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"FORMATION, FUNCTIONING AND EFFECT ON SOME CHEMICAL AND BIOCHEMICAL RHIZOSPHERE PROPERTIES OF CLUSTER ROOTS OF A SOUTH AMERICAN PROTEACEAE: *Embothrium coccineum*"

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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"FORMATION, FUNCTIONING AND EFFECT ON THE RHIZOSPHERE OF CLUSTER ROOTS OF A SOUTH AMERICAN PROTEACEAE: *Embothrium coccineum*"

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Summary and Outline of this Doctoral Thesis

Proteaceae species in south-western Australia thrive on extremely phosphorus-impoverished soils, employing a phosphorus-mining strategy involving carboxylate and phosphatases released by cluster roots, which solubilise and mineralise P making it more available for plants.This is particularly important in old, climatically buffered and infertile landscapes, nevertheless, it is also possible to find species with specialised cluster roots on young volcanic soils such as those of southern Chile, which contain high levels of total phosphorus (P), but where its availability is low. In Chapter I, we present a general introduction of this Doctoral Thesis, indicating the hypothesis and goals of this study.

In Chapter II, we discuss the role of cluster roots and other adaptive traits in Proteaceae species growing in P-deficient soils, with special emphasis on Southern south-American Proteaceae. According to the literature reviewed, there are several differences between Chilean and South-Western Australian Proteaceae species, which could be related with the contrasting soils in which the species grow, because the latter are adapted to ancient, highly weathered and extremely P poor soils, whereas Chilean Proteaceae grow in volcanic soils, which are younger and with higher fertilily. The differences in the habitat in which these species have evolved could result in differences in formation and function of cluster roots, P-use efficiency and tolerance to develop symptoms of P toxicity. Some of these aspects were evaluated in this Doctoral Thesis, using *Embothrium coccineum* (R. et J. Forst.), as a model species.

In Chapter III is shown the effect of P in cluster-root formation and functioning *E. coccineum*. For this, four-month-old seedlings were grown for 4 weeks in hydroponic cultures with 1 μM P or 50 μM P. The number of cluster roots, relative height increment, biomass distribution, cluster root/total root biomass ratio, foliar [P], root acid phosphatase activity and root carboxylate-exudation rates were determined. The results of this experiment showed that seedlings growing at 50 μM P presented 10-, 1.3- and 3.3-fold greater increase in relative height, total dry mass and foliar [P], respectively, compared with those grown at1 μM P. However, seedlings grown at 1 μM P showed a 5-, 16-, 1.7- and 1.3-fold greater number of cluster roots, cluster root/total root biomass ratio, phosphatase activity and total carboxylate exudation, respectively, as compared

with those grown at 50 μM P. The conclusions are: A low P supply promotes the initiation, growth and metabolic activity of cluster roots which is in accordance with reports on Proteaceae species occurring in ancient and highly weathered soils.

In chaper IV, we hypothesised that the functioning of cluster roots of *E. coccineum* differs from that of south-western Australian Proteaceae species, in accordance with the difference in soil P status. With more P to be gained from the soil with high levels of total P, we expect less investment in biomass and more release of carboxylates. Furthermore, we hypothesised that *E. coccineum* regulates its phosphorus-uptake capacity, avoiding P toxicity when grown at elevated P levels. To test these hypotheses, *E. coccineum* seedlings were grown at a range of phosphorus supplies in nutrient solution.

We found that *E. coccineum* allocated at least five times less biomass to cluster roots and that released at least nine times more carboxylates per unit cluster root weight compared with south-western Australian species (e.g., Banksia, Hakea). The highest P supply caused a growth inhibition and high leaf [P], without symptoms of P toxicity. We accept our hypotheses on the functioning of cluster roots and the high capacity to reduce the net P uptake in plants grown at a high phosphorus supply.

This novel combination of traits indicates divergent functioning of Proteaceae species from southern South America, exposed to frequent P input due to volcanic activity, in contrast with the functioning of south-western Australian Proteaceae species that thrive on severely phosphorus-impoverished soils. These traits could explain the functioning of *E. coccineum* on soils that are rich in total P, but with a low concentration of available P.

Finally, in Chapter V, we show the effect of cluster roots of *E.coccineum*, growing under natural conditions, on soil chemical and biochemical properties associated with the rhizosphere. For this purpose, acid phosphatase (P-ase), β-glucosidase, hydrolysis of fluorescein diacetate (FDA), dehydrogenase and phosphorus (P) lability in rhizosphere of cluster roots at different development stages (juvenile, mature, semisenescent, senescent) and bulk soil were determined. In addition, we measured total P and Mn concentrations in cluster roots at different development stages. The results show that senescing cluster roots presented the highest P-ase, β-glucosidase,

FDA and dehydrogenase, being 2.6-, 4.6-, 3.3- and 25.8-fold greater, respectively in this development stage compared with those in mature cluster roots.

The results regarding P fractionation showed that cluster roots modified the lability of different rhizosphere P fractions; the inorganic P fraction was significantly greater in the rhizosphere of mature cluster roots (62%) than in rhizosphere soil during other stages (on average: 47%). At this developmental stage, cluster roots showed the highest total [P], suggesting that mature cluster roots exhibit the greatest P uptake. We conclude that cluster roots of *E. coccineum* chemically and biochemically modified the rhizosphere, increasing P availability.

CHAPTER I

General Introduction

General Introduction

Phosphorus (P) is an essential element that commonly limits plant growth because it is linked to Ca^{+2} (neutral or calcareous soils) and Fe^{+3} or Al^{+3} (acid soils), forming organic or inorganic compounds scarcely available to plants (Stevenson & Cole, 1999). As a consequence, plants have developed some root physiological strategies for P acquisition, for example, nonmycorrihzal species forming cluster (or proteoid) roots, which are ephemeral clusters of fine rootlets around a main axis (Purnell 1960; Lamont 2003), living about three weeks. These roots occur in most species belonging to the Proteaceae, but similar root structures occur in species in other families (Luis et al. 1991; Lamont, 2003; Lambers et al. 2006). The role of these specialised roots in plants is widely known, as they can exude large amounts of citrate and malate (carboxylates), and phosphatases, to promote inorganic phosphate (Pi) solubilization and mobilization from the soil's mineral bound Pi.

Cluster roots are a response to P deficiency and have mainly been studied in Australian and South African Proteaceae species (Lamont, 2003; Lambers et al. 2006), where the main centres of diversity are found (Pate, Verboom & Galloway, 2001; Barker et al., 2007). Many species are renowned for their capacity to thrive on some of the world's most ancient and phosphorus- (P) impoverished soils (Lambers et al., 2010; Lambers et al., 2013a). However, for Proteaceae species growing in young, frequently disturbed and more fertile landscapes, very little information on the factors controlling cluster-root formation is available (Donoso-Ñanculao et al. 2010; Morales 2004; Ramírez et al 1990; 2005; Zúñiga-Feest et al. 2010). In southern South America, *Embothrium coccineum* (Proteaceae) grows in volcanic soils characterised by a high total P content (> 1500 mg kg^{-1}), but due to the high reactivity of this element with soil colloids, its availability is low for plants. Thus, cluster-root formation together with their functioning (e.g., release of phosphatases and carboxylates) are probably crucial strategies for P acquisition by Proteaceae species when growing in such habitats (see Chapter III).

Some Proteaceae species adapted to low-P soils develop foliar symptoms of P toxicity when grown at soil P levels just above those in their natural habitat (Grundon, 1972; Grose, 1989; Lambers et al., 2002; Shane, McCully & Lambers, 2004b; Hawkins et al., 2008; de Campos et al., 2013). Many authors have concluded that it is due to these Proteaceae species have lost their

capacity to down-regulate their P-uptake systems (Shane et al., 2004b; de Campos et al. 2013) in these extremely poor environments. In contrast, Proteaceae species in Chile, e.g., *E. coccineum*, occur on volcanic soils, which contain high levels of total P, compared with those in south-western Australian soils (<80 mg P kg^{-1}) (Lambers et al., 2006), no symptoms of P toxicity have ever been recorded for seedlings of *E. coccineum* growing in nutrient solution with 50 μM P (See Chapter III). These results suggest that *E. coccineum* is lesser sensitive to P toxicity than some other Proteaceae species occurring in old and infertile soils in Australia and South Africa (Lambers et al., 2013a). Until now, P-uptake in Southern South American Proteaceae has not been evaluated; therefore, this was tested on specific goal 2 (See Chapter IV).

Organic compounds released into the rhizosphere are available as substrate for microorganisms, and rapidly assimilated into microbial biomass (Pinton et al., 2001; Ryan et al., 2001; Gregory 2006). Besides, microorganisms are able to assimilate nutrients that are released by plants, generating a strong competition for nutrient uptake. To avoid the decomposition of organic compound and uptake competition by solubilised P, Weisskopf et al. (2006) proposed that *Lupinus albus* has developed three mechanisms: i) strong acidification of the cluster-root rhizosphere, decreasing bacterial abundance, because most bacteria are sensitive to acidic environments; ii) exudation of phenolic compounds that induce fungal sporulation, thus reducing their potential citrate consumption by fungi ; iii) exudation of chitinase and glucanase, which are enzymes that degrade fungal cell walls, just prior to citrate exudation. It is unknown if similar mechanisms are operating in other species bearing cluster roots.

Release of organic acids is associated with several changes in the rhizosphere, such as nutrients mobilization, decrease in pH, and metal detoxification (such as Al^{+3}, Ni^{+2}) through chelate formation with organic anions (Jones, 1998). However, most studies involving these changes have been carried out under controlled conditions, which may be quite far from what happens in field conditions. So far, how cluster roots are movilising P from non-labile to more labile fractions in the rhizosphere from plants growing in their natural conditions is still unknow. In this study, we investigated how cluster roots from adult plants of *E. coccineum* growing in volcanic soils, affect chemical (P lability) and biochemical activities in the rhizosphere (see Chapter V).

According to the previous background, we addressed the following hypotheses:

a) *Embothrium coccineum* exhibits similar physiological response to low P as cluster-rooted species growing in severely P-impoverished soils.

b) *Embothrium coccineum* has a strong capacity to down-regulate its P-uptake systems, thus avoiding P toxicity when grown at a high P supply.

c) Similar mechanisms to *Lupinus albus*, related to decreasing microbial activity in active cluster roots, are operating in *E. coccineum*. Additionally, we hypothesize that exuding cluster roots mobilise P from non-labile fractions to more labile fractions in the soil, enhancing its bioavailability.

General goal

To study the formation and functioning of cluster roots and their influence on some biochemical activities and phosphorus bioavailability in the rhizosphere of South American Proteaceae species: *Embotrium coccineum*

Specific goals

a) To study the effects of different phosphorus levels on carboxylates and acid phosphatase released by roots of *Embothrium coccineum.*

b) To evaluate the effect of P supply on the development of P-toxicity symptoms in *Embothrium coccineum* seedlings and the mechanism involved.

c) To assess the effect of cluster roots at different development stages, on chemical and biochemical activities associated to the rhizosphere soil of *Embothrium coccineum.*

CHAPTER II

REVIEW: Proteaceae species growing in phosphorus-deficient soils: *A focus on South American Proteaceae*

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Abstract

Australian Proteaceae has proven to be highly adapted to soil extremely phosphorus P deficient soil. This is possible because the majority of Proteaceae family members have cluster roots. These root structures exude large amounts of organic acids and acid phosphatase, which solubilise and mineralise P making it more available for plants. This is particularly important in old, climatically buffered and infertile landscapes, like soils in south-western Australia and South Africa. However, it is also possible to find species with specialised cluster roots on young soils such as those of southern Chile. The aim of this review is to discuss the role of cluster roots and other adaptive traits in Proteaceae species growing in P-deficient soils, with special emphasis on Southern south-American Proteaceae.

Cluster-root formation in Chilean Proteaceae is dependent on P availability in soil, showing higher root biomass when there is P deficiency. In addition, lower photosynthetic rates and lower [P] in seeds have been reported in Chilean Proteaceae compared with Australian Proteaceae, suggesting a divergent functioning in this family. The differences between Chilean and Australian Proteaceae could be due to a high adaptation to very infertile landscapes in Australia, whereas the former grow in younger and more fertile soils.

Keywords: acid phosphatase, organic acids, photosynthesis, phosphorus-deficient soils, seeds

Introduction

Cluster roots (or proteid roots) are considered as one of the three major strategies that some plants have to increase nutrient uptake, together with the association with mycorrhizal fungi and N_2-fixing bacteria (Skene, 1998).These root structure are described as dense clusters of fine rootlets around a main axis (Purnell, 1960), being found in most species belonging to the Proteaceae family, with fewer species in nine other families: Betulaceae, Casuarinaceae, Cyperaceae, Elaeagnaceae, Fabaceae, Myricaceae, Restionaceae (Shane & Lambers, 2005; Lambers & Shane, 2007).

The role of cluster roots in plant is widely known as these roots can cause changes in the rhizosphere and thus increase their ability to acquire nutrients (Marschner et al. 1986; Grierson et al. 1989).This is particularly important in P-deficient soils, because it is frequently the second mineral nutrient limiting plant growth after nitrogen (N). On the other hand, global P reserves (non-renewable resources) are being depleted, as they are worldwide exploited for fertilizer production every year (Cordell et al. 2009).

Modern agriculture requires annual fertilizers rich in P, since most of it (80-90%) may be adsorbed to soil colloids, hence not available to plants (Jones & Brassington, 1998). However, due to high prices and problems associated with fertilizer application (groundwater eutrophication), it is necessary to pay greater attention to plant species that are more efficient in P acquisition, such as cluster-bearing species. They can be used potentially for future crops or alternatively, if it is feasible to find the genes involved in the formation and functioning of cluster roots, they could be isolated and introduced into crops that lack this adaptative root mechanism (Lambers et al., 2006; Lambers& Shane, 2007).

Morphology, physiology and functions of cluster roots have been studied mainly in *Lupinus albus* L. (Gardner et al. 1983; Johnson et al., 1994; Johnson et al., 1996; Neumann et al., 1999; Neumann & Römheld, 1999; Kihara et al., 2003) and in some Australian and South African Proteaceae (Lamont, 2003; Shane et al., 2004a; Lambers et al., 2006), whose soils, in which these species grow, are highly P deficient (Lambers et al., 2008; Lambers et al., 2010).

However, there are few studies on Chilean Proteaceae species, where soils have high P contents, although it is scarcely available. The aim of this review is to discuss the role of cluster roots and

other adaptive advantages in Proteaceae species growing in P-deficient soils, with special emphasis on Proteaceae growing in young volcanic soils.

1. Cluster roots and their importance in P acquisition

Cluster roots may exude large amounts of carboxylates (Neuman et al. 1999; Neuman& R□meld, 1999; Peñalozaet al. 2002 Kihara et al. 2003; Shane et al. 2004a; Delgado et al. 2013, 2014), and acid phosphatase (Reddell et al., 1997; Gilbert et al., 1999; Neumann et al., 1999; Delgado et al 2013) compared with non-cluster roots. The carboxylates exuded from roots are important for promoting P mobilisation, because they form stable complexes with cations that are bound to phosphates (e.g., Al^{3+}, Fe^{3+} and Ca^{2+}) or displace phosphate from the soil matrix by ligand exchange (Jones et al., 1998; Shane & Lambers, 2005). On the other hand, acid phosphatases are very important because they can hydrolyze organic P compounds to inorganic P (Pi), being Pi the available form for plants (Bieleski, 1973; Duff et al., 1994; Ryan et al., 2001).

The higher exudation rate in cluster roots compared with non-cluster roots is due to metabolic changes occurring in the former. Several authors have reported metabolic changes in enzymes as phosphoenolpyruvate carboxylase (PEPC) and citrate synthase (CS), which are involved in the biosynthesis of organic anions exuded by cluster roots (Table 1). These metabolic changes lead to high exudation rates of carboxylates, being much greater in cluster roots compared with non-cluster roots and with other species that have no root adaptation (Roelofs et al. 2001; Ryan et al. 2001).

Table 1. Metabolic change in cluster roots under P deficiency

Species	Metabolic change	References
Lupinus albus varUltra	↑ PEPC activity ↑ CS activity	Johnson et al. (1994)
*Lupinus albus*var Ultra	↑ PEPC activity	Johnson et al. (1996)
Lupinus albus cv. Amiga	↑ PEPC activity	Neumann& Römheld (1999)
Lupinus albus cv. Amiga	↑ PEPC activity	Neumann et al. (1999)
*Lupinus albus*cv. Kievskij Mutant	↑ PEPC activity	Kihara et al. (2003)
Hakea prostrata	↑ PEPC activity	Shane et al. (2004a)
Lupinus albus cv. Victoria-Baer	↑ PEPC activity	Peñaloza et al. (2005)

PEPC: phosphoenolpyruvate carboxylase, CS: citrate synthase, ↑: Enhanced

Delgado et al. (2013) showed that cluster roots of *E. coccineum* exuded mainly citrate and malate. This strategy of carboxylate exudation is probably very important in southern Chilean soils, which are derived from volcanic ashes and are characterized by low levels of available P, due to their high adsorption capacity to oxides/hydroxides of Fe and Al (Borie & Rubio, 2003).
On the other hand, acid phosphatase exudation as well as exuded carboxlylate is probably very important for P acquisition in these soils, because organic P (Po) constitutes more than 50% total P (Borie & Rubio, 2003; Pinochet et al., 2001). Increased acid phosphatase activity in response to P-deficient conditions has been reported in a great number of species (Bieleski and Johnson, 1971; Tadano & Sakai, 1991; Duff et al., 1994). Nevertheless, large amounts exuded by cluster roots compared with non-cluster roots shows that these structures are highly adapted to increasing P availability.

2. Cluster roots in Chilean Proteaceae

Cluster roots have been mainly studied in Australian and South African Proteaceae, where the soils, on which these species have evolved, are considered among the poorest in the world (Lambers et al., 2008; Lambers et al., 2010). In this sense, Lambers et al. (2010) highlights the presence of species with specialised root clusters (mainly Proteaceae) in ancient and highly weathered soils such as South Western Australia soils, whereas in soils with greater P contents, species with these specialised nutrient-acquisition strategies are very uncommon. Besides, Lambers et al. (2008) suggest that the mining strategy ("from root of nutrient-solubilising or hydrolysing exudates") dominates in ancient soils, where most P is sorbed to soil particles, while the scavenging strategy ("mycorrhizal symbioses") is common in younger soils, where [P] in the soil solution is greater. However, although southern Chilean soils are young, it is also possible to find species with specialised cluster roots.
In Chile and Argentina, there are six species of Proteaceae family: *Embothrium coccineum* (J.R.Forst), *Gevuina avellana* (Mol), *Lomatia dentata* (Ruiz et Pavón) R. Br., *Orites myrtoidea* (Poepp. & Endl.) Benth.& Hook. f ex B. D. Jacks., *Lomatia ferruginea* (Cav.) R. Br., *Lomatia hirsuta* (Lam.) Diels. ex Macbr., all having cluster roots (Gonzalez, 1990). However, scarce information is available about the role of these roots in species growing in volcanic soils.

Lamont (1972) suggest that the variability on morphology, physiology and functioning of cluster roots would depend on plant age and soil type in which they grow. That is why it is important to study Proteaceae species growing in contrasting soils to the Proteaceae that have been studied so far. Some morphological characteristics of cluster roots in Proteaceae species growing in Chile can be observed in Table 2. It is noteworthy that cluster sizes varies within species, being in *G. avellana* higher than other Proteaceae species, reaching even sizes up to over 8 cm long in adult individuals (Ramirez et al. 2004).

In 54 representative seedlings of South African Proteaceae, the sizes of individual clusters ranged from 0.2 to 7.5 cm long and 0.1–3.4 cm wide (Lamont, 1983). On the other hand, in 10-month old seedling of *Hakea* species (Australian Proteaceae) cluster root size ranged from 6.6 to 13.0 cm long. However, in adult plants of *Hakea prostrata*, they reached about 20 cm long and 7 cm wide (Lamont, 1972).

Tabla 2. Cluster-root morphology in Proteaceae species growing in Chile (adapted from Gonzalez, 1990).

Species	Length (cm)	Diameter (cm)	% of cluster roots of total root weight	Nº rootlets per cm	Nº rootlets per cluster	Length rootlets (cm)
Gevuina avellana	2.47	1.33	76.0	478	1181	0.73
Embothrium coccineum	2.08	0.59	5.0	304	632	0.39
Lomatia dentata	1.60	0.45	8.1	130	208	0.43
Lomatia ferruginea	1.30	0.46	8.9	131	170	0.22
Lomatia hirsuta	1.01	0.28	0.7	100	101	0.07
Orites myrtoidea	0.8	0.47	ND	160	128	0.24

4. Factors that induce cluster-root formation

4.1 Nutritional deficiencies

Phosphorus deficiency is one of the most commonly reported factors in the induction of cluster-root formation in addition to N and iron (Fe) (Arahou& Diem, 1997; Keerthisinghe et al., 1998;

Neumann et al., 2000; Lamont 2003; Shane & Lambers, 2005; Hue, 2009). Recently, Delgado et al. (2013) reported higher number of cluster roots in *E. coccineum* when plantsare exposed to P deficiency and Piper et. al. (2013) found that cluster-root formation and growth in *Embothrium coccineum* were stimulated by low soil N concentrations, suggesting that cluster roots could also promote N–acquisition. These records are agree with those on other species that have cluster roots, such as *Casuarina cunninghamiana* Miq (Reddell et al., 1997), *Grevillea robusta* (Skene et al., 1996), *Morella cerifera* (Louis et al., 1990), *Banksia grandis* (Lambers et al., 2002), *Hakea sericea* (Sousa et al., 2007), where an adequate P, N and/or Fe supply inhibits cluster-root formation. Other species such as *Lupinus albus* (Johnson et al. 1996; Shu et al. 2007), *Casuarina glauca and Casuarina cunninghamiana* (Zaid et al., 2003) significantly reduce the number of cluster roots, when they grow under P-sufficient conditions.

