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REGULAR ARTICLE

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

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Abstract
Background and aim Cluster roots have a profound effect on their rhizosphere. Our aim was to determine the effect of cluster roots of *Embothrium coccineum*, growing under natural conditions, on soil enzyme activities and phosphorus (P) lability in its rhizosphere.
Methods We determined enzyme activities: acid phosphatase (P-ase), dehydrogenase and β-glucosidase, and the rate of hydrolysis of fluorescein diacetate (FDA), as well as P fractions in the cluster root rhizosphere at different cluster-root developmental stages (juvenile, mature, semi-senescent, senescent), in the non-cluster root rhizosphere, and in bulk soil. In addition, the concentrations of total P and manganese Mn in roots was measured.
Results 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 the inorganic P (Pi) fraction was 15 % greater in the rhizosphere of mature cluster roots than in that of other stages. Mature cluster roots showed the highest total [P], suggesting the fastest P uptake.
Conclusion Cluster roots of *E. coccineum* modified their rhizosphere depending on their developmental stage, presenting lower soil enzyme activities at the mature stage than at other development stages. 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

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Introduction

Cluster roots are a plant strategy to increase nutrient acquisition, involving release of large amounts of organic compounds (e.g., citrate, malate) that mobilise phosphorus (P) through solubilisation of P sorbed onto soil particles, making it available to be taken up by plants (Dinkelaker et al. 1995; Shane and Lambers 2005a; Skene 1998). These roots occur in most species belonging to the Proteaceae, but similar root structures

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occur in species in other families (Lambers et al. 2006; Lamont 2003), especially in actinorhizal species (Louis et al. 1991).

Cluster roots are dense clusters of fine rootlets around a main axis (Purnell 1960); they are ephemeral structures, living about 3 weeks, showing rapid carboxylate release 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. Forst & G. 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 slow. Several authors reported that these differences in carboxylate-exudation rates as dependent on cluster-root developmental stage lead to strong changes in bacterial community structure in the rhizosphere of cluster roots (Marschner et al. 2005; 2002; Weisskopf et al. 2005). On the other hand, 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 (Goh et al. 2013; Martínez-Viveros et al. 2010). Wenzel et al. (1994) have found P-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) occur in the rhizosphere of exuding cluster roots, thus enhancing nutrient availability, or if microorganism are inhibited and thus prevented from 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 (Gregory 2006; Pinton et al. 2001; Ryan and Delhaize 2001). Besides, microorganisms may assimilate nutrients that are mobilised by plants, generating strong competition for nutrient uptake. However, Weisskopf et al. (2006) proposed that *Lupinus albus* has three mechanisms curtailing 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 potential citrate consumption by fungi; iii) exudation of chitinase and glucanase (enzymes degrading fungal cell walls), expressed just prior to citrate exudation. It is not known if similar mechanisms operate in other species bearing cluster roots. Therefore, the first question we address is: do cluster roots of *E. coccineam* (Proteaceae) function 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 those in a natural environment. So far, it is unknown how cluster roots affect P lability in the rhizosphere in plants growing under natural conditions (in this study, volcanic soils). Therefore, the second question we address is: do exuding cluster roots mobilise P from non-labile fractions to more labile fractions in the soil?

To answer the questions raised above, we studied the rhizosphere of cluster roots (at different cluster-root development stages) of *E. coccineum,* a Proteaceae 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 of *E. coccineum* on chemical (P lability) and biochemical activities (soil enzymes of both plant and microbial origin) associated with cluster roots.

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 forests with 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 chemical characteristics and soil texture of the soil (derived from recent volcanic ash) were determined according to Sadzawka et al. (2004) and Schlatter et al. (2003), respectively (Table 1). The study area was a zone where *E. coccineum* forms pure second-growth forests. Samples of roots and soils were collected around the stem at

Table 1 Chemical analyses[a] and texture[b] of soil collected in the natural habitat of *Embothrium coccineum* at Puerto Chalupa, Antillanca, X Región de los Lagos, Chile

N ($mgkg^{-1}$)	37
Olsen-P ($mgkg^{-1}$)	4
Total P ($mgkg^{-1}$)	2575
K ($mgkg^{-1}$)	43
pH (H_2O)	5.32
Soil organic matter (%)	15
K ($cmol^{+}kg^{-1}$)	0.11
Na ($cmol^{+}kg^{-1}$)	0.04
Ca ($cmol^{+}kg^{-1}$)	0.57
Mg ($cmol^{+}kg^{-1}$)	0.16
Al ($cmol^{+}kg^{-1}$)	0.07
Aluminium saturation (%)	7.37
Base saturation (cmol+/kg)	0.88
Soil texture	Silt Loam (clay 15 %, sand 20 %, silt 65 %)

