REGULAR ARTICLE

Cluster root formation and function vary in two species with contrasting geographic ranges

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Abstract

Aims Southern South American Proteaceae can occupy soils that are rich in total phosphorus (P) but poor in available P (for example volcanic soils) thanks to their cluster roots (CR), which mine soil P. However, some southern South American Proteaceae occur in a wide range of soil nutrition. We hypothesized that CR formation and function are more responsive to nutrient soil availability in the widely-distributed *Embothrium coccineum* than in the narrowly-distributed *Orites myrtoidea*, which exclusively occurs in recent volcanic depositions.

Methods Survival, growth rate, CR formation (number, biomass) and function (carboxylate exudation, phosphatase activity) were evaluated in seedlings of both species after five months of growth in either a volcanic or organic substrate.

Results E. coccineum exhibited full survival in both substrates, but had significantly lower growth, higher CR formation, higher CR citrate and malate exudation, and higher phosphatase activity in the volcanic substrate. By contrast, *O. myrtoidea* had similar growth rate in both substrates but 73% lower survival and null CR formation in the organic compared to the volcanic substrate.

Conclusions Variation in soil nutrient availability caused variation in growth and CR formation and function in a southern South American Proteaceae species of wider distribution, but not in a narrowly-distributed counterpart.

Keywords Carboxylate exudation · *Embothrium coccineum* · *Orites myrtoidea* · Phosphatase activity · Volcanic depositions

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Introduction

Phosphorus (P) is an important macronutrient for plants, and low P availability limits plant survival and growth (Lambers et al. 2012; Shen et al. 2011). Species of the Proteaceae family produce a special root structure called proteoid roots or cluster roots (CR) (Lamont 1982). CR

are structures with many densely packed lateral rootlets (Purnell 1960). In comparison with non-cluster roots, CR are highly efficient at mobilizing nutrients that may be poorly-available, especially P (Delgado et al. 2014; Lambers et al. 2018). These radicular adaptations increase the availability of sparingly soluble soil P mainly by releasing organic acid anions (for example citrate and malate) and ectoenzymes (acid phosphatase) into the rhizosphere (Lambers et al. 2008; Neumann and Martinoia 2002; Watt and Evans 2003). Proteaceae species are most abundant and widely distributed in old impoverished soils of areas with Mediterranean climates in South Africa and South-Western Australia (Lambers et al. 2008, 2010); however, there are six southern South American Proteaceae species. These few species are distributed in Mediterranean, temperate and cold environments, and mainly grow in soils of volcanic origin and/or recent volcanic depositions including pumice and tephra (Donoso 2006; Hechenleitner et al. 2005). The soils where southern South American Proteaceae species are found are younger and richer in total phosphorus (P) than those in South Africa and South Western Australia, but they may be low in P availability (Delgado et al. 2014; Lambers et al. 2012). Information about the formation and function of CR is mainly available for species found in South Western Australia where both total P content and P availability are low (for example Lambers et al. 2006; Muler et al. 2014; Teste et al. 2014), but the role of CR in young soils and recent volcanic depositions remains less explored.

Previous studies indicate that CR formation in southern South American Proteaceae occurs in plants growing in volcanic substrates (Ramírez et al. 1990; Piper et al. 2013; Zúñiga-Feest et al. 2018). Also, significant differences have been identified in CR function comparing Proteaceae species from different origins around the world. Recent investigations have found that exudation of citrate and malate by the southern South American Proteaceae species *Embothrium coccineum* is approximately 22 fold higher than that reported for the Australian species *Hakea prostrata* growing in similar hydroponic conditions, while the biomass investment in CRs is significantly lower in *E. coccineum* (5% in *E. coccineum* and 25% in *H. prostrata*) (Delgado et al. 2014; Shane et al. 2004a). Under the same experimental conditions, Delgado et al. (2014) found that *E. coccineum* exudes more carboxylates per unit cluster