On the other hand, some authors reported that cluster-root formation would be governed by internal [P] in the shoot rather than by external P in soil (Marschner et al. 1987; Louis et al. 1990; Shane et al. 2003; Shan & Lambers, 2006; Li et al. 2010). In 2-month-old seedlings of *Morella cerifera,* Louis et al. (1990) reported that foliar P application at high concentrations inhibits cluster-root formation. The same behaviour has been observed in *Lupinus albus*, where at a low [P] in the shoot shows a greater number of cluster roots and higher citrate exudation rate compared with plants with a good shoot P status (Shane et al., 2003).

In contrast, Donoso et al. (2010) reported that in *E. coccineum* growing under different soil conditions (high and low organic matter) no positive correlation between [P] in leaves and cluster-root number was found.This was probably because it was evaluated in soil with no largesdifferences in P availability, because in hydroponic cultures, Delgado et al. (2014) did note that at higher [P] in leaves, the cluster roots were supressed.

4.2 Seasonal variation

Seasonal variation is closely related to cluster-root formation. In species of Proteaceae in Mediterranean climates, cluster-root formation occurs in the wet winter season (Lamont, 1976; 1983; Lamont& Bergl, 1991; Shane & Lambers, 2005). On the other hand, cluster-root formation

in *E. coccineum* from temperate rain forests occurs in spring and summer seasons, which are concomitant with the longest period of growth (Zúñiga-Feest et al., 2009).

Donoso et al. (2010) also noted a seasonal variation in cluster-root formation in *E. coccineum* which depends on soil organic matter content in the field. These authors explain that high organic matter content favors the retention of soil moisture in summer, but in winter it may cause low drainage under flooding conditions. Furthermore, low organic matter soil could keep ideal soil moisture to cluster-root formation in the rainy season, but would be unable to keep moisture in the dry summer, showing unsuitable conditions for cluster-root formation.

In this regard, it is noteworthy that water is essential for parent root growth, being a prequisite for cluster root formation (Lamont, 2003). Lamont (1976) reported that cluster and non-cluster roots are produced only during winter-spring, although dormant roots can be induced to form new root structures in summer, when sufficient water is applied to that part of the root system. Jeschke and Pate (1995) have reported that slow growth rates, absorption and storage of internal P in *Banksia prionotes* (Proteaceae) occur mainly in the winter season for remobilization and use during spring - summer time, when leaf expansion occurs.

Preliminary results obtained in our laboratory show that there is a seasonal variation in cluster-root formation and phosphatase activity, which is greater in cluster roots than in non-cluster roots (60% higher) during summer. However, these results are only shown when the roots are cut. Therefore, further research is needed to evaluate possible seasonal variation.

4.3 Organic matter content in soils

Organic matter (OM) has several properties that enhance soil characteristics: it stabilizes structure, improves water retention, increases soil temperature and cation exchange capacity, it has a buffering capacity and is an important reservoir of nutrients (Bohn et al., 1993). Due to this, root development of plants is favored in soils with high levels of organic matter.

In this regard, it is noteworthy that in Australian Proteaceae, cluster roots are concentrated in OM-rich surface soil horizons with much higher levels of nutrients than deeper profiles, thus ensuring maximal nutrient uptake by cluster roots (Lamont, 1973; 2003).

In *Lupinus albus*, cluster-root distribution in the soil profile has been reported to be strongly influenced by localized OM application (Li et al., 2010). These authors point out that the positive

effect of OM on cluster-root formation is explained by P release from decomposition of OM or phytate, which is due to the activity of microorganisms.

In this sense, Shane & Lambers (2005) suggest that many free-living rhizosphere bacteria (e.g. *Pseudomonas*) release auxins that may induce cluster-root formation or enhance their growth. It is known that organic matter promotes microbiological activity (Insam & Domsch, 1988); therefore, it is likely that both OM (as a source of nutrients) and stimuli caused by microorganisms are some of the factors involved in cluster-root formation.

In Chilean Proteaceae, a greater cluster-root number in seedlings of *E. coccineum* has been observed growing under soil with high OM compared with seedlings growing under soil with low OM content (Donoso et al., 2010). Moreover, a larger number of cluster roots was observed in 9-month-old seedlings of *G. avellana* growing in young volcanic ash soils (Andisols), which are characterized by high OM content and low available P, whereas there was a lower number of cluster roots in soils with low OM and available P (Ultisols), but with a higher biomass (Krause, 1996). This author suggests that cluster-root formation is favored in soils with high OM content, although there are fewer cluster roots, but a larger biomass when this source of nutrients is not available.

4.4 Endogenous factors

Auxins are plant hormones that induce stem and root growth, acting as marker chemicals that regulate plant growth (Taiz &Zeiger, 2002). Foliar application of auxin induces cluster-root formation in seedlings of *Grevillea robusta* Cunn. Ex. R. Br. (Proteaceae) and *L. Albus* grown under sufficient P conditions that normally suppress the development of cluster roots (Skene & James, 2000; Gilbert et al. 2000). On the other hand, in seedlings of *E. coccineum* it was observed that auxin application to the leaves of seedlings grown under P deficiency increased cluster-root number and biomass (Donoso, 2011). Nevertheless, an increase in these parameters was not observed in seedlings grown with sufficient P supply. Besides, foliar application of auxin transport inhibitor reduced cluster-root number in seedlings grown under P deficiency conditions (Donoso, 2011). These authors concluded that auxin controls their formation.

5. Nutritional strategies in Proteaceae species

Phosphorus is a vital element in all living organisms. It is a component of nucleic acids, phospholipids, sugar phosphates, it is involved in all processes that require energy in form ATP and allows activation of many enzymes (Taiz & Zeiger, 2002; Marschner & Rengel, 2007).

In photosynthesis, P is important, since this element participates in many intermediate steps on carbon fixation. Thus, P is required in chloroplasts for the production and export of triosephosphates, essential for sugar and starch formation (Taiz & Zeiger, 2002).

Phosphorus limitation in plants may cause a decrease in photosynthetic rates (Terry & Ulrich, 1973; Shane et al., 2003a; Maathuis, 2009). On the other hand, soil P limitation can influence the distribution of species in the landscape, since the most sensitive plants to P limitation may be excluded from old, climatically buffered and infertile landscapes (Lambers et al., 2006; 2008; 2010).

In this sense, it is remarkable that Australian Proteaceae (*Banksia* sp.) growing on extremely P-deficient soils exhibit a very high P-use efficiency, as they present high rates of photosynthesis to extremely low concentrations of foliar P compared with leaf P in other non-Proteaceae species (Denton et al., 2007). Lambers et al. (2012b) stated that adaptive physiology of leaves from some Australian Proteaceae to support P-use efficiency in P-impoverished soils could be partially explained because theses species replace phospholipids with galactolipids and sulfolipids during leaf development to achieve a high photosynthetic phosphorus-use-efficiency.

In Chilean Proteaceae, unlike Australian Proteaceae, leaf [P] are relatively high, as shown in Table 3. According to Lambers et al (2011), it is noteworthy that Chilean species have very high leaf [P] compared with species elsewhere in the world.

Table 3. Leaf phosphorus (P) concentrations in South American and Australian Proteaceae

Species	Family	Leaf P concentration (mg g^{-1} DW)	References
Embothrium coccineum	Chilean Proteaceae	2.40	Alberdi et al. (1977)
		1.00 – 1.80	Zúñiga et al. (2009)
Gevuina avellana	Chilean Proteaceae	2.20	
Lomatia ferruginea	Chilean Proteaceae	1.78	Alberdi et al. (1977)
Lomatia hirsuta	Chilean Proteaceae	1.62	
Lomatia dentata	Chilean Proteaceae	0.60	Dielh et al. (2003)
		1.03	Alberdi et al. (1977)
Banksia sp.	Australian Proteacea	0.14 - 0.32	Denton et al. (2007)
Other woody species	Non-Proteaceae	1.38 - 3.95	Alberdi et al. (1977)
(Chilean native species)		0.50 – 1.90	Dielh et al. (2003, 2008)

However, photosynthesis rate of Chilean Proteaceae is much lower compared with that of Australian Proteaceae (*Banksia* sp.) (Table 4). This shows that the former are less efficient in P use in photosynthesis process.

Table 4. Photosynthesis rate (at 1500 µmol m^{-2} s^{-1}) in Chilean and Australian Proteaceae

Species	Age (years)	Season	Photosynthesis (µmol CO_2 m^{-2} s^{-1})	References
E. coccineum	2-3	Summer	12.5	Alberdi et al. (2009)
	2-3	Winter	8.5	
	Adults	Spring	7.3	Zúñiga-Feest (year?)
G. avellana	Adults	Spring	6.3	(Unpublished data)
L. ferruginea	2-3	Summer	6.0	Alberdi et al. (2009)
	2-3	Winter	5.0	
Banksia sp.	1	Spring	13.8-21.7	Denton et al. (2007)

Groom & Lamont (2010) reported that Proteaceae species tend to produce seeds with high [P]; seed P reserves may be very important for seedling establishment, when P in soil is limiting growth. These authors compared Proteaceae seeds from two Mediterranean floristic regions: south-west of Australia and the Cape of South Africa. In these regions, seeds from south-western Australia, where soils are more P-impoverished, have a higher weight and [P] (seed mass = 85.5 ± 23.3 mg DW; seed P = 13.2 ± 0.8 mg g^{-1} DW) than those from the Cape (seed mass = 53.4 ± 11.2 mg DW; seed P = 5.8 ± 0.8 mg g^{-1} DW).

Recent results obtained by Delgado et al. (2014) revealed that six of southern South American Proteaceae species have low values of seed mass and [P] compared with south-western Australian

and South African Proteaceae (Seed mass = 11.5 ± 1.4 mg DW (excluding seed mass of *G. avellana* which was 354 ± 11 mg DW); Seed P = 3.2 ± 0.4 mg P g^{-1} DW, on average).

These records suggests that the investigated South American Proteaceae species have not evolved to accumulate large P reserves in their seeds, unlike south-western Australian and South African Proteaceae species, which evolved in old, climatically-buffered, infertile landscapes (Hopper, 2009). Unlike Australian soils where Proteaceae commonly grow, total P is in the range of 30–80 mg kg^{-1}, but available P is < 2 mg kg^{-1} (McArthur cited by Lambers et al., 2008; Denton et al., 2007), high concentrations of total P are found in Chilean volcanic soils, being an average 1,854 and 2,582 mg kg^{-1} for uncultivated and cultivated soils (Borie & Rubio, 2003), where plant-available P varies between 1 to 45 mg kg^{-1}, respectively.

According to the unpublished results obtained in our laboratory, where soil P (Olsen), seed mass, [P] and P content in seeds were determined, there were no correlations between soil P (P Olsen) and [P] in seeds from contrasting geographical locations in Chile (35-55ºS). Moreover, it was observed that locations with low soil P availability showed no significant differences in seeds [P] compared with locations that have high available P in the soil. This suggests that to some extent, *E. coccineum* is able to accumulate P in seeds regardless of P availability in the soil.

Despite the records on *E. coccineum*, more information is still needed about other Chilean Proteaceae and woody species of temperate rainforests in order to be more conclusive about the possible adaptive advantages that Proteaceae species have in P acquisition and use efficient, as it has been raised for Australian Proteaceae. Some adaptive advantages proposed for *Banksia* sp. (Proteaceae) in internal P use efficiency are: *1)* ability to achieve high carbon gains at low [P] in leaf; *2)* long P residence times in leaves; *3)* P re-mobilization from senescent leaves to newer leaves; and *4)* ability to concentrate P in seed to ensure seedlings survival in poor P soils (Denton et al. 2007).

Regarding nutrient acquisition, it has been observed that cluster roots of Proteaceae are more effective in Pi uptake compared with non-cluster roots (Sousa et al. 2007; Shane et al. 2004c). On the other hand, a low capacity has been recorded to down-regulate P uptake at elevated external or internal [P] in *Hakea prostrata*, which may reflect an adaptive advantage in their P-impoverished environments (Shane et al. 2004b, c). However, this low capacity to down-regulate P uptake would not be a desired trait for crops growing in high P available soils, because they may develop symptoms of P toxicity (Hawkins et al. 2008, Lambers et al. 2011)

Hakea prostrata develops symptoms of toxicity (chlorotic and necrotic leaves and extrusion of ropey strands of material from the stomata) at the highest P-supply rates and when [P] in leaves is approximately 10 mg g^{-1} DM, whose value is "associated with development of P-toxicity symptoms in many other Proteaceae" (Shane et al., 2004a). However, Delgado et al. (2014) found no symptoms of P toxicity (chlorotic and necrotic leaves) in seedlings of *E. coccineum* growing at elevaled P in nutrient solution. These authors indicated that this species is less sensitive to P toxicity because has a high ability to regulate their P uptake. Similar results found Shane & Lambers (2006) in *Grevillea crithmifolia* R. Br. (Australian Proteaceae), where a resistance for developing symptoms of P toxicity was observed due to this species regulates its net P-uptake rates at elevated P supply. These authors concluded that, unlike *H. prostrata*, *G. crithmifolia* occurring in places with more nutrients availability, suggesting that the difference between these species in their susceptibility to developing P toxicity symptoms is probably related to P availability in the soils

According to the literature reviewed above, the differences shown between Chilean and Australian Proteaceae could be due to the soil type, in which these species grow. According to the theory proposed by Hopper (2009), natural selection pressure (infertile soils) could have acted in old, climatically buffered, infertile landscapes (like south-western Australia, South Africa and Venezuela's Pantepui Highlands), resulting in special nutritional and other biological traits in species that grow in these contrasting soils. One of these ecophysiological adaptations is cluster roots, which are highly efficient in nutrient acquisition, as discussed above. The study of physiology and other possible adaptive advantages (cluster-root formation, exudation, P-use efficiency, [P] in seed, etc) in Proteaceae growing in young and more fertile soils have been poorly studied and require more attention to understand their ecological role in the environment where they grow.

6.Concluding remarks

The differences between Chilean and South-Western Australian Proteaceae could be related with the contrasting soils in which the species grow, because the latter are adapted to ancient, highly weathered and extremely P poor soils, whereas Chilean Proteaceae grow in volcanic soils, which are younger and more fertile. The differences in the habitat in which these species have evolved could result in differences in formation and function of cluster roots, P-use efficiency and tolerance to develop P toxicity symptoms. This question and others related to Proteaceae growing in volcanic soils need to be studied for a better knowledge of the habitat, where these plants grow.

CHAPTER III

The effect of phosphorus on cluster-root formation and functioning of Embothrium coccineum (R. et J. Forst.)

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Abstract

Embothrium coccineum (R. et J. Forst.) is a Proteaceae species from the southern part of South America. South-central Chilean soils are younger and contain more phosphorus (P) than soils in Australia and South Africa, where Proteaceae are common. Phosphorus deficiency is the main factor promoting cluster-root formation in Proteaceae. It is not known, however, whether this also applies to *E. coccineum*, which grows on soils with higher P content.

Four-month-old seedlings were grown for 4 weeks in hydroponic cultures with 1 μM P or 50 μM P. The number of cluster roots, relative height increment, biomass distribution, cluster root/total root biomass ratio, foliar [P], root acid phosphatase activity and root carboxylate-exudation rates were determined.

The results shows that seedlings growing at 50 μM P showed a 10-, 1.3- and 3.3-fold greater increase in relative height, total dry mass and foliar P concentration, respectively, compared with those grown at1 μM P. However, seedlings grown at 1 μM P showed a 5-, 16-, 1.7- and 1.3-fold greater number of cluster roots, cluster root/total root biomass ratio, phosphatase activity and total carboxylate exudation, respectively, as compared with those grown at 50 μM P.

We concluded that a low P supply promotes the initiation, growth and metabolic activity of cluster roots which is in accordance with reports on Proteaceae species occurring in ancient and highly weathered soils.

Keywords: Carboxylate exudation, Chilean Proteaceae, phosphatase, phosphorus

Introduction

Phosphorus (P) is an essential element in all living organisms and involved in many processes requiring energy (Marschner, 2012). In soils this element is linked to Ca^{+2} (neutral or calcareous soils) and Fe^{+3} or Al^{+3} (acid soils), forming organic or inorganic compounds scarcely available to plants (Stevenson & Cole, 1999). As a consequence of low levels of available P, plants have developed root physiological strategies for P acquisition, including i) associations with symbiotic or free-living microorganisms, ii) the exudation of carboxylates and phosphatases, which enable solubilisation and/or mineralisation of insoluble phosphates; and, iii) the modification of root geometry and architecture to increase soil exploration (Richardson et al. 2011). Some plants can develop one or more of such strategies. This is the case of plants forming cluster (or proteoid) roots, which are dense clusters of fine rootlets around a main axis (Purnell 1960; Lamont 2003). The importance of cluster roots is that these structures chemically alter the rhizosphere, increasing a plant's ability to acquire sparingly available nutrients (Marschner et al. 1986; 1987, Grierson & Attiwill 1989). Cluster roots exude large amounts of carboxylates (Lambers et al. 2002; Shane &Lambers 2005) and acid phosphatase (Reddell et al. 1997; Gilbert et al. 1999; Neumann et al. 1999). Carboxylates exuded from roots promote mobilisation of P from insoluble complexes (e.g., with Ca^{2+} and oxides/ hydroxides of Al^{3+} and Fe^{3+} or displacing phosphate from the soil matrix by ligand exchange (Jones et al. 1998). In addition, acid phosphatases hydrolyse organic P compounds releasing inorganic P (Pi), the form of P available to plants (Bieleski 1973; Duff et al. 1994; Ryan et al. 2001).

Cluster roots in Proteaceae are a response to P deficiency and have mainly been studied in Australian and South African Proteaceae species (Lamont, 2003; Lambers et al. 2006), growing in old, severely P-impoverished soils (Lambers et al. 2006, 2010). For Proteaceae species growing in young, frequently disturbed and more fertile landscapes, very little information on the factors controlling cluster-root formation is available (Donoso-Ñanculao et al. 2010; Morales 2004; Ramírez et al. 1990; 2005; Zúñiga-Feest et al. 2010). In southern South America, *Embothrium coccineum* (Proteaceae) grows in volcanic soils characterised by a high total P content (> 1500 mg kg^{-1}) where P is adsorbed onto non-crystalline material in the clay fraction, oxides and hydroxides of Fe/Al and humus-Al/Fe complexes (Borie & Rubio, 2003). In these soils, organic P (Po) represents more than 50% of total P, mainly as inositol penta- and

hexaphosphates associated with Fe and Al (Borie & Rubio, 2003; Escudey et al 2001). Due to the high reactivity of with soil colloids, the availability of this element is low for plants. Thus, cluster-root formation together with their functioning (e.g., release of phosphatases and carboxylates) are probably crucial strategies for P acquisition by Proteaceae species when growing in such habitats.

The study of root ecophysiology in native plants from southern South America has scarcely been explored and there are no reports on acid phosphatase and carboxylate exudation in Proteaceae species. The study of these species bearing cluster roots growing in young soil systems which are enriched through continuous volcanic activity is essential from an ecological point of view. As Lambers et al. (2012a) proposed, these species may act as ecosystem engineers, providing P for neighbouring plants without specialised roots to access insoluble P or P that is strongly adsorbed to the soil matrix in their natural habitat. In this paper we aimed to study the effect of P availability on the formation and functioning of cluster roots (phosphatase and carboxylate exudation) of *E. coccineum.* We hypothesised that *E. coccineum* exhibits similar physiological response to low P as cluster-rooted species growing in severely P-impoverished soils.

Materials and methods

Plant material

Embothrium coccineum (R. et J. Forst.) seeds were collected in summer 2011 from the botanical garden of Universidad Austral de Chile, Valdivia (39 º 38 ′S and 73 º 5′W) and germinated in autumn. Seeds of *E. coccineum* were treated with gibberellic acid at 250 mg L^{-1} in Petri dishes in a temperature-controlled growth chamber at 20°C for 24 h to stimulate germination. Once the seeds had germinated, they were transferred to pots with organic soil to promote root production, watered to field capacity and kept in a greenhouse for four months.

Experimental design

Thirty six *E. coccineum* seedlings of uniform size were transferred from soil to 2 L plastic pots (six plants per pot and three pots per treatment) with the following nutrient solution: 3 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.5 mM $MgSO_4$, 12 µM FeEDTA, 22 µM H_3BO_3, 4 µM $MnCl_2$, 0.4 µM $ZnSO_4$, 1.6 µM $CuSO_4$, 0.05 µM Na_2MoO_4, 10 µM KH_2PO_4. Prior to the experiment, the cotyledons were removed and seedlings were kept for two weeks to acclimate to new growth conditions. Subsequently, seedlings were separated into two groups. One group was supplied with 50 µM P and another group with 1 µM P, as KH_2PO_4 (all other nutrients were kept at the same concentrations as described above). The nutrient solutions were constantly aerated, adjusted to pH 5.5 and replaced twice a week. The seedlings were grown for four weeks. The temperature in the growth chamber was 20°C and the light intensity to which the seedlings were exposed was 50 µmol m^{-2} s^{-1}. To determine the effect of P on cluster-root formation, cluster roots were counted once a week (n = 18). At the end of the experiment, carboxylates (n = 6) and phosphatases (n = 6) were collected and growth (n = 12) and leaf [P] (n = 6) measurements were carried out. Additionally, the time of cluster-root formation throughout the growing period was assessed. For this purpose, the swollen regions of roots were identified as locations of cluster-root formation keeping a photographic record from initiation to senescence.