[a] Determined according to Sadzawka et al. (2004). [b] Determined according to Schlatter et al. (2003)

the top 20 cm of soil. This collection was carried out at three locations (within an area of approx. 0.5 ha), separated by at least 100 m. These samples were collected and placed in boxes 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 collected by shaking the roots gently. This was done for cluster roots at different development stages: juvenile, mature, semi-senescent and senescent. To differentiate among different development stages, we used the size and colour of the cluster roots as described below; 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 colour (Fig. 1). In addition, rhizosphere soil from non-cluster roots (specifically from root tips) and soil that had no contact with roots (bulk soil) were collected.

Soil pH was measured using a digital pH meter (Orion 3 star pH Benchtop, Thermo Fisher Scientific Inc, Waltham, MA, USA) in soil suspended in water (ratio 1:2.5; *w/v* H_2O). Other parameters evaluated (enzyme activities, P fractionation in the rhizosphere, P and manganese (Mn) concentration in root tissue) are listed below. Data on enzyme activities and soil pH were obtained from the rhizosphere soil of the three collection sites (n=3). However, due to the large amount of rhizosphere soil used in these determinations and little amount of rhizosphere soil obtained in one of three collecting sites (site 1), rhizosphere soil collected from "Site 1" was mixed with that collected at the other two sites (2 and 3). From this bulked soil, four replicates were obtained.

Hydrolysis of fluorescein diacetate

This technique is widely used for estimating soil microbiological activities that reflect the activity of several enzymes catalysing the hydrolysis of chemical bonds such as ester (e.g., non-specific esterases, lipases) and peptide bonds (e.g., proteases), all of which hydrolyse FDA and are involved in organic matter degradation (Adam and Duncan 2001; Nannipieri et al. 2003; Ntougias et al. 2005; Schnürer and Rosswall 1982). This was 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, fluorescein absorbance was measured in a spectrophotometer at 490 nm.

Acid phosphatase

We followed the method described by Rubio et al. (1990) adapted for volcanic soils. Briefly, 1 g soil was mixed with modified universal buffer (MUB), pH 5.5 and 0.015 M p-nitrophenyl phosphate (pNPP). Subsequently, samples were 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 5.5) and 1 mL p-nitrophenyl-beta-D-