root weight than its counterparts from South-Western Australia, which has been hypothesized as an adaptive trait to survive in volcanic soils (rich in total P, but possibly with a low concentration of available P). Thus, the high organic acid anions exudation by *E. coccineum* could be required to mobilize highly adsorbed-P (not readily available) characteristic of southern South America soils (Fajardo and Piper 2015; Piper et al. 2013; Lambers et al. 2012). Consistent with the previous hypothesis, CR formation of the southern South American Proteaceae species *E. coccineum* occurs in volcanic soils (Piper et al. 2013), but it remains untested whether its CR function (for example carboxylate exudation) is stimulated or not. On the other hand, most studies examining the role of CR in southern South American Proteaceae have used *E. coccineum* as the model study species (Delgado et al. 2013, 2014; Piper et al. 2013; Zúñiga-Feest et al. 2010, 2015), leaving uncertain whether findings for this species are true in other southern South American Proteaceae. *E. coccineum* has the widest geographic distribution along Chile and Argentina. Specifically, it is found from Linares in Chile (35° S) and Ñorquinco Lake in Argentina (39° S) to Tierra del Fuego in the southern end of the South American continent (55° S) (Donoso 2006; Rovere and Chalcoff 2010). Along this huge latitudinal gradient, *E. coccineum* inhabits diverse environments, including temperate rainforests (Lusk and Corcuera 2011), lahars at the treeline (Fig. 1), the forest-steppe ecotone in Patagonia (Fajardo and Piper 2015), and waterlogged areas (F. Piper personal observation). Contrary to *E. coccineum*, other southern South American Proteaceae species have a more restricted distribution. For example, *Orites myrtoidea* is a shrub (not a tree) with the narrowest distribution amongst southern South American Proteaceae, from Linares (35° S) to Malleco Province (38° S) in Chile, while in Argentina it has only been observed in the Neuquén Province (Hechenleitner et al. 2005). Also, in contrast to *E. coccineum, O. myrtoidea* is exclusively distributed in young volcanic soils. Both species act as colonizers, particularly in disturbed areas such as recent volcanic depositions (Donoso 2006; Grubb et al. 2013; Hechenleitner et al. 2005; Pfanzelt et al. 2008). It can therefore be hypothesized that *O. myrtoidea* is locally adapted to these soils, and hence much less responsive than *E. coccineum*, or not-responsive at all, to varied soil nutrient availability.
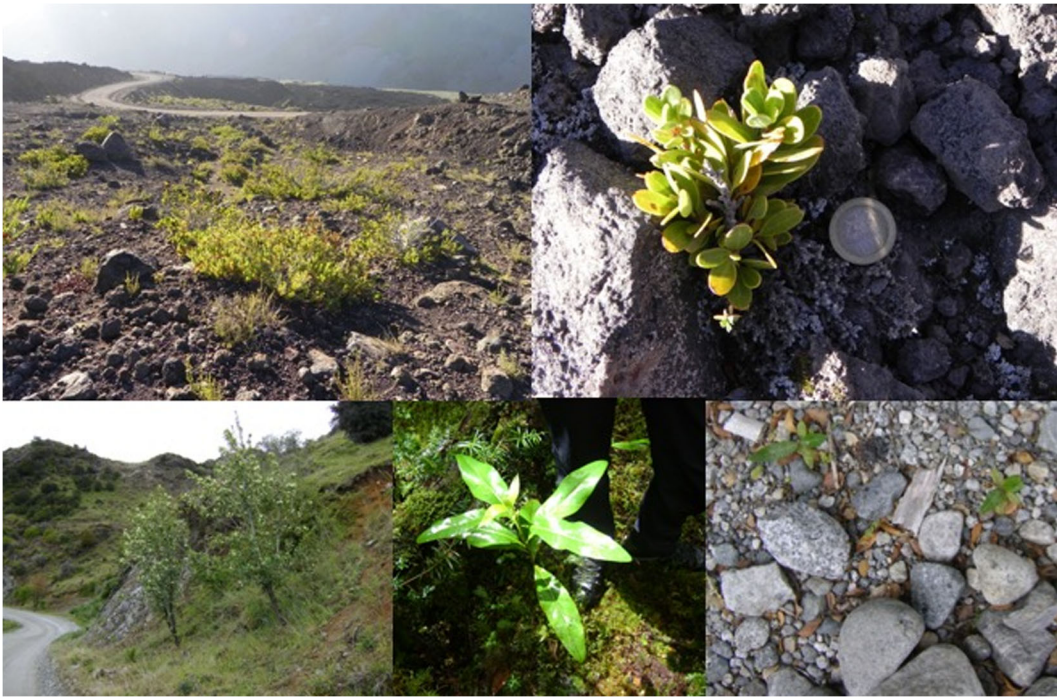
Fig. 1 *Orites myrtoidea* and *Embothrium coccineum* growing under natural conditions in Chile. *O. myrtoidea* is shown in the two upper-photographs (Antuco, Chile). This species grows exclusively on young, undeveloped volcanic substrates characterized by very low nutrient availability (for example soil analyses for Shangrilá, Termas de Chillán, where *O. myrtoidea* is a dominant species: pH 6.02 ± 0.11, organic matter content $3.64 \pm 3.30\%$, Olsen-P 6.47 ± 3.50 mg Kg^{-1}, total N $0.08 \pm 0.06\%$). *Embothrium coccineum*, by contrast, inhabits a wide range of environmental and soil conditions (lower-panel photographs)