Plant growth measurements

At the beginning and the end of the experiment the height of the stem was recorded to calculate the relative height increment as $RGRH = (\ln H_{Final} - \ln H_{Initial})/(t_{Final} - t_{Initial})$, where H = height and t = time. Additionally, numbers of cluster roots were recorded weekly. Finally, at the end of the hydroponic experiment, seedlings were separated into shoots (leaves and stems), cluster-roots and non-cluster roots, and dried at 65 °C for 48 h and weighed.

Total P concentration

Phosphorus was determined colourimetrically by the phosphoantimonylmolybdenum blue complex method described by Drummond & Maher (1995), with some modifications. In brief, dried leaves were ground and 0.5 g was ashed at 550 ºC and digested using an acid mixture (H_2O/HCl/HNO_3; 8/1/1). Subsequently, 10 mL of the acid mixture was added to the ash and

allowed to stand for 10 min. The mixture was then filtered and made to 50 mL with distilled water. Then a 1 mL subsample was mixed with a reagent containing sulfuric acid (5 M), ammonium molybdate tetrahydrate (0.038 M), potassium antimonyl tartrate (0.004 M) and ascorbic acid (0.3 M) in distilled water. Finally, the blue colour of the complex was measured spectrophotometrically at 700 nm.

Acid phosphatase activity

For a quantitative phosphatase determination the methodology described by Rubio et al. (1990) was followed. Briefly, 1 mL of p-nitrophenol phosphate (33.7 mM) was used as substrate in 25 mL of buffer solution (pH=5.5). The roots were incubated for 30 min (either an entire root system or separated into cluster roots and non-cluster roots) in a temperature-controlled water bath for one hour at 20° C. Subsequently, 5 mL of NaOH (2 M) was added to stop the reaction. Finally, the solution was filtered and the absorbance of p-nitrophenol was measured in a spectrophotometer at 400 nm.

Collection and analyses of carboxylates

The exudates were collected from entire root systems, isolated cluster roots and excised cluster and non-cluster roots. The entire root systems were submersed in a container with 50 mL 0.25 mM CaSO4 (pH=5.5). After 3 h, the solution was collected, filtered and frozen at -80 °C, and subsequently freeze-dried. The residue was resuspended in 200 μL of water for chromatography (LiChrosolv, Merck, Darmstadt, Germany) for subsequent analysis using high-performance liquid chromatography (HPLC). The same procedure was used for the excised roots. The collection of exudates at different growth stages (juvenile, mature, senescent) was performed without excising the roots. With this purpose, roots were isolated in Eppendorf tubes, where roots were submersed in 1 mL of 0.25 mM $CaSO_4$ (pH=5.5), while the remainder of the root systems was kept in aerated nutrient solution. These solutions were also freeze-dried.

Chromatographic analysis was carried out in a HPLC system (Merck-Hitachi model L-4200, Darmstadt, Germany) equipped with a UV–visible detector and a Sphere Column Heater (Phenomenex Terma model TS-130). Separation was made in a C-18 reverse phase column (LiChrospher 100 RP-18, 5 mm particle size, Merck, Darmstadt, Germany). The mobile phase

was 93% (v/v) 25 mM KH_2PO_4 at pH 2.5 and 7% (v/v) methanol with a flow rate of 1 mL min^{-1} according to the method developed by Cawthray (2003). Detection of the carboxylates was at 210 nm. Retention times were compared with citrate and malate as standards to determine the exuded carboxylates.

Statistical analysis

To determine if there were significant differences in number of clusters roots between two P treatments with time (weeks), a two-way ANOVA was carried out. For height increment, total dry mass, cluster-root mass to total root biomass ratio, and acid phosphatase activity as dependent on P supply during growth, Student's t-tests were applied. For biomass distribution and rate of carboxylate exudation, one-way ANOVAs were applied with post-hoc Tukey tests to identify significant differences. All analyses were performed with Origin 8.0 software (OriginLab corporation, Northampton, United Kingdom). Differences between the values were considered significant at a p-value ≤ 0.05.

Results

The effect of P on growth and cluster-root formation

After two weeks of growth in a hydroponic solution, both groups of seedlings supplied with either 1 µM P or 50 µM P, began to form cluster roots. Significant differences became evident in the number of cluster roots at the end of the experiment, where the mean number of clusters of the plants grown at 1 µM P was 5 per plant, whereas at 50 µM P this was only 0.5 per plant (Fig. 1).

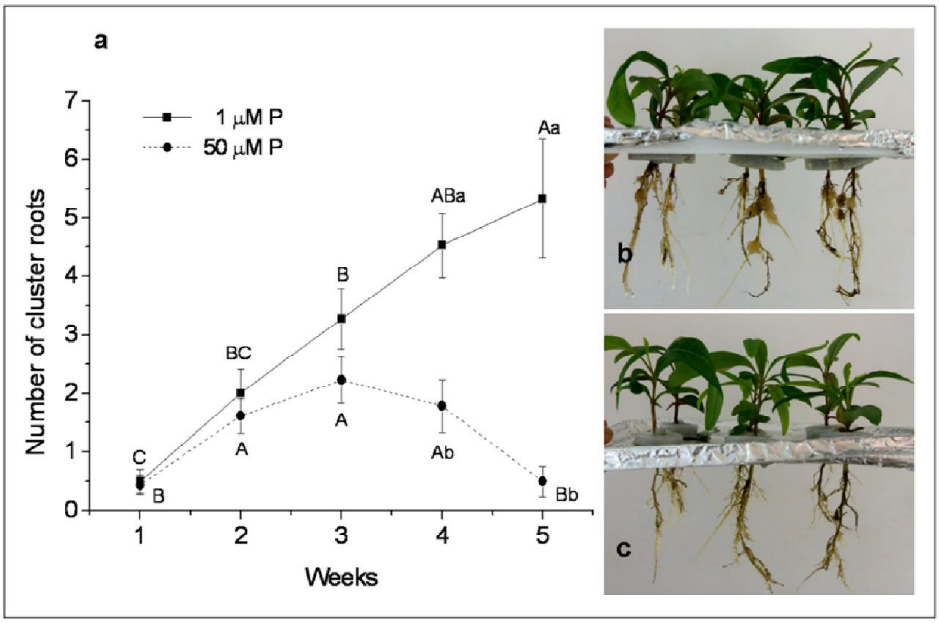

Fig. 1 a) Number of cluster roots of *Embothrium coccineum* when roots of four-months old seedlings were supplied with 1 µM P (continuous line) or 50 µM P (dashed line). Each value corresponds to a mean of 18 samples ± standard error. Capital letters indicate significant differences between weeks and lower-case letters indicate significant differences between treatments ($P \leq 0.05$). b) and c) Root systems of seedlings of *E. coccineum* growing in hydroponic culture after 28 days at 1 µM P and 50 µM P, respectively.

Seedlings grown at 50 µM P had a 90% and 27% higher relative height increment and total dry mass, respectively, compared with plants grown at 1 µM P (Fig. 2a, b). The shoot represented ca. 80% of the total plant dry weight at both P concentrations (Fig. 3a), but the root system differed between P concentrations in the growth medium.

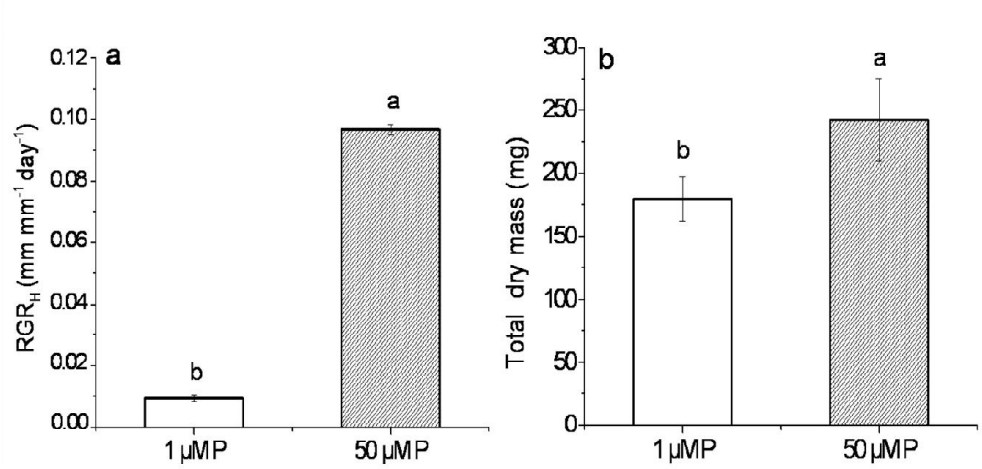

Fig. 2 Effect of phosphorus (P) on *Embothrium coccineum* seedlings grown in hydroponic culture with 1µM P or 50 µM P. a) Relative height increment (RGRH) and b) Total dry mass after a period of one month. Each value corresponds to a mean of 12 samples ± standard error. Lower-case letters indicate significant differences between treatments ($P \leq 0.05$).

In seedlings grown at 1 µM P, there was significantly less biomass allocated to non-cluster roots (12%) compared with that of plants grown at 50 µM P (16%). However, there was more biomass allocated to cluster roots (2.5%) compared with that of plants grown at 50 µM P (0.09%). Seedlings grown at 1 µM P showed a higher cluster-root/total root dry biomass ratio (Fig. 3b) than seedlings grown at 50 µM P.

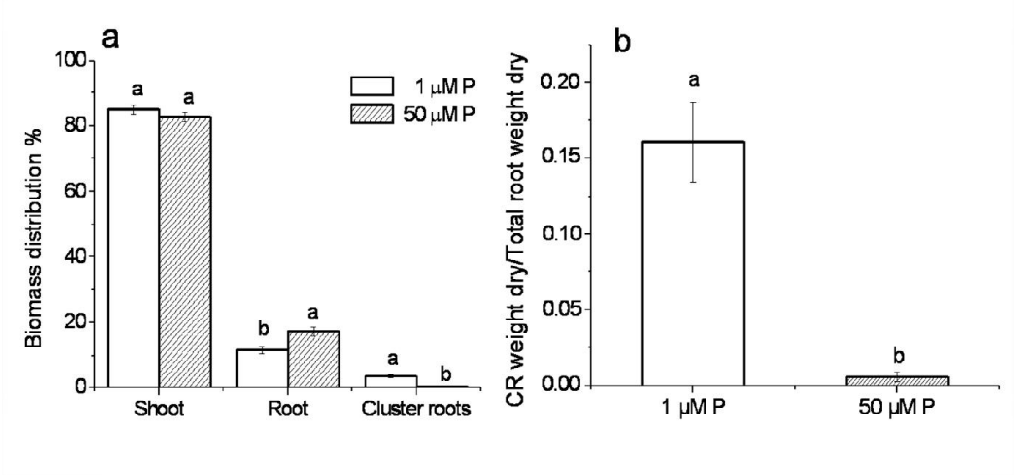

Fig. 3 Effect of phosphorus (P) on a) percentage of biomass distribution and b) cluster root (CR)/total root dry weight ratio of *Embothrium coccineum* seedlings grown for four weeks in hydroponic culture with 1 μM P (white bar) or 50 μM P (dark bar). Each value corresponds to a mean of 12 samples ± standard error. Lower-case letters indicate significant differences between treatments ($P \leq 0.05$).

Leaf P concentration

When grown at 50 μM P, leaf [P] was three times greater (2.6 ± 0.3 mg g^{-1} dry mass) than that of leaves of seedlings grown at 1 μM P (0.8 ± 0.1 mg g^{-1} dry mass). At low P supply, seedlings had green leaves (Fig. 1), without any P-deficiency symptoms.

Root exudates

Seedlings grown at 1 μM P had a higher phosphatase activity than those grown at 50 μM P (Fig. 4a). Moreover, phosphatase activity was observed in young roots and showed a higher level in cluster roots (Fig. 5b), being 2.5 times higher compared with that in non-cluster roots (Fig. 4b).

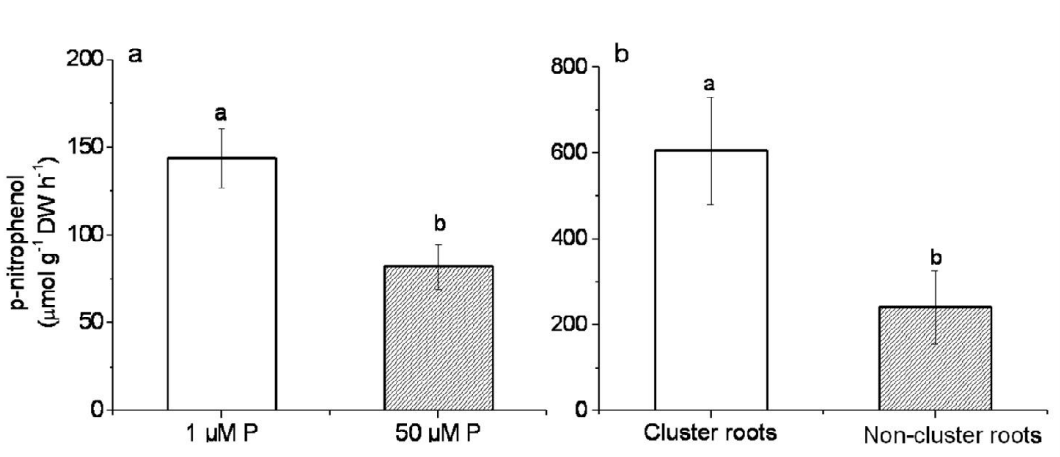

Fig. 4 Acid phosphatase activity in *Embothrium coccineum* seedlings grown for one month in hydroponic culture. a) Entire root systems of seedlings grown at 1 µM P or 50 µM P. b) Excised cluster and non-cluster roots of seedlings grown at 1 µM P. Each value corresponds to a mean of six samples ± standard error. Letters indicate significant differences between treatments (a) and tissues (b) ($P \leq 0.05$).

Seedlings grown at 1 µM P also had a higher rate of exudation of malate and citrate compared with plants grown at 50 µM P (Fig. 5a). Samples from excised cluster and non-cluster roots had greater amounts of most organic acids than samples from whole root systems. The carboxylate exudation rate from cluster roots was significantly higher than that from non-cluster roots (Fig. 5b).

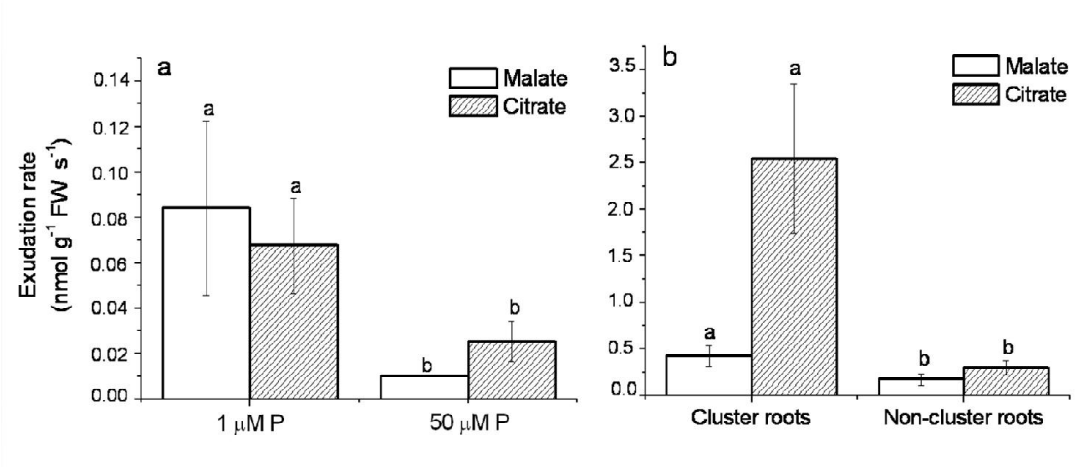

Fig. 5 Carboxylate-exudation rate of *Embothrium coccineum* seedlings grown for one month in hydroponic culture. a) Entire root systems of seedlings grown at 1 μM P or 50 μM P. b) Excised cluster and non-cluster roots of seedlings grown at 1 μM P. Each value corresponds to a mean of six samples ± standard error. Lower-case letters indicate significant differences between carboxylates ($P \leq 0.05$). Asterisks indicates significant differences between treatments (a) or tissues (b).

The time from cluster-root initiation to senescence was around 30 days (Fig. 6a). Cluster roots remained mature for approximately nine days. We observed the fastest carboxylate exudation rates in juvenile and mature cluster roots (0.73 and 0.72 nmol g^{-1} FW s^{-1}, respectively), while the exudation rate was significantly less in senescent cluster roots (0.08 nmol g^{-1} FW s^{-1}) and in non-cluster roots it was not detected (Fig. 6b).

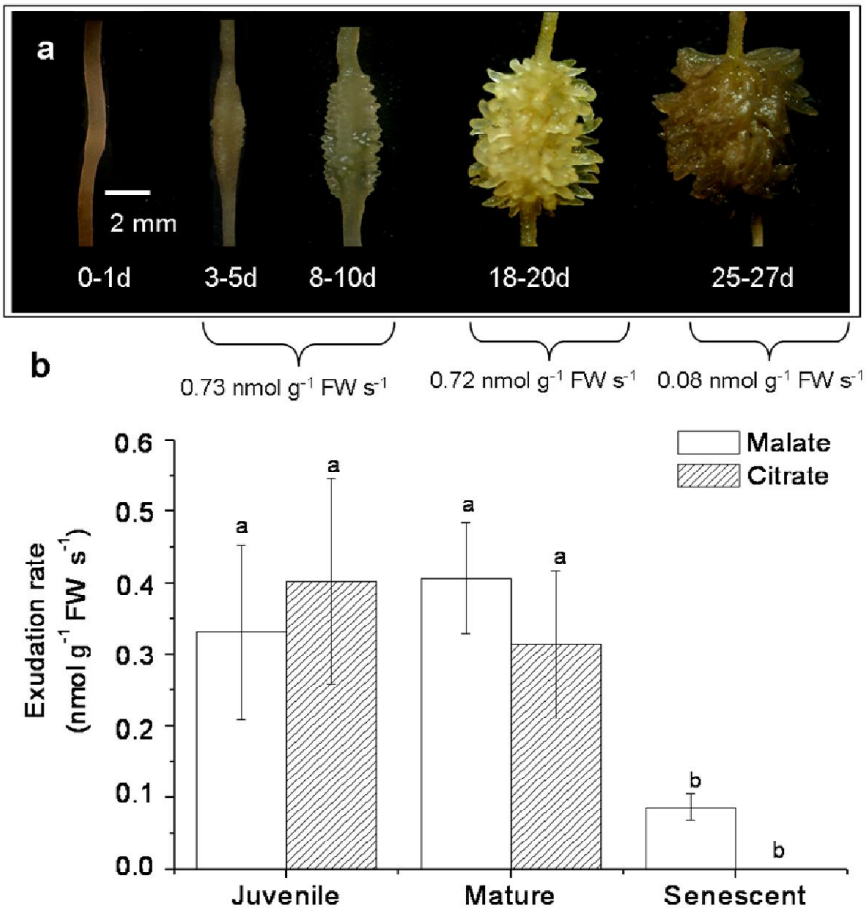

Fig. 6 Development and functioning of cluster roots of *Embothrium coccineum* seedlings grown hydroponically at 1 µM P. a) Photographic record of cluster-root development from initiation to senescence. b) Carboxylates exudation rate at different developmental stages (nmol g^{-1} FW^{-s} of each development stages: Juvenile, matures and senescent). Each value corresponds to a mean of 6 samples ± standard error.

Discussion

Phosphorus deficiency is the main factor for the formation of cluster roots in all species bearing these root structures such as occurs, for example, in *Casuarina cunninghamiana* Miq (Reddell et al. 1997), *Grevillea robusta* (Skene et al. 1996), *Morella cerifera* (Louis et al. 1990), *Banksia grandis* (Lambers et al. 2002), *Hakea sericea* (Sousa et al. 2007) and *Hakea prostrata* R.Br.

(Shane et al. 2003). Conversely, where a high P supply inhibits cluster-root formation. According to the results obtained in this study, this also applies to *E. coccineum*, which grows in soils with higher total P and a wide range of available P (Souto et al. 2009; Lambers et al. 2012a). Therefore, it responds according to the same classical model of other Proteaceae species naturally occurring on old and infertile soils (Lamont 2003; Shane &Lambers 2005).

It is interesting to highlight that at the beginning of the experiment both at low and high P supply cluster roots were formed. This might be because the young plants were P-deficient, due to cotyledon removal before the experiment. The addition of P to the nutrient solution would promote cluster-root initiation according to the model proposed by Shane at al. (2003). These authors indicate that the maximum response in relation to cluster-root initiation appears at a low P supply, rather than when no P is supplied. We observed that cluster-root formation in response to both P treatments started in the third week of the experiment, being significantly different towards the end of the experiment (Fig. 1).