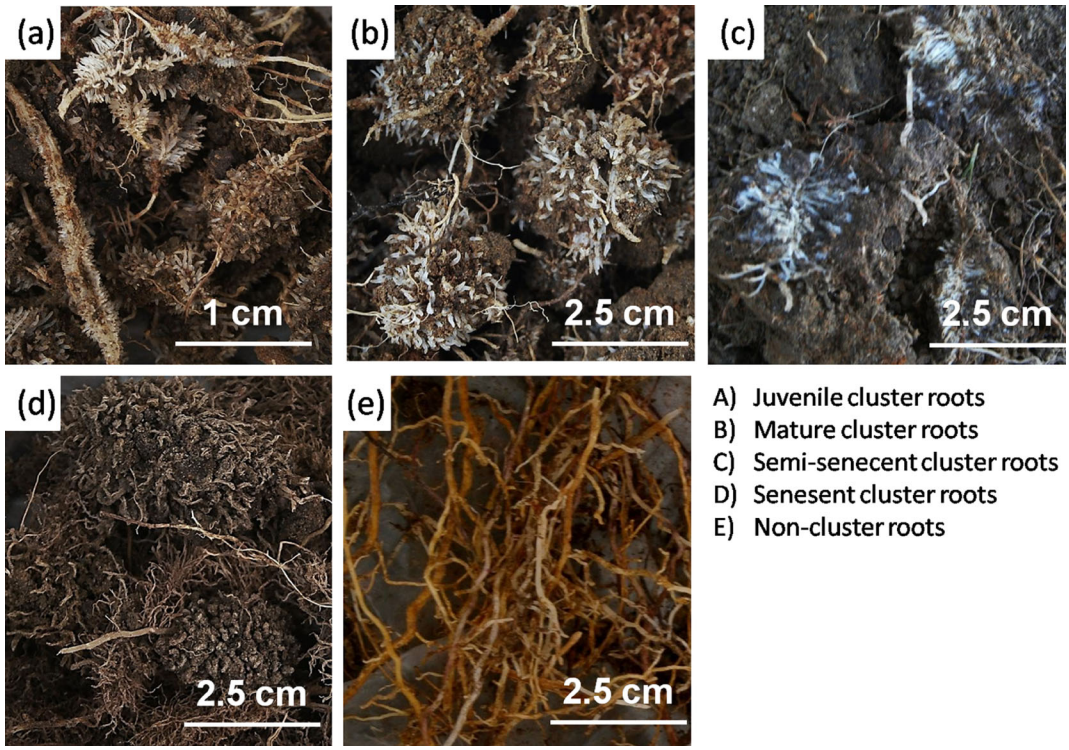

Fig. 1 Soil attached to juvenile (**a**), mature (**b**), semi-senescent (**c**) and senescent cluster roots (**d**) and non-cluster roots of *Embothrium coccineum* growing in their natural habitat

glucoside (25 mM) solution. After incubation at 37 °C for 1 h, 1 mL $CaCl_2$ solution (0.5 M) was added, stirred and filterd. The filtrate was mixed with 4 mL NaOH (0.5 M) and filtered again. 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 °C 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 0.5 M $NaHCO_3$ was added and shaken for 16 h, and then centrifuged at 5000*g* for 20 min (labile P pool). The supernatant was stored for later P analysis. The soil residue was shaken with 0.1 M NaOH (moderately-labile P pool, supposedly associated with Fe and Al minerals), as described above. Again, the supernatant was stored, and the second soil residue was further 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 P fraction, dried at 30 °C, 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 P 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 of each supernatant

was determined according to 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, formic acid (26 M) and sulfuric acid (0.5 M). The solutions were neutralised with 2 M NaOH, and Pt was determined using the ascorbic acid molybdenum blue method. Additionally, final residue was also digested and P determined as described.

Determination of P and Mn concentration in root tissue

To determine P and Mn concentrations, root tissue was carefully cleaned by hand, and then rinsed with abundant deionised water. After that, roots were dried (60 °C for 2 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, UK).

Statistical analysis

To determine if there were significant differences among five rhizosphere soils and bulk soil evaluated, 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 developmental 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 FDA 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).

Phosphorus fractionation

The total soil P concentration was, on average, 2,539 mg P kg^{-1} dry soil. 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 Po (38 %), most P being Pi (62 %) (Table 3).

The results regarding P fractionation showed that cluster roots modified the lability of different rhizosphere P fractions. In the rhizosphere of mature cluster roots, we found that labile P in the inorganic fraction was slightly greater (81 mg P kg^{-1} dry soil) than that in the other tested soil (on average 59 mg P kg^{-1} dry soil). However, this difference was significantly greater (459 mg P kg^{-1} dry soil) when compared with the fractions of "moderately-labile" P of the rest of the soils that were investigated (on average 255 mg P kg^{-1} dry soil). The non-labile P fraction represented only a small proportion of the total Pi, ranging from 8 to 14 %, 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 Pi and 31 % of Pt, respectively (Fig. 2a).

Regarding Po, the labile fraction was 2.3-fold greater (on average 147 mg P kg^{-1} dry soil) than the labile fraction of Pi (on average 63 mg P kg^{-1} dry soil), 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 P kg^{-1} dry soil) than of those for the non-labile fraction of Pi (130 mg P kg^{-1} dry soil).

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

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 (root tip) and bulk soil

	Acid phosphatase (μmolnitrophenol g^{-1} h^{-1})	β-glucosidase	FDA hydrolysis (μg fluorescein g^{-1} h^{-1})	Dehydrogenase (μgtriphenyl 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 ($P \leq 0.05$)

rhizosphere showed the lowest values (315 mg P kg^{-1} dry soil) compared with the other evaluated soils (on average; 680 mg P kg^{-1} dry soil) (Fig. 2b).

Total P and Mn concentrations in roots of *E. 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.

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 enzymes for the mineralisation of some organic compounds, thus participating in nutrient cycling (Nannipieri et al. 2003). This is the case for acid phosphatases, which hydrolyse P esters, releasing Pi, the form of P absorbed by 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 bulk soil which agrees with findings of Marschner et al. (2005) in three *Banksia* species.