In this study, we examined the CR formation and function (exudation of organic acid anions and phosphatase activity) of two southern South American Proteaceae species with contrasting geographic distributions (*E. coccineum* and *O. myrtoidea*) growing in recent volcanic depositions (pumice, with low nutrient availability) or in organic substrate (high nutrient availability). We tested the hypothesis that CR formation and function are stimulated in both species when they grow on pumice. If so, they should produce more CR and release more organic acid anions and acid phosphatase at the rhizospheric zone when they grow on recent volcanic substrate than when they grow on organic substrate. Furthermore, we hypothesized that responses of growth and CR formation and function to the type of substrate depend on the species' geographic range. Specifically, we anticipated that the widely-distributed species *E. coccineum* will be more responsive to different substrate types than *O. myrtoidea*, and will show higher variation in growth and CR formation and function.

Materials and methods

Plant material

Seedlings of *Embothrium coccineum* (J. R. Forst. et G. Forst.) and *Orites myrtoidea* (Poepp. & Endl.) Benth. & Hook. F. ex. B.D. Jacks were obtained after seed collection in February 2012 (austral summer). *E. coccineum* seeds were collected in Pichiquillaipe (Región de Los Lagos, 41°31' S and 72°45' W) and germinated in July 2012. *O. myrtoidea* seeds were collected in Antuco (Parque Nacional Laguna del Laja, 37°22′-37°28' S and 71°16′-71°26' W) and germinated in April 2012. *O. myrtoidea* seeds were germinated before *E. coccineum* because their germination is slower, and their growth rate is lower than that of *E. coccineum*. Before germination, seeds of both species were maintained at 4 °C in darkness, were treated with a bactericide-fungicide solution (Captán 2.7 g L^{-1} + Agrep 0.5 g L^{-1} + Benlate 1.8 g L^{-1}) and were exposed

to pre-germination treatments (250 mg L^{-1} of gibberellic acid at 20 °C for 24 h). Once seeds germinated (2–3 mm radicle), the seedlings of both species were transplanted to 250 mL pots containing organic substrate of commercial origin (ANASAC®) (Table 1). Pots were maintained for approximately three weeks in a growth chamber (16/8 h photoperiod, 150 μmol photons m^{-2} s^{-1} PAR, 20 °C, 80% RH). Then, seedlings of both species were transported to the greenhouse and kept there for approximately four months until the experiment began.