Seedlings growing at the higher [P] showed a greater height increment and total dry mass. Their lower total dry mass increment compared with a higher RGRH observed in our study was probably due to the very low light intensity at which the seedlings were grown (50 μmol m-2 s-1) and the short duration of the experiment (four weeks). Seedlings grown at 50 μM P had thin leaves and stems, which was not enough to contribute to great differences in total dry biomass compared with RGRH (Fig. 2a, b). Despite these limitations in our experiment, we did observe significant differences in cluster-root formation, RGRH and total dry biomass between the two P treatments. The [P] in leaves suppressing the formation of cluster roots varies widely among species. For example, leaf [P] of *Hakea prostrata* grown at 10 μM P, which inhibits cluster-root formation, was 2.8 mg P g^{-1} leaf DM (Shane et al. 2004b). This leaf [P] is similar to that found in *E. coccineum* (2.6 mg P g^{-1}), despite a higher [P] in nutrient solution. Several authors have reported a low capacity to down-regulate P-uptake in Australian Proteaceae species (de Campos et al. 2013; Shane et al. 2004b), whereas our results suggest the opposite, since no P toxicity symptoms were observed in plants grown at 50 μM P. Nevertheless, a further thorough evaluation of this trait in Chilean Proteaceae species, which naturally grow in soils with a high total P content is needed.

We found that a low P supply increased acid phosphatase activity in roots of *E. coccineum*, similar to reports for several other species (Bieleski & Johnson 1972; Tadano & Sakai 1991; Duff et al. 1994). In addition, we observed larger amounts of phosphatase released by cluster roots compared with non-cluster roots, showing that these structures are highly adapted to access organic P, as has been suggested in other studies (Reddell et al. 1997; Gilbert et al. 1999; Neumann et al. 1999). It is important to highlight that phosphatase activity was considerably higher (6-fold) in excised tissues, raising the possibility that the intracellular phosphatase leaked into the medium when cells were damaged. However, these results are similar to those in other reports that have evaluated the phosphatase activity in cluster roots (*Casuarina cunninghamiana*, Reddell et al. 1997; *Lupinus albus*, Gilbert et al. 1999; Neumann et al. 1999) and dauciform roots (*Caustis blakei*, Playsted et al. 2006). This is the first report contrasting the activity of whole root system and excised roots.

In our study, we found a greater phosphatase activity compared with that reported in other studies mentioned above. Probably, acid phosphatase is crucial in Chilean Proteaceae growing on volcanic soils, because in these soils organic P constitutes more than 50% of total P (> 1500 mg kg^{-1}) (Borie &Rubio 2003; Pinochet et al. 2001). In relation to carboxylates exuded from roots, we observed that under P deficiency the exudation rates from cluster roots of *E. coccineum* were similar to those observed for several Australian Proteaceae species growing under P-deficient conditions, with exudation rates between 1.0 - 2.5 nmol g^{-1} FW s^{-1}. These values are considerably higher than those reported for a variety of crop species (Table 2 in Roelofs et al. 2001). When the root system was separated into cluster and non-cluster roots, the exudation of citric acid from cluster roots was significantly greater than that from non-cluster roots, as has been reported for *Hakea prostata* (Shane et al. 2004c) and *Lupinus albus* (Neumann et al. 1999; Neumann &Römheld 1999; Peñaloza et al. 2002; Kihara et al. 2003).

The development of cluster roots from initiation to senescence took approximately four weeks, which is slightly more than reported for several Hakea species (Lamont 1972; Dinkelaker et al. 1995; Shane et al. 2004a). The juvenile and mature cluster roots showed the highest exudation rate compared with senescent cluster-roots. This result differs from observations on *L. albus* and *H. prostrata*, where the highest exudation rate was for mature clusters. In *L. albus*, citrate efflux begins when cluster roots have just stopped growing, around 3 to 4 d after their emergence (Watt

& Evans, 1999), releasing large amounts of citric acid (0.32 nmol g^{-1} FW s^{-1}) when exposed to P deficiency compared with P-sufficient seedlings, where no exudation was detected (Neumann et al. 1999). In the case of *H. prostrata*, the peak of exudation rates (citrate + malate: 0.7 nmol g^{-1} FW s^{-1}) was observed from 12 to 13 days after emergence (Shane et al. 2004c). Our results showed no exudation peak in mature cluster roots, possibly because we did not excise the roots as commonly done in other studies. Probably the different techniques to collect exudates (excised and intact roots) significantly affect the results. However, we confirm that in both cases the cluster roots exuded greater amounts of carboxylates than non-cluster roots.

Concluding remarks

Like other Proteaceae species naturally occurring in highly weathered soils, *E. coccineum* exhibited more pronounced cluster-root formation and activity under P deficiency. This is the first report for Chilean Proteaceae species on acid phosphatase and carboxylate exudation, showing that both are released in large amounts by cluster roots.

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CHAPTER IV

Divergent functioning of Proteaceae species: the South American Embothrium coccineum displays a combination of adaptive traits to survive in high-phosphorus soils

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Abstract

Proteaceae species in south-western Australia thrive on phosphorus (P)-impoverished soils, employing a P-mining strategy involving carboxylate-releasing cluster roots. Some develop symptoms of P toxicity at slightly elevated soil [P], due to their low capacity to down-regulate P uptake. In contrast, Proteaceae species in Chile, e.g., *Embothrium coccineum* J.R. Forst. & G. Forst., occur on volcanic soils, which contain high levels of total P, but P availability is low.

We hypothesised that the functioning of cluster roots of *E. coccineum* differs from that of south-western Australian Proteaceae species, in accordance with the difference in soil P status. With more P to be gained from the soil with high levels of total P, we expect less investment in biomass and more release of carboxylates. Furthermore, we hypothesised that *E. coccineum* regulates its P-uptake capacity, avoiding phosphorus toxicity when grown at elevated P levels. To test these hypotheses, *E. coccineum* seedlings were grown at a range of P supplies in nutrient solution.

We show that *E. coccineum* allocated at least five times less biomass to cluster roots that released at least nine times more carboxylates per unit cluster root weight compared with south-western Australian species (e.g., *Banksia*, *Hakea*). The highest P supply caused a growth inhibition and high leaf [P], without symptoms of P toxicity. We accept our hypotheses on the functioning of cluster roots and the high capacity to reduce the net P uptake in plants grown at a high P supply.

This novel combination of traits indicates divergent functioning of Proteaceae species from southern South America, exposed to frequent P input due to volcanic activity, in contrast with the functioning of south-western Australian Proteaceae species that thrive on severely P-impoverished soils. These traits could explain the functioning of *E. coccineum* on soils that are rich in total P, but with a low concentration of available P.

Keywords: carboxylates, cluster roots, phosphorus uptake, phosphorus toxicity, seed phosphorus content, severely phosphorus-impoverished soils, trade-off, young volcanic soils.

Introduction

Proteaceae are a predominantly southern-hemisphere family with a Gondwanan origin, with their main centres of diversity in Australia and South Africa (Pate, Verboom & Galloway, 2001; Barker et al., 2007). Many species are renowned for their capacity to thrive on some of the world's most ancient and phosphorus- (P) impoverished soils (Lambers et al., 2010; Lambers et al., 2013a). This is possible, in part, because the vast majority of Proteaceae species develop dense clusters of short-lived, fine, hairy rootlets at intervals along main root axes (Purnell, 1960; Lamont, 2003). These cluster roots release large amounts of carboxylates in an exudative burst (Shane & Lambers, 2005; Shane et al., 2004a). The role of carboxylate (e.g., citrate, malate) exudation from cluster roots in forming complexes with Ca^{2+} and oxides/hydroxides of Al^{3+} and Fe^{3+} during P solubilisation to enhance the acquisition by plants has been studied extensively (Gardner, Parbery & Barber, 1981; Dinkelaker, Hengeler & Marschner, 1995; Ryan, Delhaize & Jones, 2001; Lambers et al., 2006).

Some Proteaceae species adapted to low-P soils develop foliar symptoms of P toxicity when grown at soil P levels just above those in their natural habitat (Grundon, 1972; Grose, 1989; Lambers et al., 2002; Shane, McCully & Lambers, 2004b; Hawkins et al., 2008; de Campos et al., 2013). *Hakea prostrata* R.Br. develops symptoms of P toxicity when the leaf [P] is approximately 10 mg P g^{-1} dry mass (DM), which is a value "associated with development of P-toxicity symptoms in many other Proteaceae" (Shane et al., 2004b). These authors showed that development of P toxicity is due to the species' low capacity to down-regulate its P-uptake systems. The closely related *Grevillea crithmifolia*, which occurs on slightly richer soils, shows down-regulation of its P-uptake capacity and no signs of P toxicity when grown at higher P supply (Shane & Lambers, 2006). Similar differences have been observed in a comparison of South African Proteaceae (Shane, Cramer & Lambers, 2008). Mild symptoms of P toxicity (i.e. yellow spots on some leaves and drying and curling of leaf tips) were reported for *Banksia attenuata* R.Br. and *B. menziesii* R.Br. plants grown at 10 µM P in nutrient solution (de Campos et al., 2013). As did Shane et al. (2004b), the authors concluded that this was due to these species having a low capacity to down-regulate their P-uptake systems. A high capacity to down-regulate its P-uptake capacity is crucial for any species that occurs in a habitat with a high P availability to avoid P toxicity (Lambers et al., 2006).

The southern South American Proteaceae species *Embothrium coccineum* J.R. Forst. & G. Forst. grows in volcanic soils with high levels of total P, >1,500 mg kg^{-1} (Borie & Rubio, 2003) compared with those in south-western Australian soils (<80 mg kg^{-1}) (Lambers et al., 2006), where Proteaceae species are abundant (Pate et al., 2001). In volcanic South American soils, P has high reactivity with soil colloids, being adsorbed onto non-crystalline material in the clay fraction (allophane), oxides and hydroxides of Fe and Al and humus complexes with Al and-Fe (Borie & Rubio, 2003). No symptoms of P toxicity have ever been recorded for seedlings *of E. coccineum* grown in sand and fertilised with nutrient solution containing 1,000 μM P (Zúñiga-Feest, Delgado & Alberdi, 2010), nor in seedlings growing in nutrient solution with 50 μM P (Delgado et al., 2013). These results suggest that *E. coccineum* is less sensitive to P toxicity than some other Proteaceae species occurring in old and infertile soils in Australia and South Africa (Lambers et al., 2013a). Our aim was to test the effects of an increased P supply on the development of P-toxicity symptoms, carboxylate exudation and P uptake by roots of *E. coccineum* seedlings. We hypothesised that *E. coccineum* has a strong capacity to down-regulate its P-uptake systems, thus avoiding P toxicity when grown at a high P supply. To test this hypothesis, we grew seedling in nutrient solution at a range of [P], as done before with both P-sensitive and P-insensitive Proteaceae species, and measured their P-uptake capacity. We also hypothesised that *E. coccineum*, which naturally occurs on soils with a high total [P] but a low P availability, will exhibit less investment in cluster-root biomass, but show faster rates of carboxylate exudation compared with that of species endemic to severely P-impoverished soils. This hypothesis was tested by measuring the investment of biomass in cluster roots and the rate of release of carboxylates from these cluster roots, and comparing the results with information in the literature on species from south-western Australia.

Materials and methods

Collection, dry weight and P content of southern South American Proteaceae seeds

In order to compare seed P reserves of southern South American Proteaceae species with those of south-western Australian species, which produce large seeds with high P content (Groom & Lamont, 2010), we collected seeds from six Proteaceae species during 2012 and 2013 growing in Chile: i) *Embothrium coccineum* J.R. Forst. & G. Forst., ii) *Gevuina avellana* Mol., iii) *Lomatia hirsuta* Lam. from Parque Katalapi, Región de los Lagos (41° 31' S – 72° 45' W), iv) *Orites myrtoidea* Poepp. & Endl. from Antuco, Región del Bio-Bio (37º 20' S – 71º 41' W), v) *Lomatia dentata* Ruiz et Pavon R.Br. from the Universidad Austral de Chile, Región de los Ríos (39°48' S - 73° 15' W) and vi) *Lomatia ferruginea* Cav. R. Br. from Puyehue (40º 76' S – 72º 5' W). Average seed weight for each species was calculated from batches of 30 seeds (n = 3), and [P] determined as outlined below.

Plant material and growth conditions

Seeds of *E. coccineum* were sent to the plant growth facilities at the University of Western Australia where they were sown in standard native plant soil mixture (pine bark, coco peat, river sand (w/w/w; 5:2:3), pH 5.5).

After two months, 42 seedlings of uniform size were selected and the soil was washed from the root systems. Each plant was transferred to a 2 L pot containing continuously-aerated nutrient solution: 200 µM $Ca(NO_3)_2$, 200 µM K2SO_4, 54 µM $MgSO_4$, 20 µM KCl, 0.24 µM $MnSO_4$, 0.1 µM $ZnSO_4$, µM 0.018 $CuSO_4$, 2.4 µM H_3BO_3, 0.03 µM Na_2MoO_4, 10 µM FeEDTA (pH 5.6). Root temperature was maintained at 18 to 20 ^{0}C by placing the pots in a root-cooling tank. Seedlings were assigned to one of six P treatments (supplied as KH_2PO_4): 1, 10, 25, 50, 100 and 250 µM for two months (n = 6 plants per treatment). Entire nutrient solutions including the P treatments were replaced daily. Plants were cultivated in glasshouses as above during spring (average conditions, 11/35 ºC day/night; 13/11 h photoperiod at 710 µmol m^{-2} s^{-1} photosynthetically active radiation).

Plant growth measurements

Height and total dry weight were determined for each plant at the beginning and end of the experiment. Relative growth rates (RGR) were calculated as described previously (Barrow, 1977): i.e. RGR = (ln XFinal – ln XInitial)/(tFinal − tInitial); where X = height (cm) or dry weight (g) and t = time (days). Cluster-root growth was assessed by counting the number of cluster roots produced by each plant per week.

Measurement of net P-uptake rates by intact root systems

Net P-uptake rates were determined as reported previously (Shane, Szota & Lambers, 2004c). Briefly, 24 h before the final harvest, plants were transferred to individual pots containing basal nutrient solution lacking P. The following day, basal nutrient solutions were replaced and supplemented with 5 μM P (supplied as KH_2PO_4). Following thorough mixing of the solution, samples (1 mL) from each pot were taken at t = 0 and then at 30-min intervals between 9:30 and 11:30 h. The [P] of each sample was determined using the malachite green method (Motomizu, Wakimoto & Toei, 1983). All P-depletion profiles were linear with respect to time.

Collection of root exudates

This was conducted using intact, whole root systems of transpiring plants or excised cluster roots, and non-cluster roots. Excised cluster roots were assigned to one of three stages of cluster-root development, as defined previously by Delgado et al. (2013) according to the number of days following rootlet initiation (DAI), i.e. i) juvenile 12 DAI, ii) mature 13 to 25 DAI, and senescent: > 25 DAI. Non-cluster roots were defined as un-branched regions of young main root axes up to 3 cm proximal from the tips. Root systems or excised roots were incubated for 3 h in basal nutrient solution (18–20 °C as above) lacking P. After this period, solutions were syringe-filtered to 0.2 μm and stored at -20 °C. Lyophilised samples were resuspended in 93% (v/v) 25 mM KH_2PO_4 + 7% (v/v) methanol (pH 2.5) for HPLC analysis.

Carboxylate identification and quantification

Chromatographic analysis was carried out by HPLC (Waters/Millipore, Milford, MA, USA) 600E dual head pump, 717 plus auto-sampler and a 996 photo-diode array (PDA) detector. Analysis was performed on an Altima C-18 column (250 mm x 4.6 mm, I.D.) with 5 μm particle size (Alltech Associates, Deerfield, IL, USA). Mobile phase (93% (v/v) 25 mM KH_2PO_4 + 7% (v/v) methanol (pH 2.5)) was passed through the column at 1 mL min^{-1}. Detection of carboxylates was at 210 nm. Carboxylate identification and quantification was carried out as previously described (Cawthray, 2003).

Determination of tissue total P concentrations

At the end of the experiment, plants were separated into leaves, stems, cluster roots and non-cluster roots, and dried to constant weight at 65 °C. Tissue or seed samples were powdered using a mortar and pestle, and total P determinations were carried out after acid digestion using the malachite green method (Shane et al., 2004c).

Determination of plant-available and total P concentrations in soil

'Plant-available' (Olsen) soil P was measured by extracting soil with 0.5 M $NaHCO_3$, at pH 8.5, according to Olsen & Sommers (1982) and determined colourimetrically by the phosphoantimonylmolybdenum blue complex method (Drummond & Maher, 1995). Total soil P was determined after digestion of 1 g of soil, using nitric acid and perchloric acid (1:1). Subsequently, the acid mixture was filtered and made to 250 mL with deionised water. Then, 5 mL subsample was mixed with a reagent containing sulfuric acid (0.5 M), ammonium molybdate tetrahydrate (4 mM), potassium antimonyl tartrate (0.5 mM) and ascorbic acid (30 mM) in deionised water. Finally, the blue colour of the complex was measured spectrophotometrically at 880 nm, as described above.

Phosphorus desorption into the soil solution as affected by citrate

To examine the effect of citric acid on P desorption into the soil solution, citric acid solutions were prepared at 0, 1, 5, 10, 20, 50, 100, 500 and 1000 μM in 5 mM KCl (pH 4.5). Five mL of each citrate solution was added to 1 g (n=6) of steam-sterilised air-dried Karakatta sand (this soil is a typical severely P-impoverished soil in the region upon which numerous species of Proteaceae thrive). Details of the soil characteristics and preparations prior to be used in the experiment are given in Suriyagoda et al. (2012). Samples were rotated on an end-over-end shaker at 30 rpm for 20 min and then centrifuged (16,000 g for 20 min). The resultant supernatants were syringe-filtered to 0.45 μm. Citric acid identification and quantification and [P] were done for clarified extracts as described above. In order to best describe the citrate adsorption and P desorption of the soil with the equilibrium soil solution citrate concentration, non-linear model fitting was performed using PROC NLIN in SAS, and the best fitted model was judged by the coefficient of determination (R^2).

Statistical analysis

To determine if there were significant differences in relative growth rates in height and biomass, carboxlylate exudation rate, Net P-uptake and tissue [P] of *E. coccineum* seedlings as dependent on P supply during growth, one-way ANOVAs were applied with post-hoc Tukey tests. The same test was applied to determine significant differences in dry weight, [P] and content of seeds of six South American Proteaceae species. To determine significant differences in number of cluster a factorial ANOVA was carried out, using the main factor (number of cluster roots) and their interaction: P supply (1-250 μM P) and Time (weeks). All analyses were performed with Origin 8.0 software (OriginLab corporation, Northampton, United Kingdom). Differences among the values were considered significant at p-value ≤ 0.05.

Results

Variation in seed mass, P concentration and content

Mean seed dry weight (DW) ranged from 5.3 mg to 17.2 mg amongst the six species (Table 1). *Gevuina avellana* showed by far the largest seed mass and the lowest seed [P]; its higher total P content was accounted for by its very large dry mass compared with that of the other species. Average seed [P] of the six species was 3.2 mg g^{-1} DW.

Table 1. Dry weight, phosphorus (P) concentrations and contents of seeds of six South American Proteaceae species and total and 'plant-available' (Olsen) soil P in the top 20 cm in the natural habitat in Chile.

Species	Weight* (mg $seed^{-1}$)	[P] (mg g^{-1})	Total seed P (μg)	Total soil P (mg kg^{-1})	pH (H_2O)	Olsen P (mg kg^{-1})
Embothrium coccineum	17.2 (0.6) b	4.7 (0.2) a	80.6 (4.8) b	1648	4.4 - 5.6	0.1 – 2.6*
Orites myrtoidea	11.3 (0.5) c	3.4 (0.2) ab	37.9 (0.7) c	4300	5.4	5.2
Gevuina avellana	354.2 (11.1) a	2.2 (0.2) b	762.6 (110.8) a	1648	4.4 - 5.6	0.1 – 2.6*
Lomatia ferruginea	8.8 (0.2) cd	3.2 (0.4) ab	28.4 (2.6) cd	2575	5.2	2
Lomatia dentata	6.3 (0.1) d	2.2 (0.2) b	14.1 (1.4) d	1957	5.6	13.2**
Lomatia hirsuta	5.3 (0.1) d	3.6 (1.2) b	18.5 (6.5) d	1648	4.4 - 5.6	0.1 – 2.6*

Each value represents the mean (SE in parentheses, $n = 3$). Values not sharing the same lower case letter within each column are significantly different ($P \leq 0.05$).

* Data from Donoso-Ñanculao *et al.* (2010).

** Data from Krause (1996).

Soil P concentrations in the natural habitat

In Chile, *Orites myrtoidea* only grows on the Andes Mountains (37-39°S). Total and 'plant-available' P from the soil where this species grows was as high as 4,300 mg kg^{-1} and only 5.2 mg kg^{-1}, respectively. The other Proteaceae species occur in both Chile and Argentina (36° S- 44°S) (Steubing, Alberdi & Wenzel, 1983); total soil P was considerably higher than that in the habitat of south-western Australian and South African Proteaceae, but 'plant-available' soil P in their habitats were relatively low (Table 1).

Influence of P supply on growth and cluster-root formation of E. coccineum

Two months following initiation of P treatments, the relative growth rates for height and biomass accumulation (RGRH and RGRB, respectively) were maximal for seedlings supplied with 10 μM P during growth (Fig. 1). However, RGRH and RGRB were reduced significantly only for seedlings grown at the highest P supply (250 μM).