We found that the rhizosphere of senescent cluster roots showed the highest 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.

Table 3 pH, total, inorganic (Pi) and organic (Po) phosphorus concentrations per unit dry rhizosphere soil of cluster roots at different developmental 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	38	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 (0.2) ab	1038 (44) b	39	1553 (46) a	61	2591

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

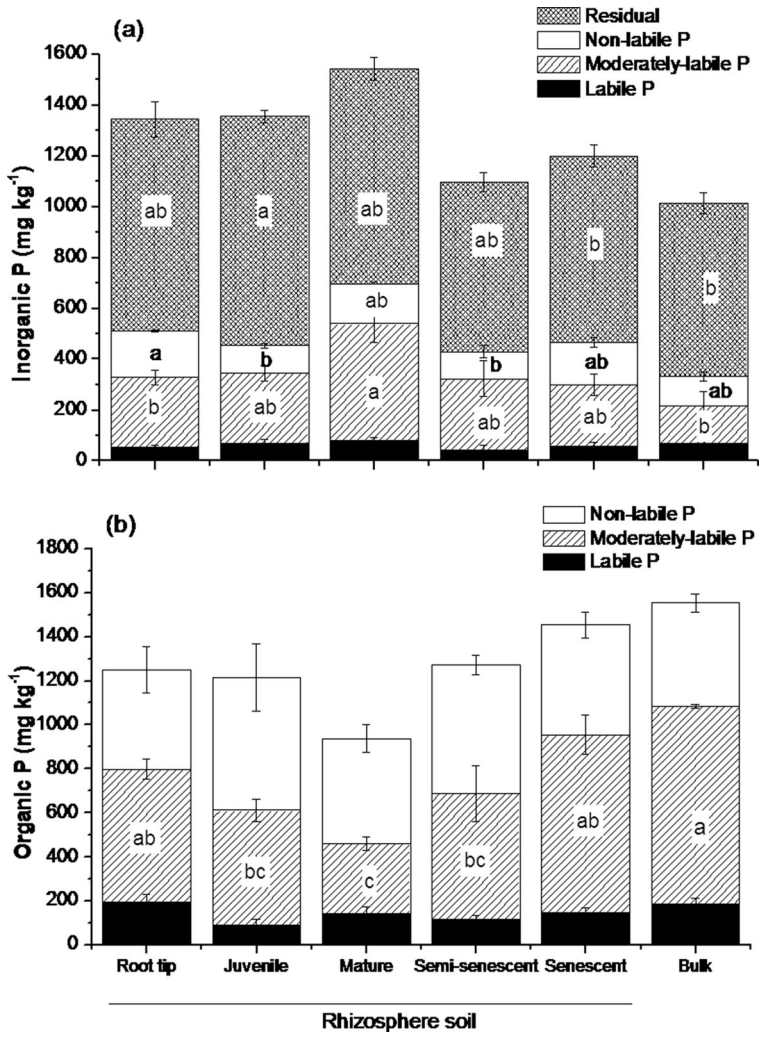

Fig. 2 Phosphorus (P) fractionation. **a** Inorganic P and **b** Organic P of rhizosphere soil of cluster roots of *Embothrium coccineum* at different developmental stages (juvenile, mature, semi-senescent, senescent), non-cluster roots (root tip) and bulk soil. Each value corresponds to a mean of four samples±standard error. Values not sharing the same the lowercase letter indicate significant differences among different soil evaluated ($P \leq 0.05$). * Not all residual fraction correspond to inorganic P, nevertheless, most of this (70 %) corresponds to inorganic form