Experimental design

On December 5th, 2012 (late spring in the Southern Hemisphere), we transplanted thirty seedlings of E. coccineum and O. myrtoidea to 1 L pots filled with either an organic or a volcanic substrate (one plant per pot; 15 seedlings per species and substrate type) at the Universidad Austral de Chile (Valdivia, Chile) (Table 1). At this time, seedlings of E. coccineum were 5 months old and 8.97 ± 0.46 cm tall ($n = 30$), while seedlings of O. myrtoidea were 8 months old and 2.05 ± 0.10 cm tall ($n = 30$). The volcanic substrate was extracted from the vicinity of Mocho-Choshuenco volcano (39° 54' S, 72° 2' W, 200 m a.s.l.), which last erupted in 1864 (Rawson et al. 2015). The substrates differed in

Table 1 Chemical compositions of volcanic (pumice) and organic substrate

Properties	Volcanic	Organic
pH H_2O	6.57	5.25
pH $CaCl_2$	5.77	4.66
mineral N (mg kg^{-1})	8.40	43.40
Olsen-P (mg kg^{-1})	1.30	17.30
Total N (%)	0.03	0.33
Total P (mg kg^{-1})	217	1500
Organic Matter (%)	0.53	22.38
extractable Al (mg kg^{-1})	19.68	4.99
exchangeable Al (cmol$^+$ kg^{-1})	0.008	0.039
Al saturation (%)	0.18	0.11
Ca (cmol$^+$ kg^{-1})	2.49	25.62
Mg (cmol$^+$ kg^{-1})	0.83	6.68
K (cmol$^+$ kg^{-1})	0.38	1.73
Na (cmol$^+$ kg^{-1})	0.67	0.65
Base saturation (cmol$^+$ kg^{-1})	4.37	34.68

their chemical compositions; the volcanic substrate had a lower content of mineral N, available P, total P, and a higher percentage of Al saturation than the organic substrate (Table 1).

During the experiment, seedlings were regularly watered with tap water and were not fertilized. The pots were randomly distributed in the greenhouse and placement was changed weekly. The average daily temperature in the greenhouse during the experiment was 15.3 °C, and the average daily light intensity was 300 μmol photons m^{-2} s^{-1}. These conditions were recorded with a datalogger (HOBO Pendant®, ONSET Corporation). The experiment ended in April 30th, 2013 (late growing season in the southern hemisphere), when all seedlings were harvested.

Biomass determinations

Just before starting the experiment, six seedlings of both species were selected for the determination of initial dry biomass and initial height. The stems, leaves, non-cluster roots, and cluster roots of these seedlings were separated; then, the plant material was dried at 70 °C for 48 h (Venticell MMM, Poland) and weighed on a scale with a precision of 0.0001 g. (RADWAG, AS 220 C/2). Initial dry mass of the E. coccineum seedlings was 0.093 ± 0.007 g ($n = 6$) and was 0.022 ± 0.001 g (n = 6) for O. myrtoidea. At the end of the experiment, the percentage of survival was recorded for each treatment. A seedling was considered dead when all its shoots were dry and senescent. For the surviving seedlings, we measured seedling height and total dry weight to determine the relative growth rates in terms of biomass (RGRb) or height (RGRh) as described previously by Barrow (1977): RGR = (ln X Final - ln X Initial)/(t Final - t Initial); where X = height (cm) or dry weight (g) and t = time (days). The root systems were gently washed, and the total number of cluster roots was registered according to development stage: young, mature or senescent. The development stages were identified according to Delgado et al. (2013): young cluster roots were white and small (E. coccineum: ≤ 1 cm; O. myrtoidea: ≤ 0.5 cm diameter); mature cluster roots were white or light brown (brownish) and larger than young cluster roots (E. coccineum: ≥ 1 cm; O. myrtoidea: ≥ 0.5 cm); senescent cluster roots were dark brown and dehydrated. The plant material was then dried and weighed as previously described. Finally,

shoot/root ratios, CR/total root dry weight ratios, and CR /total biomass dry weight ratios were calculated.