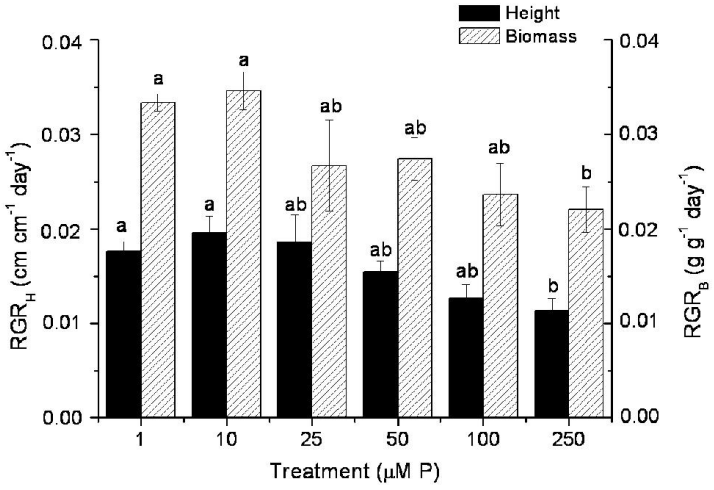

Figure 1. Influence of different phosphorus (P) supplies on relative growth rates for plant height (RGRH) or biomass (RGRB) of *Embothrium coccineum* seedlings. Each value represents the mean, bars are SE (n = 6). Values not sharing the same the lower case letter within each black or hatched bars are significantly different ($P \leq 0.05$).

As [P] supplied for growth was increased, there was a steady increase in biomass allocated to shoots, while biomass allocation to the roots decreased (Fig. 2). By the end of the experiment, cluster roots were present only on plants supplied with 1 or 10 μM P, comprising less than 5% of the total plant biomass (Fig. 2).

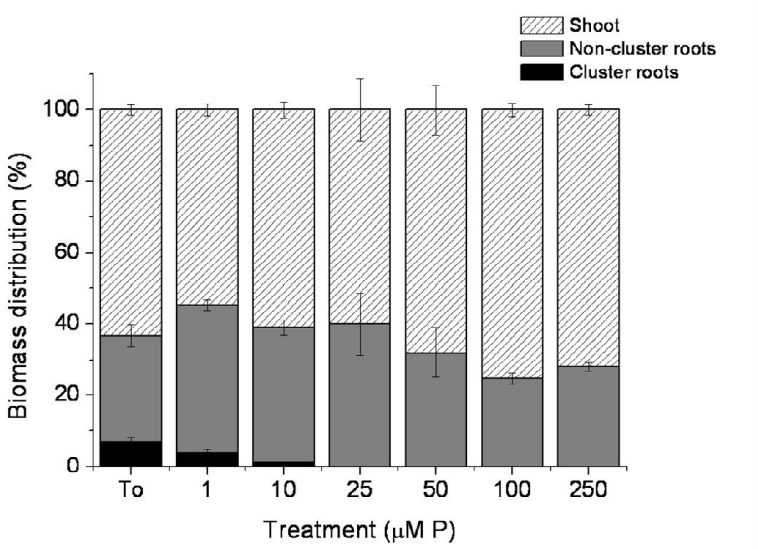

Figure 2. Percentage biomass distribution of *Embothrium coccineum* seedlings prior to the start of the experiment (To) and in seedlings grown for two months at a phosphorus (P) supply of 1 to 250 µM. Each value represent the mean, bars are SE (n = 6).

Significant differences were found in cluster-root number and their interaction (P supply and time) based on factorial Anova (p-value ≤0.05). The reduction in numbers of cluster roots with increasing P supply took approximately 5 weeks (Fig. 3a).

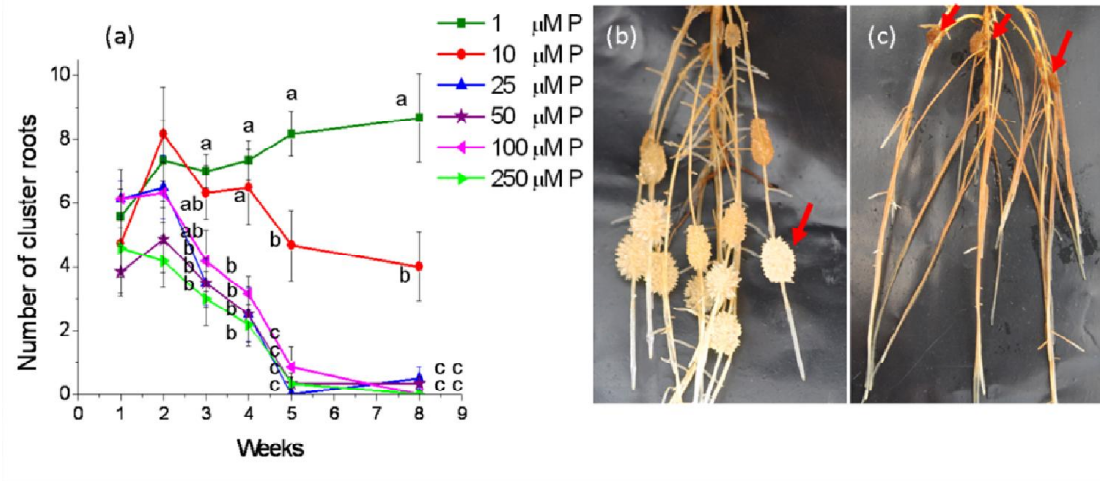

Figure 3. Variation in the number of cluster roots developed on *Embothrium coccineum* seedlings cultivated over 8 weeks at different phosphorus (P) supplies. (a) Total number of cluster roots

present. Bars are SE (n = 6). Values not sharing the same the lower case letter within each week are significantly different ($P \leq 0.05$). (b, c) Representative root systems of a six-month-old seedling following growth over two months at different P supply. (b) Plant supplied with 1 μM P showing sustained development of young cluster roots (arrows), and (c) root system of a plant supplied with 25 μM P showing only the decayed remains of cluster roots (arrows) that were present at the beginning the P treatments.

Influence of P supply on tissue P concentration of E. coccineum

Seedlings cultivated for three weeks at 10 to 250 μM P shed 10 to 40% of their leaves (see Fig. S1a in supporting information). Shed leaves, had eight-fold greater leaf [P] compared with mature fully-expanded leaves at the end of the experiment (Table 2). Prior to shedding, these leaves showed necrosis, with yellow and black spots on the foliage (Fig. S1b, c). The [P] of mature fully-expanded leaves and stems increased six- and nine-fold, respectively, as the P supplied for growth was increased from 1 to 250 μM (Table 2). Root [P] increased from 1.0 to 2.3 mg P g^{-1} DW.

Table 2. Tissue phosphorus (P) concentrations of *Embothrium coccineum* seedlings grown at 1, 10, 25, 50, 100 or 250 μM P supply.

	P concentration (mg g^{-1} DW)					
Treatment	Cotyledons	Shed leaves	Mature leaves	Stems	Non-cluster roots	Cluster roots
pre-treatment*	0.3 (0.0)	--	1.1 (0.2) ab	1.0 (0.2) c	4.9 (0.6) a	1.5 (0.5) a
1 μM	-	--	0.5 (0.1) b	0.8 (0.2) c	1.0 (0.1) bc	0.5 (0.1) b
10 μM	-	12.9 (2.1) a	1.2 (0.2) ab	1.9 (0.4) bc	0.8 (0.1) c	0.4 (0.1) b
25 μM	-	15.8 (0.8) a	1.3 (0.1) ab	2.9 (0.9) b	1.9 (0.4) b	---
50 μM	-	17.2 (1.6) a	2.9 (0.7) a	6.9 (1.1) a	1.9 (0.5) b	---
100 μM	-	18.2 (2.1) a	2.1 (0.6) ab	6.3 (0.9) ab	1.5 (0.1) bc	---
250 μM	-	19.2 (0.5) a	3.2 (1.1) a	7.2 (1.3) a	2.3 (0.6) b	---

All values represent means (SE in parentheses, $n = 4$). Values not sharing the same the lower case letter within each column are significantly different ($P \leq 0.05$).

*Pre-treatment: Four-month old seedlings grown in a soil mixture (pine bark, coco peat and river sand; 5:2:3) were transferred to nutrient solution with the indicated P concentration.

- Cotyledons were removed at the beginning of the experiment

-- Seedlings did not shed leaves

--- Seedlings grown over 10 μM P did not develop cluster roots.

Seedlings supplied with 1 μM P had the lowest leaf [P], while seedlings supplied with 50 or 250 μM P had a significantly higher leaf [P] concentration (Table 2). Seedlings provided with 10, 25 or 100 μM P had a mean leaf [P] of 1.5 mg P g^{-1}, but this was not significantly different from either the higher or lower mean leaf [P] (Table 2). These results show great variability in seedling response to different P supply, even at the same P treatment, especially at higher P supply. However, regardless of [P] supplied to seedlings, there were no symptoms of P toxicity.

Influence of P supply on rates of carboxylate exudation

Fast carboxylate-exudation rates were determined for plants grown at 1 μM P (Fig. 4), compared with seedlings grown at higher P levels. Malic and citric acid were the major carboxylates exuded, with only trace amounts of cis-aconitic, fumaric, and trans-aconitic acid detected (Fig. 4).

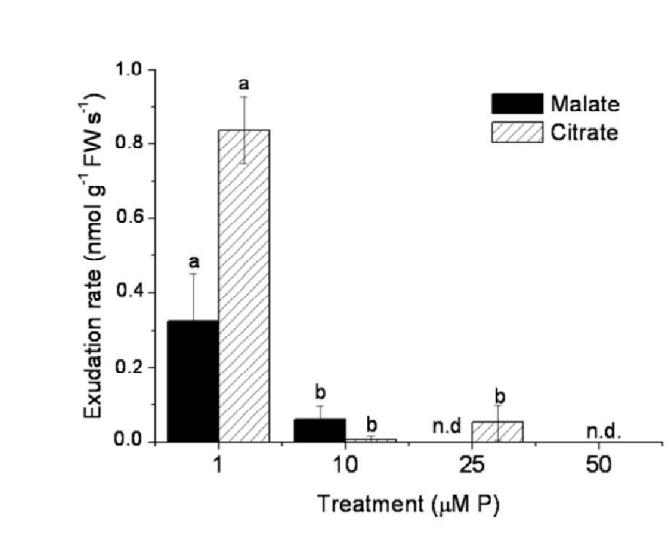

Figure 4. Rates of carboxylate exudation from intact root systems of transpiring plants after two-months growth at different phosphorus (P) supplies of *Embothrium coccineum* seedlings. All values represent means ±SE (n = 3). Values not sharing the same lower case letter within each black or hatched bars are significantly different ($P \leq 0.05$). n.d = not detected.

Similar results were obtained for excised cluster roots; however no citric acid was detected in exudates of non-cluster roots (Table 3).

Table 3. Rates of citrate and malate exudation from excised cluster roots and non-cluster roots of *Embothrium coccineum* after two months of phosphorus (P) treatment at 1 or 50 μM P.

Treatment	Tissue	Exudation rate (nmol g^{-1} FW s^{-1})	
		Malate	Citrate
1 μM P	Juvenile cluster roots	0.1 (0.1) b	2.9 (1.5) b
1 μM P	Mature cluster roots	1.3 (0.6) a	14.0 (5.3) a
1 μM P	Senescent cluster roots	0.0 (1.0) b	ND
1 μM P	Non-cluster roots	1.0 (0.1) a	ND
50 μM P*	Non-cluster roots	0.6 (0.3) b	ND

All values represent means (SE in parentheses, $n = 3$). Values not sharing the same the lower case letter within each column are significantly different ($P \leq 0.05$).

ND: Not detected

* Seedlings grown at 50 μM P did not develop cluster roots.

Influence of P supply on P-uptake rates

Net P-uptake rates measured at 5 μM P for all plants, irrespective of the [P] in the nutrient solution during growth, were significantly reduced for plants grown at high [P] in the nutrient solution compared with plants grown at 1 μM P (Fig. 5). This reduction in P-uptake capacity shows that *E. coccineum* has the capacity to down-regulate its high-affinity P-transport system which avoided P-toxicity symptoms and allowed 100% seedling survival.

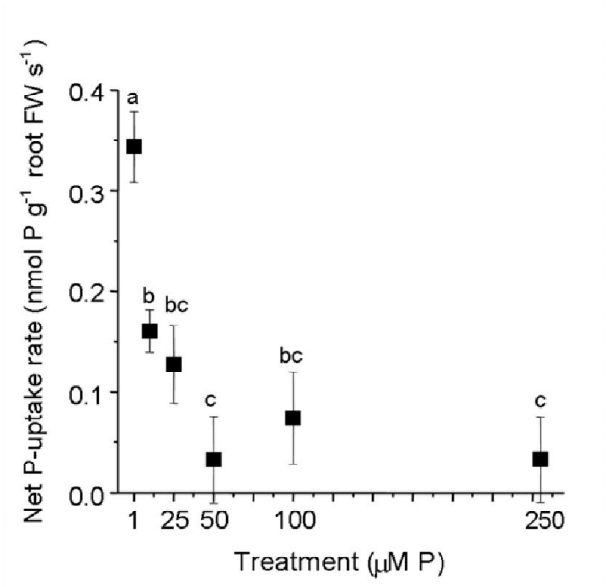

Figure 5. Net phosphorus- (P) uptake rates from intact root systems of transpiring plants of *Embothrium coccineum* seedlings determined at 5 μM P for plants grown for two months at different P supplies, ranging from 1 to 250 μM. Since the same concentration was used during P-uptake measurements, the results compare P-uptake capacity, rather than the P-uptake rate during growth at different concentrations. Each point represent the mean and bars are SE (n = 6). Values not sharing the same lower case letter are significantly different. All plants in all treatments appeared healthy, without any foliar symptoms of P toxicity.

Citrate adsorption and P desorption

When citric acid concentrations in the soil solution were increased, the amount of citrate adsorbed onto the low-P south-western Australian soil increased, but at a very slow rate, even at a high equilibrium soil solution citric acid concentration (Fig. 6b). In the absence of citric acid in soil solution, solution [P] were 0.1 mg P kg^{-1} soil DW, while with the sorption of citrate onto soil, the soil solution [P] increased to 0.5 mg P kg^{-1} soil DW (Fig. 6b).

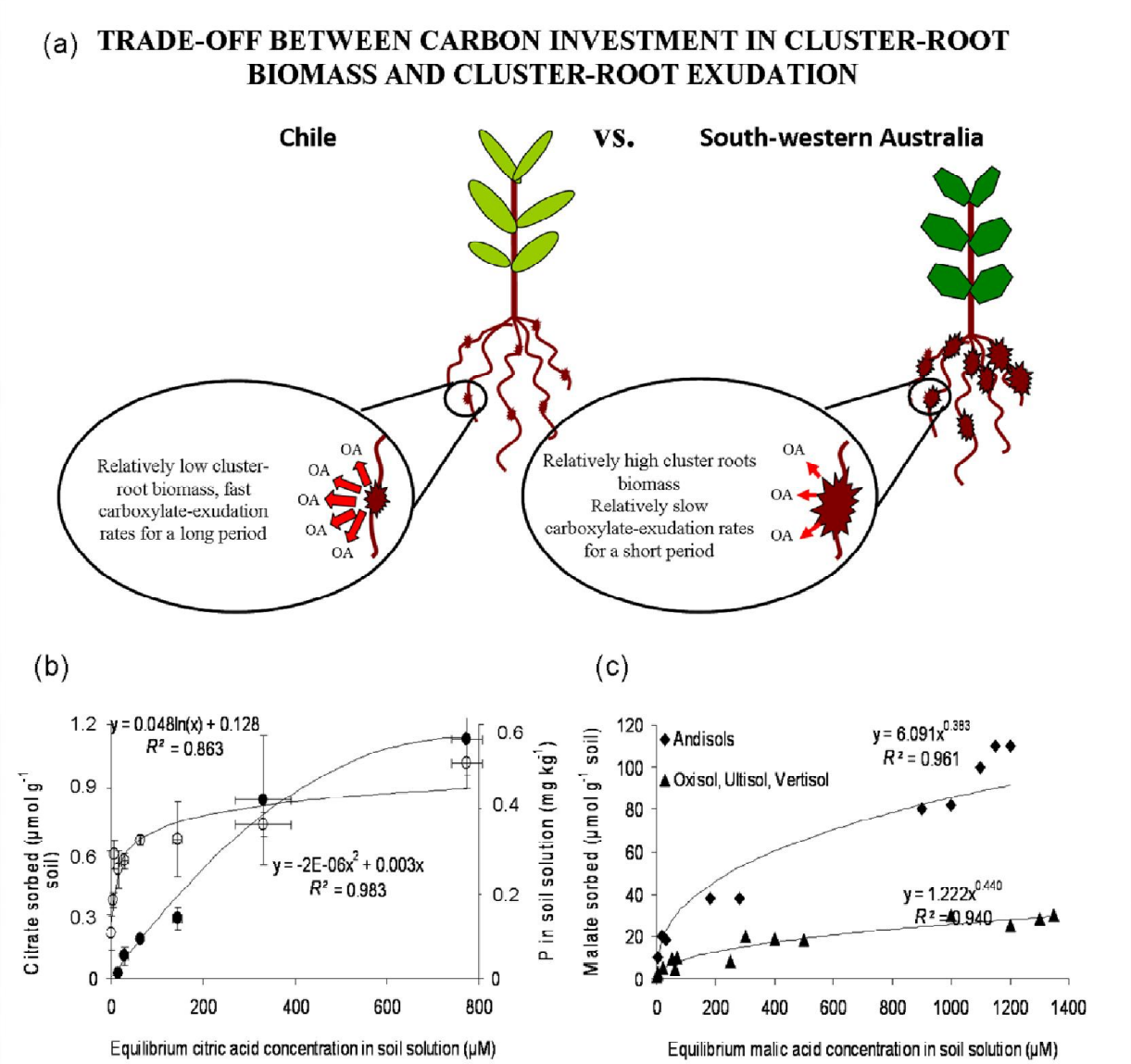

Figure 6. Model summarising the experimental present data on *Embothrium coccineum* and literature data on *Hakea prostrata*, showing divergent investment in and functioning of cluster roots of a Proteaceae species from Chile versus that of species in south-western Australia. (a) Cluster-root production and citrate release as dependent on soil characteristics in *Embothrium coccineum* (left) and *Hakea prostrata* (right). (b) The amount of citrate sorbed onto soil (black circles) and phosphorus (P) desorbed into soil solution (white circles) as dependent on the equilibrium citric acid concentration in the soil solution for a low-P south-western Australian soil (total P concentration=60 mg kg^{-1}), and their fitted relationships (lines). Each value represents the

mean; bars are SE (n = 4). (c) Sorption of malate on five mineralogically diverse soils from Hawaii; reproduced from Hue (1991).

Discussion

In accordance with our hypotheses, *E. coccineum*, which naturally occurs on high-P soils with low P availability, exhibited traits that differ remarkably from those described for Proteaceae species occurring on severely P-impoverished soils in Australia and South Africa.

Influence of P supply on growth and tissue P concentration of E. coccineum

At high P supply, *E. coccineum* seedlings initially showed reduced growth (height and biomass) and symptoms of P toxicity. However, all seedlings recovered, showing a RGR at an optimum P supply of 0.033 g g^{-1} day^{-1} over a period of 60 days. This value is only slightly higher than that of the south-western Australian *Banksia grandis* Willd. (0.026 g g^{-1} day^{-1}) grown in soil supplied with an optimum level of P over a period of 63 days (Barrow, 1977). Therefore, the recovery of *E. coccineum* seedlings after having been exposed to high P supply was not due to rapid growth, but rather to suppression of its P-uptake capacity, as discussed below.

After three weeks of growth at a high P supply, *E. coccineum* seedlings showed symptoms of P toxicity. However, seedlings recovered after they shed some of their older leaves with very high [P] (>10 mg P g^{-1} DW), a "value associated with development of P-toxicity symptoms in many other Proteaceae" (Shane et al., 2004b), and began to produce new leaves. This result reveals the capacity of this species to acclimate to high-P conditions. In this regard, it is noteworthy that *E. coccineum* has been reported as a facultative evergreen species, since it is capable of shedding its leaves under stress conditions, for example cold or drought (Escobar et al., 2006). So far, there are no reports on the ability of *E. coccineum* to shed its leaves under high-P conditions, but we do know that leaves of *E. coccineum* can show a very low P-remobilisation efficiency in its natural habitat (Lambers et al., 2012a).

In seedlings grown at high P supply, the [P] in mature leaves of *E. coccineum* was maintained below toxic levels, showing lower values than those in the stem. Several authors have suggested that the stems or roots act as a sink for excess P, thus reducing P-toxicity symptoms in Proteaceae (e.g., *Banksia ericifolia* L.f. (Parks, Haigh & Cresswell, 2000); *Hakea prostrata* (Shane et al.,

2004b); *Grevillea crithmifolia* R.Br. (Shane & Lambers, 2006). Parks et al. (2000) and Shane et al. (2004b) concluded that when stems or roots exceed their P-storage capacity, the leaf [P] increases to toxic levels (>10 mg P g^{-1} DW), leading to visible P-toxicity symptoms. In our study, the [P] in stem was much higher than that in the P-sensitive species *B. ericifolia* and *H. prostata* (4 and 1.5-fold higher, respectively), suggesting that *E. coccineum* has a greater P-storage capacity in its stem (Table 2). In *G. crithmifolia*, a P-tolerant species, stem [P] was not measured. However, the authors reported that 'excess' P was allocated to roots. The [P] in roots of *E. coccineum* was 3.5-fold lower than that in *G. crithmifolia*, but as other authors have suggested, we propose that both roots and stems may have a function in buffering leaf [P], thus avoiding P toxicity. Phosphorus concentrations in *E. coccineum* show significant seasonal variation, being higher in the stem during winter, corresponding to around 70% of total P content of the plants (Zúñiga-Feest et al., 2009); the P content in the stem then declines when shoot growth increases in spring. This shows that *E. coccineum* can function at a range of P availabilities.