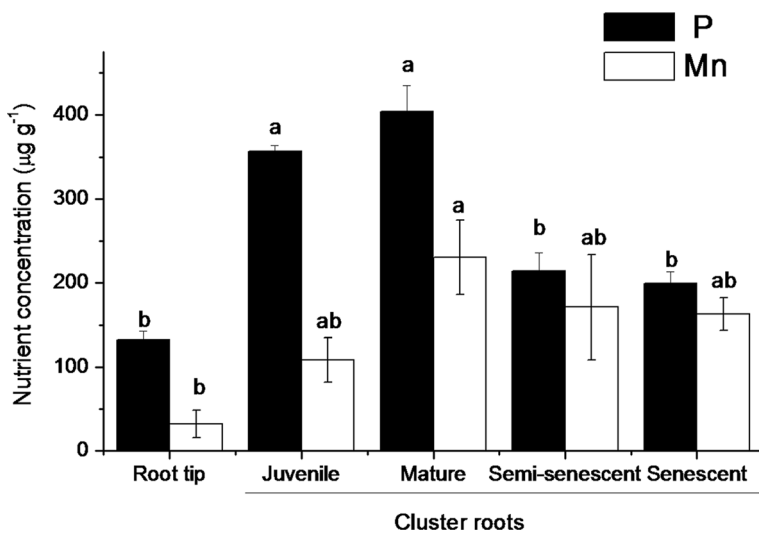

Fig. 3 Total phosphorus (P) and manganese (Mn) concentrations in roots of *Embothrium coccineum* growing in their natural habitat. Each value corresponds to a mean (± SE) (n=3). Values not sharing the same the lowercase letter indicate significant differences among different root tissue evaluated ($P \leq 0.05$)

The high percentage of organic P found in the 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 slower than mineralisation rates. Besides, an increased organic P concentration in this rhizosphere zone could be a consequence of direct release of organic substrate from decomposing roots as has been shown for roots of wheat, *Triticum aestivum*, where the release of ^{32}P from labelled roots in leachates ranged from 63 to 81 % depending of incubation temperature (10 to 55 °C) of seedlings roots (Martin and Cunningham 1973).

Tarafdar and Junk (1987) found high phosphatase activity in the rhizosphere of *T. aestivum* which was associated with a high concentration of Pi, suggesting a higher mineralisation rate than plant P uptake. In this regard, we found high Pi (labile and moderately labile fractions) in the rhizosphere of mature cluster roots, and, consequently, this root tissue had the highest [P] compared with the other evaluated cluster-root stages, suggesting that there is a very rapid 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 the 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 hydrolysis 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. 2013; 2014), the microbial activity of this root tissue increases significantly by the decomposition. In contrast, in the rhizosphere of mature cluster roots, dehydrogenase activities were significantly lower than those in the other evaluated rhizosphere soils and bulk soil. A similar trend was observed in the values of FDA hydrolysis in the rhizosphere of juvenile and mature cluster roots, which were significantly lower than those in the others rhizospheres evaluated, but higher than those in the bulk soil. These results indicate that in the rhizosphere of the more active roots (juvenile and mature) microbial activity is low, which is in line with results reported by Weisskopf et al. (2005), who showed a decreased bacterial abundance at the mature stage of cluster roots. At this stage, the fastest citrate and proton-exudation rate occur (Neumann and Römheld 1999; Shane et al. 2004a; Watt and Evans 1999), with consequent P solubilisation. Weisskopf et al. (2006) suggested that it is a complex strategy of *L. albus* to protect released organic anions against microbial degradation, since most bacteria are inhibited in acidic environments. The same authors found higher activity of chitinase and glucanase at the immature stage ("the stage preceding the rapid citrate excretion"); both enzymes are involved in degrading fungal cell walls, suggesting that this species also exhibits an efficient way to inhibit fungal growth, which may use citrate and malate as carbon sources. Unlike this finding related to chitinase activity, Wasaki et al. (2005) found that increased activity of this enzyme occurs in senescent roots which was attributed to an accumulation of this organic compounds (including citrate, acid and alkaline phosphatase) in the rhizosphere by continuous cluster-root exudation. In our study, we suggest that the higher enzyme activity found in the rhizosphere of senescent roots was mainly due to increased microbial activity as shown by the high values found in FDA hydrolysis and dehydrogenase activity, both indicators of microbial activity and well correlated with biomass and microbial respiration (Garcia et al. 1997; Schnürer and Rosswall 1982).

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). Results of this study showed a decrease in the pH in the rhizosphere of mature cluster roots compared with that during other developmental stages and, even though we did not determine the rate of carboxylate 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 indirect measurement of the release of these organic compounds, because these mobilise P (through ligand exchange and/or complexation of metal ions binding P) and micronutrients such as Mn (through high chelating and reductive capacity of some

organic acids). Thus, an increase in nutrient availability in the rhizosphere increases the possibility that these nutrients can be taken up by roots (mainly by more active roots); therefore, nutrient concentration in root tissue might be used as a proxy for cluster-root functioning (Lambers et al. 2015; Shane and Lambers 2005b). We suggest that cluster roots of *E. coccineum* function in a similar way to those of *L. albus*, where the mature cluster roots decrease the rhizosphere pH, and consequently the microbial activity, thus avoiding that carboxylates or desorbed P are consumed by microorganisms, leaving this nutrient more available to root plants.

