cluster roots are characterised and quantified separately, their influence can only be truly assessed in combination.

## Utilisation of organic P for cultivation of Proteaceae

The demonstrated ability to use sparingly soluble organic P may be applicable to commercial production of cutflower Proteaceae and *Macadamia* sp. nut crops. Due to a poorly developed capacity to down-regulate P uptake and a low tolerance to elevated tissue P, Proteaceae are prone to P toxicity when grown on post-agricultural land or when supplied with relatively modest amounts of readily available $PO_4^{2-}$ (Shane *et al.*, 2004b; Hawkins *et al.*, 2008; Hue, 2009). Due to this sensitivity to excess P-uptake, sparingly soluble forms of organic and inorganic P have been suggested as an alternative to soluble P applied by fertigation, where P would be acquired primarily by cluster root-mediated mechanisms, and uptake can be adjusted to plant P requirements by increasing or suppressing investment in cluster roots (Hawkins *et al.*, 2007). This study indicates that all Proteaceae are capable of utilising organic P, suggesting further investigation into the management of soil organic matter (e.g. addition of mulch or cultivation of a cover crop) and guidelines for use of organic fertilisers (e.g. fowl manure, bone meal) in the production of cutflower Proteaceae and *Macadamia* sp. nuts. Sparingly soluble iron and calcium phosphate provided similar growth to plants supplied with organic phytic acid salts (Chapter 3) and also deserve further investigation as a source of slow-release fertiliser for Proteaceae.

The ability of Proteaceae to utilise organic P may be of future interest to production of other crops. Availability of P is one of the most common limitations to agricultural productivity (Rhaghothama, 1999), and it has been predicted that global reserves of mineral P for fertiliser production will be depleted within 60 to 90 years (Runge-Metzger, 1995). Proposed strategies for adapting to a future shortage of P include enhancing the P acquisition ability of crops via transgenic techniques (Rhaghothama, 1999). As a significant proportion of total soil P occurs as organic matter (Tisdale *et al.*, 1993), enhancing the capacity to utilise organic P sources is a promising strategy, where the extracellular secretion of microbial phytase by transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* allowed plants to utilise sparingly soluble organic P (Richardson *et al.*, 2001; Lung *et al.*, 2005). The advanced adaptations to organic P acquisition possessed by Proteaceae may be useful in analogous future studies.

## Future research

The likely interactive effects between AP and other root-exuded compounds need to be studied further in order to fully characterise the role of root exudates in nutrient acquisition. The apparent ability to utilise organic P may be used to inform soil management protocols for cutflower Proteaceae and *Macadamia* nuts, and suggest a study of the suitability of organic P as a source of fertiliser for these crops. The novel observation of uptake and endogenous hydrolysis of organic P by *L. albus* (Chapter 4) needs to be replicated, ideally along with other cluster root-producing plants, and the mechanism of uptake and its implications characterised and explored more thoroughly.

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# Appendix

Elemental nutrient concentration for UWA solution (µM):

| | |
|---|---|
| NO3 | 0.40 |
| Ca | 0.20 |
| K | 0.20 |
| SO4 | 0.15 |
| PO4 | 0.004 |
| Mg | 0.054 |
| Mn | 0.00024 |
| Zn | 0.00010 |
| Cu | 0.00002 |
| H3BO3 | 0.00240 |
| Mo | 0.00003 |
| Fe | 0.01012 |