Influence of P supply on rates of P uptake

At the beginning of the experiment, upon a transfer to a high P supply, *E. coccineum* showed P-toxicity symptoms (See appendix S1). These initial P-toxicity effects would appear to be a typical response when plants are transferred from low to high P supply, as found by Cogliatti & Clarkson (1983) in *Solanum tuberosum* L. We hypothesise that initially *E. coccineum* did not quickly down-regulate its P-uptake capacity; however, once the seedlings had acclimated to the high P supply, they did show down-regulation of their net P-uptake capacity, allowing a seedling survival rate of 100%. The new leaves formed during the experiment did not show symptoms of P toxicity, (See Figure S2) and no significant differences in leaf [P] were found at high P levels in the nutrient solution (Table 2). This can be explained by the P-uptake rates of roots of *E. coccineum*, measured at 5 μM P, decreasing from 0.34 to 0.03 nmol P g^{-1} root FW s^{-1} in seedlings grown at 1 and 250 μM P, respectively.

The values found for P-uptake rates in *E. coccineum* were much higher than those of other Proteaceae species (Table 4), suggesting a greater ability to adapt to soil with high total P. We surmise that this was due to a small extent to the higher growth rate of *E. coccineum* and largely

to the presence of relatively more young non-woody roots (Fig. 3). Therefore, a greater proportion of the entire root system was taking up P from the nutrient solution.

Table 4. Comparison of characteristics found for *Embothrium coccineum* in this study with published data on Proteaceae species endemic to other regions in the world.

Species	Range of P treatments (μM)	Range of net P-uptake rate (nmol P g^{-1} FW s^{-1})	Mature leaf [P] (mg g^{-1} DW)	Reference
E. coccineum J.R. Forst. & G. Forst.[1]	1 - 250	0.34 - 0.03	0.49 –3.16	This study
Hakea prostrata R.Br.[2]	0 - 10	0.06 - 0.02	0.02 – 13.6	Shane *et al.* 2004c
Grevillea crithmifolia R.Br.[2]	0 - 200	0.09 – 0.04	0.15 – 2.50	Shane and Lambers (2006)
Protea compacta R.Br.[3]	0.01 - 1	0.03 - 0.02	0.1 – 0.7	Shane *et al.* (2008)
Protea obtusifolia Bueck ex Meissner[3]	0.01 - 1	0.05 - 0.03	0.2 – 1.0	Shane *et al.* (2008)
Leucadendron meridianum I.J. Williams[3]	0.01 - 1	0.08 - 0.04	0.1 – 1.2	Shane *et al.* (2008)
Banksia attenuata R.Br.[2]	0 - 10	0.04 - 0.02	2 - 16	de Campos *et al.* (2013)
B. menziesii R.Br.[2]	0 - 10	0.03 - 0.02	1 - 20	de Campos *et al.* (2013)

[1]South America, [2]south-western Australia, [3]South Africa

Evidence for a trade-off between investment of carbon in cluster-root biomass and in carboxylate exudation

Cluster-root formation was progressively inhibited after 5 weeks at high [P] in the nutrient solution, and completely inhibited above 25 μM P, in the same way as has been shown in studies on other Proteaceae species grown in nutrient solution (Shane et al., 2003; Shane et al., 2004b; Shane et al., 2004c). At 1 μM P, *E. coccineum* produced, on average, nine cluster roots per plant, and the biomass allocation to the cluster roots was quite low (less than 5% of total biomass). These results agree with other reports on *E. coccineum* (Gonzalez, 1990; Delgado et al., 2013; Donoso-Ñanculao et al., 2013; Piper et al., 2013). However, *Hakea prostrata* (Shane et al., 2004c) and *Grevillea crithmifolia* (Shane & Lambers, 2006) grown at low P supply in nutrient solution allocate approximately 25% of their total biomass to cluster roots. In a comparison of six Banksia species grown in soil, investment in cluster-root biomass was, on average, 60% of the

total biomass (range 32-75%) (Denton, Veneklaas & Lambers, 2007). Therefore, *E. coccineum* allocated much less resources to cluster-root formation than south-western Australian Proteaceae species do.

At low P supply, the carboxylate-exudation rates were faster than those at a high P supply (Fig. 4), as has been reported for other species bearing cluster roots when growing under low-P conditions (Neumann et al. 1999; Neumann & Römheld, 1999; Shane et al. 2004a; Peñaloza et al. 2005; Kihara et al., 2003). When the root system was excised and separated into cluster and non-cluster roots, the exudation of citrate from mature cluster roots was significantly greater than that of young cluster roots, while in exudates of non-cluster roots this organic anion was not detected (Table 3). Interestingly, our results for exudation of *E. coccineum* were nine times faster than those of seven Australian Proteaceae species, whose intact cluster roots showed rates of approximately 1.6 nmol g^{-1} FW s^{-1} (Roelofs et al., 2001) and 22 times higher than the rate during the exudative burst of *H. prostrata* (Shane et al., 2004a). In addition, the carboxylate exudation from mature cluster roots of *E. coccineum* (13-23 days after rootlet initiation; Delgado et al. 2013) lasted for a longer period than that in *H. prostrata*, where the peak of exudation rate (citrate + malate) was observed between 12 and 13 days after emergence (Shane et al. 2004a). We conclude that the South American *E. coccineum* invests far less biomass in cluster roots, but that these clusters release carboxylates over a longer period and at a much faster rate when compared with south-western Australian Proteaceae species (Fig. 6a).

A similar contrast between investment in and functioning of cluster roots as discussed above for *E. coccineum* and *H. prostrata* has been found for *Lupinus cosentinii* Guss. when compared with *L. albus* L. grown in sand with a sparingly soluble P form (Pearse et al., 2007). *Lupinus cosentinii* produced a small amount of cluster-root biomass (5% of total root biomass) compared with *L. albus* (47% of total root biomass). However, *L. cosentinii* produced significantly more carboxylates (253 μmol g^{-1} root DW) compared with *L. albus* (140 μmol g^{-1} root DW). In the case of *E. coccineum*, the larger amount of carboxylates exuded from relatively few clusters is associated with the form of P in the soils in which this species grows naturally. Despite large amounts of total P, this P is scarcely available, due to the low pH and the high aluminium and iron concentrations of these soils (Steubing et al. 1983; Lambers et al. 2012a). Souto, Premoli & Reich (2009) reported that 'plant-available' P in the habitats of 26 populations of *E. coccineum*in

Patagonia ranges from 0.09 to 44 mg P kg^{-1} soil, with 26% of soils sampled showing values below 1 mg P kg^{-1}. Therefore, the rapid carboxylate-exudation rate from cluster roots of *E. coccineum* might be more effective for this species that colonises young deposits of volcanoes, agricultural or forest margins, clearcuts or landslides (Escobar et al., 2006). It should be emphasised that the proposed model pertains to the population of *E. coccineum* used in our study and relates to the soil in the natural habitat of this population. Given the wide distribution of *E. coccineum* (Souto et al., 2009), it would be interesting to explore both ecotypic and phenotypic variation, as dependent on soil type in the natural habitats of this species. The main point we make in our model is that carboxylate exudation from cluster roots is not only important for plants naturally occurring on P-impoverished soils, but also when soils contain large amounts of P, of which most is poorly available for plants lacking specialised P-mining roots (Lambers et al., 2012a). The question that remains to be answered is: is there is a trade-off between carbon investment in a large amount of biomass in cluster roots that release a relatively small amount of carboxylates and a lower investment in cluster-root biomass with clusters releasing far more carboxylates as dependent on soil mineralogy? The essence of this trade-off is summarised in Fig. 6a, comparing the present results on *E. coccineum* with published information on the south-western Australian *H. prostrata*.

Our results related to citrate sorbed onto soil and P desorbed into soil solution for a south-western Australian P-impoverished soil showed that even though the equilibrium soil solution citric acid concentration was increased to very high levels, only a very small amount of citrate was adsorbed onto the sandy soil (Fig 6b). A similar trend was reported by Hue (1991) in the adsorption of malate onto oxisol, ultisol and vertisol. However, the amount adsorbed on to andisol was very high (Fig. 6c). The small amount of citrate adsorbed in sandy soils implies that a small amount of soil P is desorbed into the soil solution. In rhizosphere soil of *L. albus* clusters, the citric acid concentration in solution is 1.1 mmol kg^{-1} soil; at 20% water content, this amounts to approximately 5.5 mM (Dinkelaker, Römheld & Marschner, 1989). This is in excess of citrate required to desorb most P in a sandy soil (Fig. 6b). However, in a strongly P-sorbing andisol (Hue, 1991; Jara et al., 2006), such a high carboxylate concentration would make substantially more P available.

Phosphorus nutrition and species distribution as dependent on soil P

Phosphorus toxicity has been observed in many Australian and South African Proteaceae species (Nichols & Beardsell, 1981; Hawkins et al., 2008; Lambers et al., 2002; Shane et al., 2004b; de Campos et al., 2013). However, not all Australian and South African Proteaceae species are sensitive to elevated P supply. Shane and Lambers (2006) reported that *Grevillea crithmifolia* (a south-western Australian Proteaceae species) does not present P-toxicity symptoms when grown at 200 μM P. These authors suggested that the insensitivity to developing P-toxicity symptoms is related to *G. crithmifolia* naturally occurring in places with somewhat greater P availability. We also associate the P insensitivity of *E. coccineum* with a P-rich soil type in which this species grows naturally compared with that of P-sensitive Australian and South African Proteaceae species (see Fig. 8 in Lambers et al. (2012a)).

Southern South American species growing in their natural habitat have, on average, very high leaf [P] compared with plants in other regions in the world (Lambers et al., 2011). This contrast is particularly strong when comparing Proteaceae species from southern South America and south-western Australia (Lambers et al., 2012a). Several authors have reported that species naturally occurring in nutrient-poor soils tend to produce large seeds with high nutrient content, providing nutrients to seedlings and thus ensuring their survival and establishment (Milberg, Pérez-Fernández & Lamont, 1998; Kidson & Westoby, 2000; Vaughton & Ramsey, 2001; Groom & Lamont, 2010). Our results on dry mass and [P] in seeds of *E. coccineum* and five other Chilean Proteaceae species (Table 1) showed much lower seed mass and seed [P] than those found for Proteaceae species growing in nutrient-poor soils in south-western Australian and the Cape Floristic Region of South Africa. The values for the south-western Australian species are 86 ± 23 mg DW and 13.2 ± 0.8 mg P g^{-1} DW, and those for south-western African species are 53 ± 11 mg DW and 5.8 ± 0.8 mg P g^{-1} DW, respectively (Groom & Lamont, 2010), compared with 11.5 ± 1.4 mg DW (excluding seed mass of *G. avellana* which was 354 ± 11 mg DW) and 3.2 ± 0.4 mg P g^{-1} DW, on average, for southern South American Proteaceae species. This suggests that the investigated South American Proteaceae species have not evolved to accumulate large P reserves in their seeds, unlike south-western Australian and South African Proteaceae species, which evolved in old, climatically-buffered, infertile landscapes (Hopper, 2009). Henery & Westoby (2001) reported that plants with smaller seeds have a greater dispersal and colonisation potential.

This could also be relevant for *E. coccineum*, which has a wide geographical distribution, from 35º S to 56º S in Chile and Argentina (Escobar et al., 2006). The soils in which this colonising species grows are characterised by high total soil P compared with those in Australian soils (Lambers et al., 2008; Lambers et al., 2010; Lambers et al., 2012a), but containing low levels of 'plant-available' P, due to the strong sorption to oxides and hydroxides of Fe and Al (Borie & Rubio, 2003).

Delgado et al. (2013) found rapid exudation of carboxylates and acid phosphatase by cluster roots compared with non-cluster roots of *E. coccineum*, thus showing a similar functioning as other Proteaceae species naturally occurring in ancient and highly-weathered soils in Australia and South Africa (Shane & Lambers, 2005). Lambers et al. (2012a) proposed that the role for species with cluster roots that grow on young P-rich soil with low P availability in southern South America is to access strongly sorbed P, and then act as ecosystem engineers, providing P in leaf litter for neighbouring plants without cluster roots. That capacity of species bearing cluster roots to make the relatively unavailable P available to other plants could be an ecologically important trait in young volcanic soils (Lambers, Clements & Nelson, 2013b). This key trait shown by E. coccineum must be considered in future ecological restoration work, especially for seedling establishment through an increased P acquisition in soils with poor nutritional status, as suggested by Piper et al. (2013).

Concluding remarks

This study documents novel responses related to P nutrition in *E. coccineum* a southern South American Proteaceae species endemic to young relatively P-rich volcanic soils, with low P availability. First, we show down-regulation of net P-uptake capacity as well as P storage in woody stems occur simultaneously in plants grown at relatively high P supply. Second, we found relatively fast rates of carboxylate exudation and relatively little investment in cluster-root biomass, when compared with south-western Australian species. We surmise that this allows *E. coccineum* to effectively solubilise P from P-rich volcanic soils with high P-sorption capacity. Together, these traits explain why this Proteaceae species persist in soils with very high total P where P is relatively unavailable.

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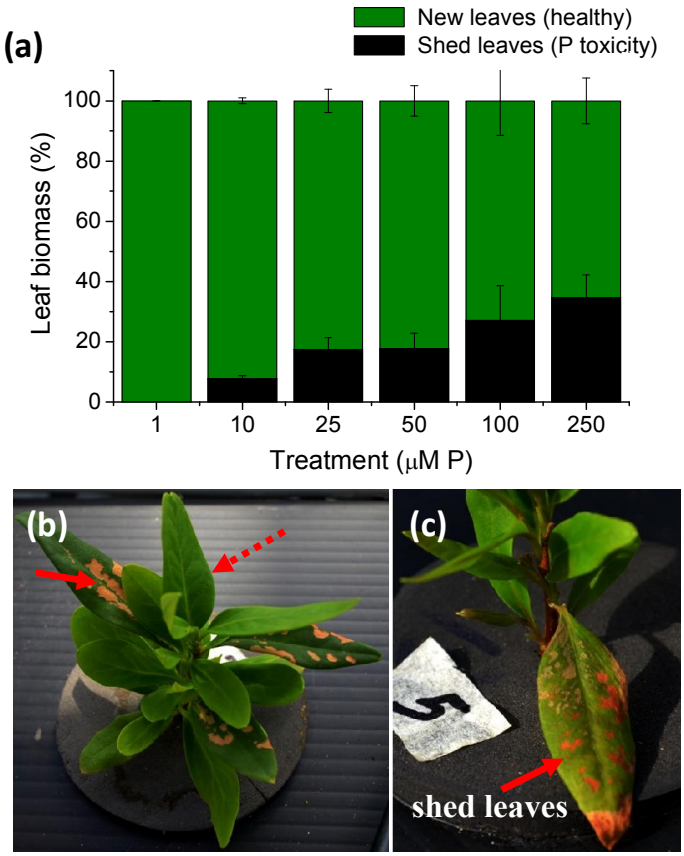

Figure S1. Influence of different phosphorus (P) supplies, ranging from 1 to 250 μM P in the nutrient solution, on the occurrence of foliar P-toxicity symptoms in *Embothrium coccineum*seedlings. (a) Percentage shed and new leaves of total leaf weight. (b) Leaf symptomsof P toxicity as indicated by dark and yellow spots (solid arrow) and new healthy leaves (dashed arrow) on a seedling supplied with 250 μM P. (c) Leaf showing symptoms of P toxicity just prior to shedding after three weeks following initiation of P treatments.

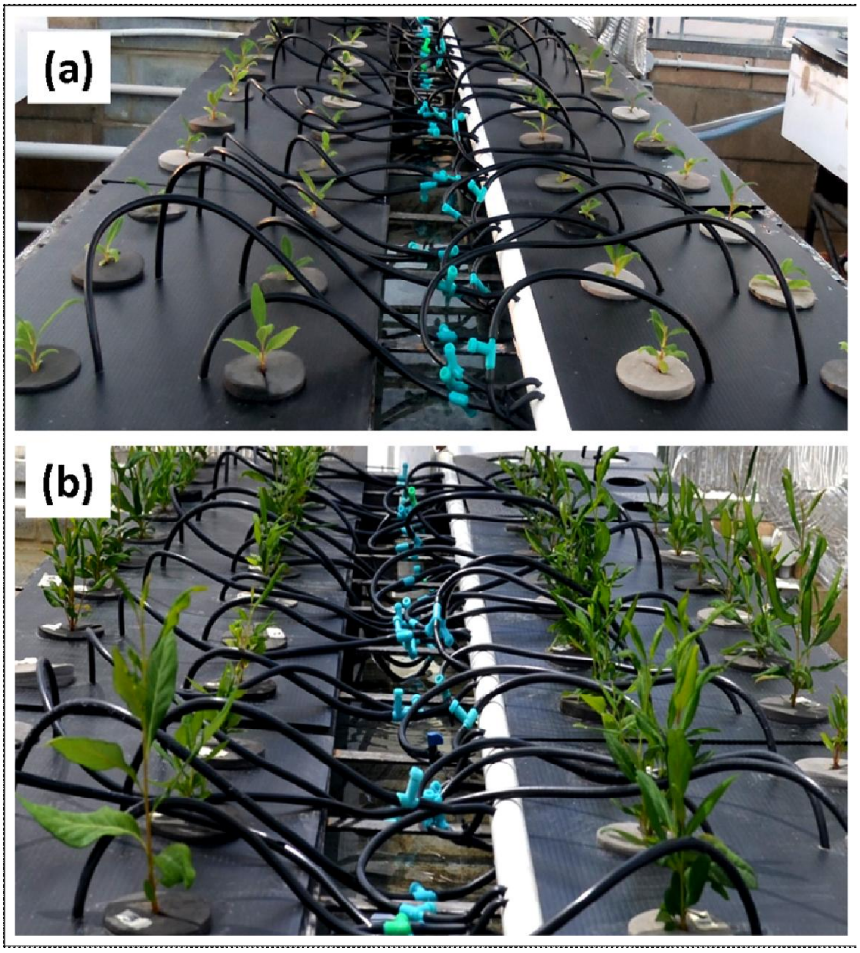

Figure S2. a) Seedling of *Embothrium coccineum* at the beginning and b) at the end of the experiment.

CHAPTER V

Cluster roots of Embothrium coccineum (Proteaceae) affect enzyme activities and phosphorus lability in the rhizosphere soil

Abstract

Cluster roots release organic compounds, enhancing plant's capacity to acquire scarcely available nutrients. Microorganisms are able to assimilate these organic compound and nutrients that are released by plants, generating competition for nutrient uptake. The development of mechanisms limiting microbial degradation of citrate exuded from cluster roots has been reported for *Lupinus albus*. However, it is not known if similar mechanisms are operating in other species. The aim of this study was to evaluate the effect of cluster roots of *Embothrium coccineum*,growing under natural conditions, on soil enzyme activities and phosphorus (P) fractions in its rhizosphere. For this purpose, we determined enzyme activities (of both plant and microbial origin): acid phosphatase (P-ase), dehydrogenase and β-glucosidase, the rate of hydrolysis of fluorescein diacetate (FDA), and P fractions in the cluster root rhizosphere at different developmental stages (juvenile, mature, semi-senescent, senescent), non-cluster root rhizosphere, and bulk soil. In addition, we measured total [P] and manganese [Mn] in roots. The results show that the rhizosphere of senescing cluster roots presented the highest P-ase, β-glucosidase and dehydrogenase activities, and fastest rate of FDA hydrolysis, being 2.6-, 4.6-, 3.3- and 25.8-fold greater, respectively, than those in the rhizosphere of mature cluster roots. The P fractionation showed that cluster roots modified P fractions in the rhizosphere, the inorganic P (Pi) fraction being significantly greater in the rhizosphere of mature cluster roots (62%) than in that of other stages (on average: 47%). Mature cluster roots showed the highest total [P], suggesting the fastest P uptake. We conclude that cluster roots of *E. coccineum* modified their rhizosphere depending on their development stage. Mature cluster roots decreased soil enzymatic activities, suggesting similar functioning to that reported for *L. albus*. In addition, mature cluster roots increased the Pi fraction in their rhizosphere, allowing the highest total root [P] at this developmental stage.

Keywords: enzymatic activities, phosphorus fractionation, volcanic soils, southern South America

Introduction

Cluster roots are one of the plant strategies to increase nutrient acquisition, involving release of large amounts of organic compounds (e.g., citrate, malate) that solubilise phosphorus (P) sorbed onto soil particles, making it available to be taken up by plants (Dinkelaker et al. 1989; Skene, 1998; Shane and Lambers, 2005).These roots occur in most species belonging to the Proteaceae, but similar root structures occur in species in other families (Lamont, 2003; Lambers et al. 2006), especially in actinorhizal species (Luis et a. 1991).

Cluster roots are dense clusters of fine rootlets around a main axis (Purnell, 1960); they are ephemeral structures, living about three weeks, showing rapid carboxylates efflux just when the rootlets have stopped growing (e.g., in *Lupinus albus*: around 3 to 4 days after their emergence, Watt and Evans, 1999; in *Hakea prostrata* R.Br., 12 to 13 days after emergence, Shane et al., 2004a; in *Embothrium coccineum* J. R. et. Forst, 13 to 25 days after emergence, Delgado et al 2014). In contrast, in juvenile or senescent cluster roots, the release of carboxylates is very low. Several authors have reported that these differences in carboxylates exudation rates as dependent on cluster-root developmental stage lead to strong changes in bacterial community structure in the rhizosphere of cluster roots (Weisskopf et al. 2008; Marschner et al., 2002; 2005). However, there is no information about how important these changes are for nutrient acquisition by plants.