In relation to Pi 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 Pi fractions (labile and moderately labile) is important, because in volcanic soils P is strongly sorbed to mineral constituents of variable charge (Al-oxides, Fe-oxides, allophone, imogolite) and a large amount of total P (~50 %) is found in organic forms, mainly as penta- and hexaphosphates, which are linked to humic compounds or mineral complexes through metal bridges with Al and/or Fe (Borie and Rubio 2003; Borie et al. 1989). Therefore, this macromolecule is highly stabile in soil, and consequently unavailable to most plants.

According to the results obtained in this study, the total P concentration in soil was quite high, but only a small amount is available (Olsen-P) for plants (Table 1). Vistoso et al. (2009) reported the kinetics of sorption of phosphate in four Chilean andisols (very close to our study site), and found that 61 % of phosphate was sorbed in the first half hour, and then the sorption was slowly increased to 97 % after 72 h. The high P retention is typical of recent volcanic ash, which usually dominates the silty loam texture (Table 1), whose limitations are low conductivity of water in unsaturated state, fast drying surface when exposed to direct radiation, high acidity and high levels of active aluminium (Schlatter et al. 2003). Due to these limitations in these soils, we suggest that cluster roots of *E. coccineum* are highly specialised structures promoting solubilisation and mineralisation processes. They do so through the exudation of large amount of organic compounds (previous studies by Delgado et al. (2014)), modifying lability of 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 mature cluster roots having the greatest [P]. Besides, [Mn] was higher compared with that in the tips of non-cluster roots. Likewise, Shane et al. (2004d), 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 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 carboxylates (e.g., malate).

Recycling and export of nutrients in plants from senescent to new organs have been studied mainly in leaves, but there are reports about on roots. In terms of P remobilisation, *H. prostrata* remobilises nearly 100 % of all P from senescing cluster roots (Shane et al. 2004a), while *E. coccineum* only remobilises approx. 50 %. Likewise, Lambers et al. (2012) reported that leaves of some Australian Proteaceae remobilise more P than Chilean Proteaceae do. The authors propose that for plants that naturally occur on soils that contain high total P, but with strong P sorption, efficient and proficient P resorption is not strongly selected for, because exudates from cluster roots can mobilise large amounts of P compared with species from south-western Australia, where soils are extremely P impoverished. In addition to this, we propose that P is not accumulated in the roots, but rapidly mobilised within the plant and utilised in new plant tissue. Overall, values of [P] in roots of *E. coccineum* were lower than those found in other species bearing similar root structure growing in hydroponics (Keerthisinghe et al. 1998; Shane et al. 2004b; Shane and Lambers 2006; Shane et al. 2004c), but similar values of [P] have been found in other roots of plants growing in soil (Menge et al. 1978; Ratnayake et al. 1978; Richardson et al. 2001).

Volcanic soils in the south of Chile are characterised by high organic matter content, where P as humus-P complexes comprises a major portion of Pt (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 nutrient-impoverished environments, on relatively nutrient-rich soils selection is for

quick recycling of nutrients from leaf litter and from senesced roots. In this study, we observed in the field that roots of *E. coccineum* produce cluster roots, showing all development stages at the same time, and we presume that mature cluster roots could be acquiring nutrients from mineralised cluster roots after senescence, as has been proposed for wheat roots, where it was shown that decomposed roots labelled with ^{32}P can release P directly into the soil solution, mainly as orthophosphate, representing a source of P for new plant growth (Martin and Cunningham 1973). This information raises new opportunities for future research.

Concluding remarks

In this study, we highlight three main contributions: first, we report that cluster roots of *E. coccineum* modified their rhizosphere depending on their developmental stage, showing lower soil enzymatic activities in exuding cluster roots (mature) than at other development stages. At the mature stage, we found the highest total root P concentration. Second, we found a slightly increased labile Pi and a significant increase of moderately-labile Pi fraction in the rhizosphere of mature cluster roots, showing important biological and biochemical changes in this rhizosphere zone. Third, we found a low P remobilisation from senescent cluster roots compared with that of *H. prostrata,* indicating that *E. coccineum* could act as an ecosystem engineer, not only through the decomposition and mineralisation of leaf litter, as proposed Lambers et al. (2012), but also at the rhizosphere level.

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