It is widely recognised that many microorganisms are capable of stimulating plant growth through a variety of mechanisms that include improvement of plant nutrition, production of phytohormones, and suppression of pathogenic microorganisms (Martinez et al. 2010; Goh et al. 2013). Wenzel et al. (1994) have found phosphorus-solubilising bacteria associated with the cluster-root rhizosphere of waratah (*Telopea speciosissima* (Sm.) R. Br). However, we do not know if such bacteria (or other microorganisms stimultating plant growth) are occurring in the rhizosphere of exuding cluster roots, where the fastest P-uptake rate has been reported (Shane et. al 2004b)or if microorganism are inhibited because they are immobilising nutrients, making them less available to plants.

Organic compounds released into the rhizosphere are available as substrate for microorganisms, and rapidly assimilated into microbial biomass (Pinton et al., 2001; Ryan et al., 2001; Gregory 2006). Besides, microorganisms are able to assimilate nutrients that are released by plants, generating a strong competition for nutrient uptake. However, Weisskopf et al. (2006) proposed

that *Lupinus albus* has three mechanisms limiting microbial degradation of citric acid exuded from its cluster roots: i) strong acidification of the cluster-root rhizosphere, decreasing bacterial abundance, because most bacteria are sensitive to acidic environments; ii) exudation of phenolic compounds that induce fungal sporulation, thus reducing their potential citrate consumption by fungi; iii) exudation of chitinase and glucanase, which are enzymes that degrade fungal cell walls, just prior to citrate exudation. It is not known if similar mechanisms are operating in other species bearing cluster roots.Therefore, the first question we address is: are cluster roots from Proteaceae species functioning similarly to cluster roots of *L. albus,* decreasing microbial activity in active cluster roots?

Release of organic acids is associated with several changes in the rhizosphere, such as mobilisation of P, Mn and iron (Fe), decrease in pH, and metal detoxification through chelation (Jones, 1998). However, most studies involving these changes have been carried out under controlled conditions, which may be quite far from what happens in natural conditions. So far, it is unknown how cluster roots are affecting P lability in the rhizosphere from plants growing in their natural conditions (in this study, volcanic soils). Therefore, the second question we address is: are the exuding cluster roots mobilising P from non-labile fractions to more labile fractions in the soil?

To answer the questions raised above, we studied in field conditions the rhizosphere of cluster roots (at different development stages) of *E. coccineum,* a Proteaceae species from the southern part of South America. This species forms small cluster roots, but with a rapid carboxylate-exudation rate (Delgado et al 2014). Thus, the aim of this study was to assess the effect of cluster roots on chemical (P lability) and biochemical activities (soil enzymes of both, plant and microbial origin) associated with cluster roots of *E. coccineum.*

Materials and Methods

Study area and soil collection

Rhizosphere soil and cluster roots *of E. coccineum* were collected in February 2014 from Puerto Chalupa, Comuna Puyehue, Región de los Lagos, Chile. The area is mainly covered by scrub (*Rubus ulmifolius*) and second-growth forestswith some mature trees such as *Drymis winteri* (J.R. et. Forster), *Lomatia hirsuta* ((Lam.) Diels. Ex Macbr), *Eucryphia cordifolia* (Cav.), *Luma*

apiculata ((DC.) Burret) and *E. coccineum.* The soil is derived from recent volcanic ash (soil Series: Puerto Octay), whose chemical characteristics are shown in Table 1.

Table 1. Chemical analyses* of soil collected in the natural habitat of *Embothrium coccineum* at Puerto Chalupa, Antillanca, X Región de los Lagos, Chile

pH (H_2O)	5.32
P Olsen (mg kg^{-1})	4
Soil organic matter (%)	15
K (mg kg^{-1})	43
N (mg kg^{-1})	37
K ($cmol^{+/}kg$)	0.11
Na ($cmol^{+/}kg$)	0.04
Ca ($cmol^{+}/kg$)	0.57
Mg ($cmol^{+}/kg$)	0.16
Al ($cmol^{+}/kg$)	0.07
Aluminium saturation (%)	7.37
CICE ($cmol^{+}/kg$)	0.95
Base saturation ($cmol^{+}/kg$)	0.88

* determined according to Sadzawka et al. (2004).

Our study area was a zone where *E. coccineum* forms pure second-growth forests, whose ages ranges from 30 to 40 years-old. Samples of roots and soils were collected around the root system at the first 20 cm of soil from the surface. This collection was carried out at three locations (within an area of approx. 1 ha), separated by at least 200 m. These samples were collected and placed in boxes refrigerated with ice bags inside, being subsequently taken to the laboratory, sieved to 1 mm and stored at 4 °C until later analysis.

To assess the effect of the roots on chemical and biochemical activities, rhizosphere soil was collectedby shaking the roots gently. The rhizosphere soil from several cluster roots were pooled at each collection site, thus obtaining three replicates. Cluster roots were differenciated in four development stages: juvenile, mature, semi-senescent and senescent. To differentiate among different development stages, the size and color of the cluster roots was used as done Marschner

et al. (2002); Juvenile: white, small and elongated shape; mature: white and rounded shape; semi-senescent: mildly-dehydrated, white or light brown and rounded shaped: senescent: thoroughly-dehydrated and dark brown color (Figure 1). In addition, rhizosphere soil was collected from non-cluster roots and soil that had no contact with roots (bulk soil).

Soil pH was measured using a digital pH meter (Orion 3 star pH Benchtop, Thermo Fisher Scientific Inc, Waltham, MA) in soil suspended in distilled water (ratio 1:2.5; w/v H_2O).

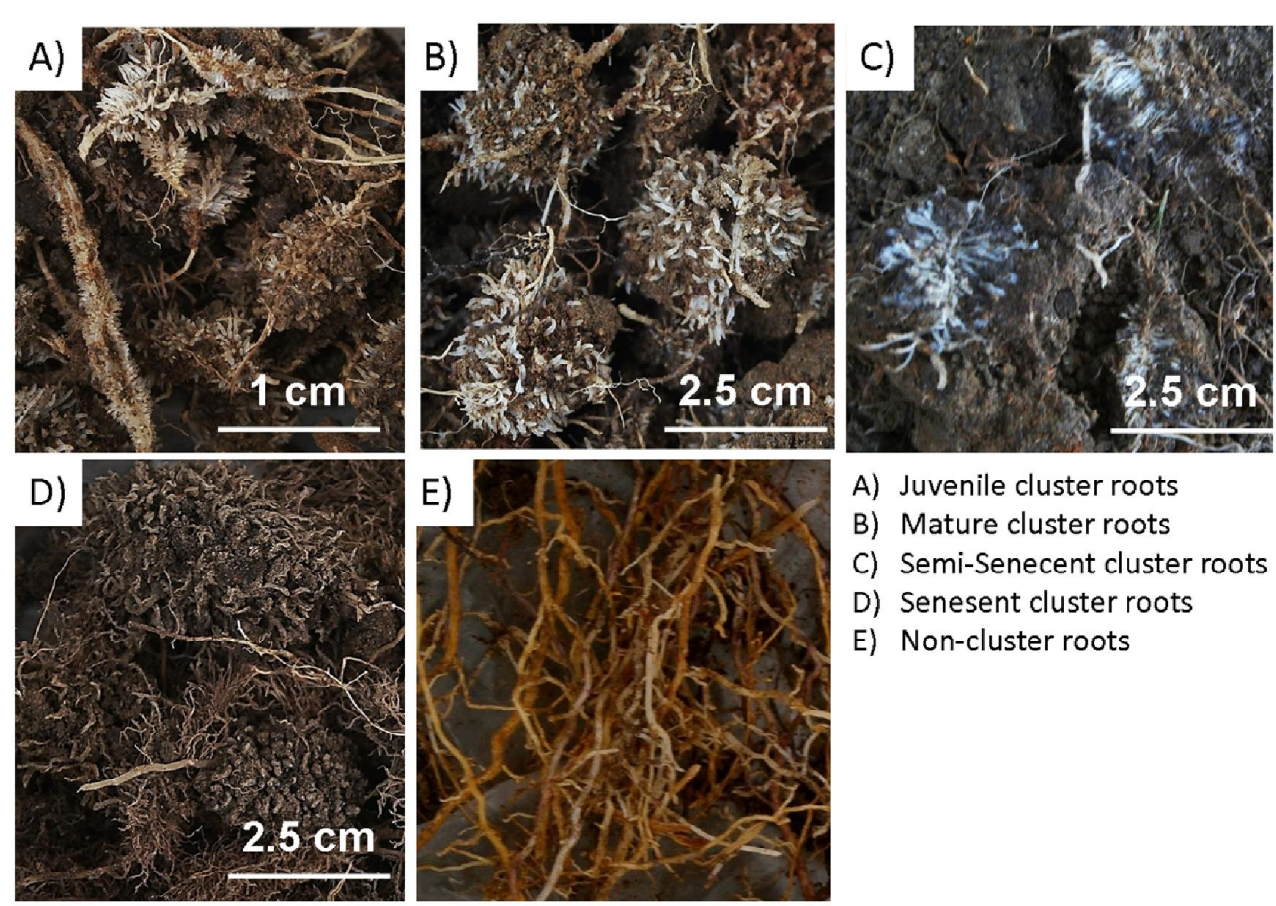

Fig 1. Soil attached to juvenile, mature, semi-senescent and senescent cluster roots (A, B, C, D) and non-cluster roots (E) of *Embothrium coccineum* growing in their natural habitat.

*Hydrolysis of fluorescein diacetate***:** this technique is widely used for estimating soil microbiological activities reflecting the activity of several enzymes (e.g., non-specific esterases, proteases and lipases) involved in organic matter degradation (Adam and Duncan, 2001). Thiswas determined using fluorescein diacetate (FDA) as described by Adam and Duncan

(2001).Briefly, 1 g rhizosphere soil was placed into 50 mL centrifuge tubes. Then, 10 mL potassium phosphate buffer (pH 7.6) was added. After 10 min, FDA was added; tubes were sealed and incubated at 30 ° C for 1 h in an orbital incubator at 100 rpm. After this, 10 mL acetone was added to each tube. Finally, absorbance was measured in a spectrophotometer at 490 nm.

Acid phosphatase: The method described by Alvear et al. (2005) was followed. Briefly, 1 g soil was mixed with modified universal buffer (MUB), pH=5.5 and 0.18 M p-nitrophenyl phosphate (pNPP). Subsequently, it was incubated at 20 ° C for 1 h. Then, 0.5 M $CaCl_2$ was added to the control and samples, which were stirred and filtered. The filtrate was mixed with NaOH (0.5 M) and filtered again. The released p-nitrophenyl (pNP) was determined spectrophotometrically at 400 nm.

β- glucosidase: this enzyme is involved in the degradation of cellulose, the main component of plant cell walls (Turner et al., 2002). This was determined according to Alef and Nannipieri (1995). Briefly, 1 g soil was incubated with 4 mL of MUB (pH 6.0) and 1 mL p-nitrophenyl-b-D-glucoside (25 mM) solution. After incubation at 37 ºC for 1 h, 1 mL $CaCl_2$ solution (0.5 M) and 4 mL NaOH (0.5 M) were added. The samples were centrifuged and the nitrophenol concentration was determined spectrophotometrically at 400 nm.

Dehydrogenase activity: this activity is considered to be a general index of biological activity on account of its role in the respiratory metabolism of microorganisms (Nannipieri et al. 2003). This was determined according to Alef and Nannipieri (1995). Briefly, 1 g soil was incubated with 1 mL of 100 mM Tris/HCl buffer (pH 7.6) containing triphenyl tetrazolium chloride (30 mM). After incubation at 30 ºC for 24 h, 8 mL acetone was added. The samples were filtered and the triphenyl formazan concentration was determined spectrophotometrically at 546 nm

Phosphorus fractionation: This was carried out according to Hedley et al. (1982), with some modifications. Briefly, 1 g soil was placed into 50 mL centrifuge tubes. Then, 30 mL of $NaHCO_3$ 0.5 M (labile P pool) was added, and the sample was shaken for 16 hours, and then centrifuged (5000 *g* for 20 min). The supernatant was stored for later analysis. The soil residue was incubated

with 0.1 M NaOH (moderately-labile P pool, associated with Fe and Al minerals), as described above. Again, the solution was stored and the second soil residue was additionally extracted with 1 M HCl (non-labile P pool, associated with Ca minerals). The final solution was stored, and the soil residue of the final extraction was called residual fraction, dried at 30ºC and stored for further analysis. For each clarified extract of sequential extraction, inorganic P (Pi) and total P (Pt) were determined as described below. The organic P (Po) was obtained by difference between Pt and Pi.

Determinations of phosphorus concentrations in soil: Inorganic P (in each extract mentioned above) was determined by spectrophotometry using the ascorbic acid molybdenum blue method described by Drummond and Maher (1995). Total P was determined following Dick and Tabatabai (1977). Briefly, 2 mL samples were digested with sodium hypobromite (NaBrO) in a sand bath at 260-280 °C until a white dry residue was obtained. This residue was resuspended in distilled water, concentrated formic acid (26 M) and sulfuric acid (0.5 M). The solutions were neutralized with 2 M NaOH, and Pt was determined using the ascorbic acid molybdenum blue method.

Determination of P and manganese (Mn) concentration in root tissue: To determine P and Mn (used as indicators of the release of organic compounds), root tissue was carefully cleaned by hand, and then rinsed with abundant deionized water. After that, roots were dried (60°C for two days), ground, ashed at 550 °C and digested using a H_2O/HCl/HNO_3 mixture (8/1/1, v/v/v). Phosphorus was determined by spectrophotometry using the ascorbic acid molybdenum blue method described by Drummond and Maher (1995). The [Mn], was determined by flame atomic absorption spectroscopy (UNICAM 969 AA spectrometer).

Statistical analysis: To determine if there were significant differences among six soil fractions, a one-way ANOVA was applied. A Tukey test was used to identify those values with significant differences. All analyses were performed with Origin 8.0 software. Differences among the values were considered to be significant at a p-value ≤ 0.05.

Results

Soil biochemical activity

Roots of *E. coccineum* significantly affected soil enzymatic activities compared with those in bulk soil. However, not all roots affected these activities in the same way, e.g., the rhizosphere of senescent cluster roots showed the greatest activity of acid phosphatase and β-glucosidase compared with those in the rhizosphere at the other development stages of cluster roots (juvenile, mature, semi-senescent) and that of non-cluster roots. The rhizosphere of juvenile and mature cluster roots showed the lowest values of phosphatase and β-glucosidase (Table 2). Roots of *E. coccineum* significantly affected soil microbial activity. The rate of fluorescein hydrolysis showed the same trend as that of activities of phosphatase and β-glucosidase, being six-fold greater in the rhizosphere of senescent cluster roots compared with that in bulk soil. In addition, they were 2.4-, 3.2-, 3.2- and 2-fold higher than those of non-cluster roots, juvenile, mature and semi-senescent cluster roots, respectively (Table 2).

Table 2.Acid phosphatase, β-glucosidase, fluorescein (FDA) hydrolysis and dehydrogenase activity in the rhizosphere soil of cluster roots of *Embothrium coccineum*at different development stages (juvenile, mature, semi-senescent, senescent), non-cluster roots and bulk soil.

	Acid phosphatase	β-glucosidase	FDA	Dehydrogenase
	(μmol nitrophenol g^{-1} h^{-1})		(μg fluorescein g^{-1} h^{-1})	(μg triphenyl formazan g^{-1} h^{-1})
Root tip	493 (13) d	596 (19) b	34 (0.6) c	8.8 (0.3) c
Juvenile	653 (29) c	311 (13) c	26 (0.9) d	2.2 (0.2) e
Mature	557 (15) dc	380 (11) c	25 (0.5) d	0.5 (0.1) f
Semi-senescent	1030 (7) b	531 (13) b	42 (0.9) b	10.5 (0.1) b
Senescent	1464 (20) a	1747 (42) a	82 (0.1) a	12.9 (0.3) a
Bulk soil	248 (15) e	125 (5) d	14 (0.6) e	4.1 (0.2) d

Each value corresponds to a mean of three samples ± standard error in brackets. Letters indicate significant differences among three soil conditions ($P \leq 0.05$).

Phosphorus fractionation

The total soil P was, on average, 2,539 mg kg^{-1}. Organic P represented, on average, 50% of total P, being the highest in the bulk soil (61%) and in the rhizosphere soil of semi-senescent (54%) and senescent cluster roots (55%). In contrast, mature cluster roots presented the lowest value of organic P (38%), most P being inorganic (62%) (Table3).

Table 3. pH, inorganic (Pi) and organic (Po) phosphorus in rhizosphere soil of cluster roots at different development stages (juvenile, mature, semi-senescent, senescent), non-cluster roots and bulk soil of *Embothrium coccineum.*

	pH (H_2O)	Pi (mg kg^{-1})	Pi (%)	Po (mg kg^{-1})	Po (%)	P Total (mg kg^{-1})
Root tip	6.1 (0.2) a	1241 (124) ab	50	1249 (31) ab	50	2490
Juvenile	5.5 (0.4) bc	1355 (34) ab	53	1217 (174) ab	47	2571
Mature	5.2 (0.4)c	1543 (70) a	62	938 (59) b	37	2481
Semi-senescent	5.7 (0.3) b	1097 (47) b	46	1271 (132) ab	54	2368
Senescent	6.1 (0.5)a	1110 (80) b	45	1453 (136) a	55	2653
Bulk	5.9 (0.3) ab	1038 (44) b	39	1553 (46) a	61	2591

Each value corresponds to a mean of three samples ± standard error in brackets. Letters indicate significant differences among three soil conditions ($P \leq 0.05$).

The results regarding P fractionation showed that cluster roots modified the lability of different rhizosphere P fractions. In the rhizosphere of mature cluster roots,it was found that labile P in the inorganic fraction, was slightly higher (81 mg P kg^{-1}) than that in the other tested soil (on average; 59 mg P kg^{-1}). However, this difference was significantly greater (459 mg P kg^{-1}) when compared with the fractions of "moderately-labile" P of the rest of the soils that were investigated (on average 255 mg kg^{-1}). The non-labile P fraction represented only a small proportion of the total inorganic P, ranging from 7.9-13.7%, showing the lowest and highest values for juvenile and senescent cluster roots, respectively. The remaining P in the residual fraction (the last residual fraction after the final acid extraction of the Hedley fractionation) represented, on average, 62% of inorganic P and 31% of total P, respectively (Figure 2A).

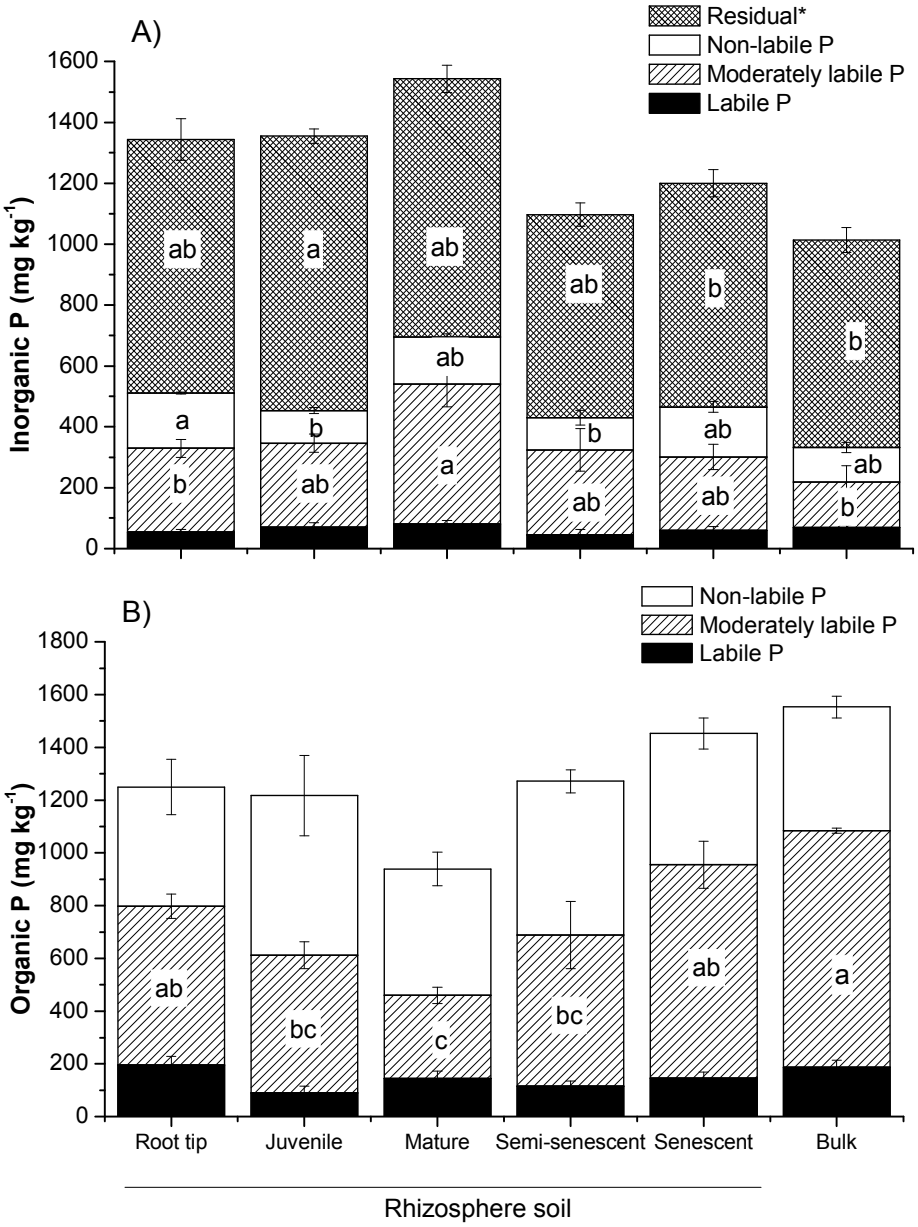

Fig. 2. Phosphorus (P) fractionation. (A) Inorganic P and (B) Organic P of rhizosphere soil of cluster roots of *Embothrium coccineum*at different development stages (juvenile, mature, semi-senescent, senescent), non-cluster roots and bulk soil. Each value corresponds to a mean of three samples ± standard error. * Not all residual fraction correspond to inorganic P, nevertheless, most of this (70%) corresponds to inorganic form (Velazquez et al. Unpublished data).

Regarding organic P, the labile fraction was 2.3-fold greater (on average 147 mg kg^{-1}) than the labile fraction of inorganic P (on average 63 mg kg^{-1}), representing 12 and 5% of total organic and inorganic P, respectively. In addition, values of the non-labile fraction of organic P were higher (514 mg kg^{-1}) than of those for the non-labile fraction of inorganic P (130 mg kg^{-1}).
No significant differences were found in the labile and non-labile P fractions among the different rhizosphere and bulk soils evaluated. However, for the moderately-labile P fraction, the mature cluster root rhizosphere showed the lowest values (315 mg kg^{-1}) compared with the other evaluated soils (on average; 680 mg kg^{-1}) (Figure 2B).

Total P and Mn concentrations in roots of Embothrium coccineum

The [P] in root tissue was significantly greater in mature cluster roots than that at the other development stages and in non-cluster roots (Fig. 3). The [Mn] in cluster roots of all development stage was invariably higher than that in tips of non-cluster roots.

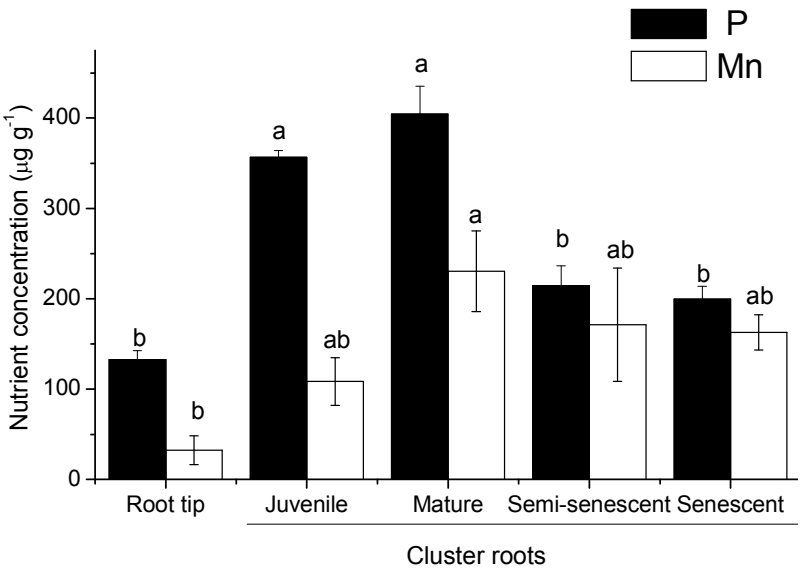

Fig. 3. Total phosphorus (P) and manganese (Mn) concentrations in roots of *Embothrium coccineum* growing in their natural habitat.Each value corresponds to a mean (± SE) (n=3). Letters indicate significant differences ($P \leq 0.05$).

Discussion

Biochemical changes in the rhizosphere of cluster roots of E. coccineum

Microorganisms may consume organic compounds (e.g., carboxylates) that are released by plants (Gregory 2006; Pinton et al. 2001; Ryan and Delhaize 2001) and may also release extracellular enzymes for the mineralisation of organic compounds, thus participating in nutrient cycling (Nannipieri et al., 2007). This is the case for acid phosphatases, which participates in P cycle hydrolysing organic P esters, releasing phosphate, the form of P available to plants (Bieleski 1973; Duff et al. 1994). In our study, we found a higher acid phosphatase activity in the cluster root rhizosphere compared with that of non-cluster roots and bulk soil, which agrees with findings of (Marschner et al. 2005). In addition, we found that the rhizosphere of senescent cluster roots showed the highest values of acid phosphatase activity. These results agree with a recent study by Tang et al. (2013), who found an increased expression of two genes encoding intracellular and extracellular phosphatases (*LaSAP1* and *LaSAP2*, respectively) in senescent cluster roots of *Lupinus albus*. These authors suggested that *LaSAP1* and *LaSAP2* are associated with a high internal P recycling and external P acquisition from mature cluster roots.

The high percentage of organic P found in rhizosphere of senescent cluster roots could be due to these roots not exuding organic acids (Delgado et al. 2014), therefore, although there is a higher phosphatase activity, solubilisation rates are much lower than mineralisation rates. Tarafdar and Junk (1987) found high phosphatase activity in the rhizosphere of *Triticum aestivum* which was associated with a high concentration of inorganic P, suggesting a higher mineralisation rate than plant P uptake. In this regard, we found that mature cluster roots had the highest P concentration compared with the other evaluated cluster root stages, suggesting that there is a very high P uptake at this development stage.

Biochemical activities are used as indicators of soil quality (Bastida et al. 2008). In our study, β-glucosidase activity was significantly higher in rhizosphere of senescent cluster roots, suggesting rapid decomposition of organic matter in this root zone. Hayano and Tubaki (1985) found that β -glucosidase is derived predominantly from soil microbial heterotrophs, in particular members of the mucorales (fungi), such as *Actinomucor* or *Mortierella*, species living as saprotrophs in soil.

In addition, we found higher values of FDA and dehydrogenase activity in the rhizosphere of senescent cluster roots, indicating that, due to the short life of these roots (approx.30 days, (Delgado et al. 2014; 2013), the microbial activity of this root tissue increases significantly by the decomposition. In contrast, in the rhizosphere of mature cluster roots, FDA and dehydrogenase activity were lower than those found in the other evaluated rhizosphere soils and bulk soil, hence showed a decrease in microbial activity at this development stage. These results are in line with those reported by Weisskopf et al. (2005), who showed a decreased bacterial abundance at the mature stage of cluster roots. In this stage the fastest citrate and proton exudation rate occurs (Neumann and Römheld 1999; Shane et al. 2004a; Watt and Evans 1999), with consequent P solubilisation. Weisskopf et al. (2006) suggest that it is a plant strategy to protect released organic anions against microbial degradation, since most bacteria are inhibited in acidic environments.

Chemical changes in the rhizosphere of cluster roots of E. coccineum

Previous results with *E. coccineum* showed a rapid carboxylate exudation rate (citrate, malate) by mature cluster roots (Delgado et al. 2014) and a strong acid exudation in cluster roots when they were placed on agar plates with bromocresol purple where acid exudation is indicated by a change in colour from purple to yellow (Zúñiga-Feest et al. 2010). Our results showed a decrease in the pH in the rhizosphere of mature cluster roots compared with that during other development stages and, even though we did not determine the rate of carboxylates exudation by cluster roots at different development stages in the present study, we measured [P] and [Mn] in these root tissues, which can be used as an indicator of the release of these organic compounds (Lambers et al. 2015). We suggest that cluster roots of *E. coccineum* are functioning in similar way that in *L. albus*, where the mature cluster roots decrease the pH and consequently the microbial activity, thus avoiding that carboxylates or desorbed P are consumed by microorganisms.

In relation to inorganic P in the rhizosphere of mature cluster roots, we found a significantly greater fraction of moderately labile and a slightly greater fraction of labile P, compared with that of the rest of rhizospheres evaluated. This increase in inorganic P fractions (labile and moderately labile) is important, because P is found mainly in organic forms in volcanic soil, mainly as penta- and hexaphosphates, which are linked to other humic compounds or mineral complexes through

metal bridges with Al and/or Fe (Borie et al. 1989). Therefore, this macromolecule is highly stabile in soil, and consequently unavailable to most plants.

Solubilisation and mineralisation are crucial processes prior to P absorption by plants, and we suggest that cluster roots are highly specialised structures promoting these processes. They do so, through the exudation of organic compounds and reducing microbial activity (avoiding the decomposition of solubilised P), modifying the different P fractions in soil and allowing P to be more available to plants.

Total P and Mn concentrations in roots of E. coccineum

Our results showed that mature cluster roots are the tissue where the greater [P] occurs. Likewise, [Mn] was higher compared with that in the tip of non-cluster roots. In this sense, Shane et al. (2004b), found that cluster roots of *H. prostrata* showed faster net P-uptake rates compared with non-cluster roots; this presumably coincides with more rapid uptake of micronutrients. Indeed, Dinkelaker et al. (1995) reported that in several species bearing cluster roots from Proteaceae and Fabaceae family, an increase in micronutrient availability was observed; this was associated with rhizosphere acidification and with strongly increased reductive capacity for Mn-oxides and Fe^{3+} through intense exudation of organic acids (e.g., malate).

Recycling and export nutrients in plants from senescent to new organs have been studied mainly in leaves, but there are fewer reports about what occurs in the roots. In terms of P remobilisation, *H. prostrata* removilise nearly 100% of [P] from senescent cluster roots to mature cluster roots (Shane et al. 2004a), while *E. coccineum* only removilise approx. 50%. Likewise, Lambers et al. (2012) it has been reported that leaves of Australian Proteaceae removilise more P than Chilean Proteaceae do. The authors propose that soils containing high total P but with strong P sorption, the efficient and proficient P resoption may not be strongly selected because exudates from cluster roots can mobilize much more P from soil than Australian counterparts, where soils are extremely P poor.

Volcanic soils in south of Chile are characterised by high organic matter content, where the P sink as humus-P complexes, compose the major portion of total-P (Borie and Zunino

1983), In this sense, Delgado et al. (2013) found that *E. coccineum* presented a greater phosphatase activity compared with that reported in other species bearing-cluster roots . We suggest that, contrary to what happens in extremely poor environments; in relatively nutrient-rich soils could be selecting species that can quickly recycle nutrients from the leaf litter, including their own senescent roots. In our study, we observed in the field that these roots grow forming groups of cluster roots, mixing all development stages, and we presume that in these roots may be feeding on themselves. These recent information raised open new opportunities to continue studying these issues in future research.

Concluding remarks

Embothrium coccineum biologically modified the rhizosphere of their cluster roots, depending on their development stage. Mature cluster roots presented the lowest activities of P-ase, β-glucosidase, FDA and dehydrogenase, whereas values were the highest for senescent cluster roots. In addition, cluster roots modified the lability of different rhizosphere P fractions, with the inorganic P fraction being significantly greater in the rhizosphere of mature cluster roots than in the rest of rhizosphere soil evaluated. At this developmental stage, we found the highest total [P] in the root tissue, suggesting that mature cluster roots show the fastest P-uptake rate, as found before in *H. prostrata* (Shane et. al 2004b).

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CHAPTER VI

General discussion, concluding remarks and future directions

General discussion, concluding remarks and future directions

Phosphorus deficiency has been reported as one of the main factors that induce cluster roots formation and its functioning (Keerthisinghe et al., 1998; Neumman et al., 2000; Lamont 2003; Shane & Lambers, 2005; Hue, 2009, Zúñiga-Feest et al. 2010; Zúñiga-Feest et al. 2014). Our results are in line with previous reports, because *E. coccineum* exhibited more pronounced cluster-root formation and activity under P deficiency.The information presentedin this thesis (Chapter III and published in *Plant and soil* 373: 765-773) corresponds to the first record in the exudation of carboxylates and acid phosphatase by cluster roots from a Chilean Proteaceae (*E. coccineum*), showing that both types of exudates are released in greater amounts by cluster roots than non-cluster roots. Additionally, we identified that malate and citrate are the most abundant carboxylatesin the exudates, similar to those reported previously in species of Proteaceae of South Western Australia, which naturally live in very old and highly leached soils. These results suggest that the release of organic compounds from roots are probably crucial strategies for P acquisition in species growing on volcanic soils, where, despite high levels of total P in the soil, the availability of this element to plants is low due to the high reactivity of P in soil colloids (Borie et al 1983;. Borie & Zunino 1983; Borie & Rubio 2003).

As mentioned in the previous chapters, we know that organic acids have been implicated in several changes associated with the rhizosphere, promoting P mobilisation from insoluble complexes (e.g., with Ca^{2+} and oxides/ hydroxides of Al^{3+} and Fe^{3+} or displacing phosphate from the soil matrix by ligand exchange) (Jones et al. 1998). However, most studies involving these changes have been carried out under controlled conditions, which may be quite far from what happens in natural conditions. So far, it was unknown how cluster roots are affecting P lability in the rhizosphere from plants growing in their natural conditions. In this sense, we found that cluster roots of *E. coccineum* growing in the field effectively modified the lability of different rhizosphere P fractions, depending on their development stage (juvenil, mature, semi-senescent or senescent). Inorganic P fraction was significantly greater in the rhizosphere of mature cluster roots than in the rhizosphere soil from other evaluated developmental stage. In mature cluster roots, we found the highest total [P] in the root, suggesting that these roots show the fastest P-uptake rate, as found before in *H. prostrata* (Shane et. al 2004). Besides, mature cluster roots decrease soil enzymatic activities (P-ase, β-glucosidase, dehydrogenase and FDA), suggesting

similar functioning to that reported for *L. albus*, where mechanisms limiting microbial degradation of citric acid, exuded from its cluster roots, have been found (Weisskopf et al. 2006).
In adittion to capacity to form cluster roots, Proteaceae species have been reported as highly efficient in P use at plant-level. For example, some of the advantages proposed for *Banksia* sp. (Australian Proteaceae) are: *i)* high photosynthesis rates at low [P] in leaves, *ii)* high remobilization of P from old to new leaves and *iii)* high capacity to concentrate P in seeds to ensure the survival of seedlings during establishment on poor soils (Denton et al., 2007). We compiled information from reports related to Proteaceae family and its cluster roots (see Chapter II), focusing on the Sourthen South American Proteaceae, and incorporating the discoveries made in this thesis. Summarising, we discuss the main differences between South Western Australian Proteaceae and Chilean Proteaceae. The former have been reported as species that thrive on phosphorus-impoverished soils, employing a phosphorus-mining strategy involving carboxylate-releasing cluster roots.

According to our findings and reports from the literature, we postulate that Proteaceae species growing in young soils containing high total P as southern South America soils in Chile (total P: > 1500 mg kg^{-1}; Borie & Rubio, 2003), are functioning differently compared to South West Australia Proteaceae, which grown in extremely P poor soils (total P: <80 mg kg^{-1}, Lambers et al, 2006), where these species are very abundant (> 600 species, Pate et al., 2001).
Some of divergent functioning found are:

i) In Chilean Proteaceae, leaf [P] are relatively higher than Australian Proteaceae, being noteworthy that Chilean species have very high leaf [P] compared with species elsewhere in the world (Lambers et al. 2011). However, photosynthesis rate of Chilean Proteaceae is much lower compared with that of Australian Proteaceae (*Banksia* sp.) (Alberdi et al. 2009, Denton et al. 2007), suggesting that the former are less efficient in P use in photosynthesis process. Lambers et al. (2012b) reported that adaptive physiology of leaves from some Australian Proteaceae to support a high photosynthetic P-use efficiency in P-impoverished soils could be partially explained because theses species replace phospholipids with galactolipids and sulfolipids during leaf development. However, it is not known whether similar mechanisms are occurring in Chilean Proteaceae.

ii) Groom & Lamont (2010) reported that Proteaceae species tend to produce seeds with high [P]; seed P reserves may be very important for seedling establishment, when P in soil is limiting growth. The [P] in the seeds of 6 species of Proteaceae living in Chile is on average 3.2 ± 0.4 mg P g^{-1} MS (see Chapter IV), value 4 and 2 times lower compared with the average value of 41 species Proteaceae of south western Australia (13.2 ± 0.8 mg P g^{-1} DM) and average value of 25 southern African Proteaceae (5.8 ± 0.8 mg P g^{-1} DM), respectively (Groom & Lamont, 2010). These data suggest that Proteaceae growing on young volcanic soils, such as those in southern Chile, have not evolved to accumulate large reserves of P in seeds as Proteaceae growing on ancient and extremely infertile soils.

iii) Many Proteaceae inhabiting in soils of south western Australia and southern Africa are sensitive to suffer symptoms of P toxicity, even at slight increases in the amounts of P in soil solution, due to some of these species have a low capacity to down-regulate its P-uptake systems (Shane et al, 2004b; Hawkins et al 2008; Campos et al 2013;). However, we found (see Chaper IV) that *E. coccinuem* grown with high [P] in solution can regulate its rate of P uptake by the roots, and shed its leaves when they exceed a [P] threshold of 12 mg g^{-1} dry weight, generating new leaves with lower levels of P, maintaining the vitality of the plant without symptoms of toxicity to P excess.

iv) We have reported that *E. coccineum* allocates relatively low cluster root biomass (<5% of the total biomass corresponds to cluster roots), compared with other Australian Proteaceae (*Grevillea crithmifolia* and *Hakea prostrata*: 25%; *Banksia* sp: 60%) (see Chaper IV). However, the carboxylates exudation rate of *E. coccineum* grown with low P supply, was 22 times greater than that reported for *Hakea prostrata*, grown under similar hydroponic conditions. We propose that exist a trade off between carbon investment in a large amount of biomass in cluster roots that release a relatively small amount of carboxylates and a lower investment in cluster-root biomass with clusters releasing far more carboxylates, which depend on soil mineralogy. However, this still requires further evaluation.

Lambers et al. (2012a) proposed that one of the possible roles of Proteaceae species growing on young volcanis soils (rich in total P, but low availability), could be to achive P that is strongly adsorbed on soil colloids and unavailable to other plants, maintain high levels of P in leaves (not remobilise) and thus, to eliminate foliage act as "ecosystem engineers", providing P in the leaf litter to neighboring plants that do not have these specialised roots. The ability of cluster roots to make P available that is unavailable to other plants could be a feature of great ecological importance in volcanic soils with high P retention (Lambers et al., 2013). However, there is little evidence about the ability of enrichment of leaf litter by the nutritional input of leaves from Proteaceae species, arising an opportunity to study these issues in future research.

As conclusios of this Doctoral Thesis, we highlight the novel response related to P nutrition in *E. coccineum*, a Proteaceae species from the southern part of South America, which grow in volcanic soils, characterised by being younger and with more total P than soils in South Western Australia (SWA) and South Africa (SA), where Proteaceae are abundant.

First, we found that *E. coccineum* sowed greater cluster-root formation realising large amounts of organic compunds (acid phosphatase and carboxylates) under P deficiency. The release of organic compounds by cluster roots occurs mainly when they have stopped growing (mature developmental stage), showing relatively fast rates of carboxylate exudation and relatively little investment in cluster-root biomass, when compared with south-western Australian species.

Second, we show that *E. coccineum* avoid P toxicity Pthrough down-regulation of net P-uptake capacity as well as P storage in woody stems occur simultaneously in plants grown at relatively high P supply.

Third, *E. coccineum* biologically modified the rhizosphere of their cluster roots, depending on their development stage. Mature cluster roots presented the lowest values of ezimatic activities (P-ase, β-glucosidase, dehydrogenase and FDA), showing similar trend decreasing bacterial abundance to *L. albus*, where mechanisms limiting microbial degradation of citric acid exuded from its cluster roots have been proposed (Weisskopf et al. 2006)

In addition, cluster roots modified the lability of different rhizosphere P fractions, with the inorganic P fraction being significantly greater in the rhizosphere of mature cluster roots than in the rest of rhizosphere soil evaluated. At this developmental stage, we found the highest total [P] in the root, suggesting that mature cluster roots show the fastest P-uptake rate, as found before in *H. prostrata* (Shane et al. 2004b).

We summarise that solubilisation and mineralisation are crucial processes prior to P absorption by plants, and we conclud that cluster roots are highly specialised structures promoting these processes. They do so, through the exudation of organic compounds and reducing microbial activity (avoiding carbon decomposition and consumption of solubilised P), modifying the different P fractions in soil and allowing P be more available to plants. Together, these traits explain why this Proteaceae species persist in soils with very high total P where P is relatively unavailable.

From the background reported in this Thesis, new questions arise: Do are all species from Proteaceae family, growing on young volcanic soils with high contents of P, "ecosystem engineers"? Are there different strategies to use or acquire P among Protecaeae species? Do are cluster roots with less biomass exuding more carboxylates rates than cluster roots with greater biomass? Is there a relationship between carbon invested in biomass cluster roots and carboxylate exudation rate, does it depend on the mineralogical characteristics of the soil? These questions raised open new opportunities to continue studying these issues in future research.

In addition, we know that cluster roots modify chemically and biochemically their rhizosphere, nevertheless, physical properties such as soil aggregation, aggregate stability, porosity, etc., still require to be studied, as these are key factors controling erosion in areas that are highly vulnerable to this process.

The knowledge of these aspects allows us understand the auto-ecology of Proteaceae species and the role playing in the environment where they grow, being able to propose these species to recover degraded soils (P deficient, eroded) through reforestation, or to be used in intercropping systems, in order to facilitate P acquisition of species that lack these root adaptations, eventually enhancing productivity of neighboring species and reducing the use/cost of fertiliser.

Finally, the knowledge given in this Doctoral Thesis can be extrapolated to other crops of species bearing cluster roots e.g. *Lupinus albus*, of great importance in regional agriculture in southernof Chile, where this species is grown by smallfarmers, mostly belonging to the Mapuche ethnic. The main uses are as nutriment forthe poultry, in the dairies and salmon industry. However, as mentioned above, the species bearing cluster roots have great potential to be used for recovering soil with high nutrient fixation (As volcanic soils) as they can, extract, mobilise and take up nutrients that other species can not, which gives an important scientific and social value to the country.

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