Collection and analysis of carboxylates

Because the highest carboxylate exudation rate in *E. coccineum* has been found for mature CR (Delgado et al. 2013, 2014), we only collected the exudates from mature CR. For this, the CR were gently removed from the root system. We selected three independent *E. coccineum* seedlings per treatment and one mature CR per seedling for these measurements. For *O. myrtoidea*, only seedlings that grew in the volcanic substrate were selected since this species did not form CR in the organic substrate. Each mature CR was separated from the rest of the root under a solution of 10 mL of 0.25 mM $CaSO_4$ (calcium sulphate) (pH 5.5) and were then incubated for 2 h on a shaker at 150 rpm as described by Delgado et al. (2014). Afterwards, the solutions were syringe-filtered (0.22 μm pore size), frozen in liquid nitrogen, and stored at −18 °C. Then the samples were lyophilized and re-suspended in a solution of 93% (v/v) 25 mM KH_2PO_4 (potassium phosphate monobasic) and 7% (v/v) methanol (pH 2.5) for chromatographic acid determination (High Performance Liquid Chromatographer, HPLC) at the Universidad de la Frontera, Temuco, Chile. Chromatographic analysis was carried out in a HPLC system (Jasco, USA) equipped with a UV-visible detector. Separation was achieved using 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 of pH 2.5 and 7% (v/v) methanol with a flow rate of 1 mL min^{-1} (Cawthray 2003). Carboxylates were detected at 210 nm and retention times were compared with citrate, isocitrate, oxalate, succinate, and malate as standards.

Acid phosphatase activity

Acid phosphatase activity was quantified following the methodology described by Rubio et al. (1990). For these measurements, three *E. coccineum* seedlings per treatment were selected, and the same three *O. myrtoidea* seedlings used to analyse the carboxylate exudates were selected. Each whole root system was separately incubated in a water bath for 30 min at 20 °C in 25 mL of modified universal buffer (MUB): pH 5.5 (100 mM $C_4H_{11}NO_3$ (tris hydroxymethyl aminomethane),

100 mM $C_4H_4O_4$ (maleic acid), 67 mM $C_6H_8O_7*H_2O$ (citric acid monohydrate), 102 mM H_3BO_3 (boric acid), 1 M NaOH (sodium hydroxide) and 1 mL 33.7 mM of PNPP (ρ-nitrophenyl phosphate). Subsequently, 5 mL of NaOH (2 M) were added, and the solution was filtered. Finally, the absorbance was measured at 400 nm with a spectrophotometer (UV-2000 Spectrophotometer SP, Shanghai Spectrum Instruments Co., China), and values were interpolated on a calibration curve prepared with PNP (ρ-nitrophenol) as a standard.

Variation in the concentration of rhizospheric Olsen P during the experiment

To evaluate the effectiveness of the P-mining by the whole root systems and considering that some mined P could not have necessarily been acquired by the plants but rather could have remained in the rhizosphere, we determined the P availability in the rhizosphere soil at the end of the experiment. For this, seedlings were carefully removed from the pot in order to retain the substrate attached to the root system (rhizospheric soil). Then, the rhizospheric soil was collected, transferred to plastic bags, sieved and dried following Sadzawka et al. (2004), and then maintained at 4 °C for approximately. 3 months, until chemical determinations were made. The concentration of Olsen P in the rhizospheric soil (0–1 cm from the root) of the whole root system was determined by the method described by Olsen et al. (1954). These values were compared with the initial Olsen-P concentrations of both substrates used in the assay (Table 1): [(final Olsen P – initial Olsen P)/initial Olsen P *100].

Leaf P concentration

At the end of the experiment, P concentration was estimated in the leaves formed during the experiment. The leaves were washed with distilled water and later dried at 70 °C for 48 h (Venticell MMM, Poland). Dried samples were ground to a powder and P concentrations were determined colorimetrically using the vanadophosphomolybdate method, as described by Sadzawka et al. (2004).

Statistical analyses

The effects of species and substrate type on the relative growth rates, shoot/root ratios, leaf P concentration, and

Olsen P variation (%) were analysed using a two-way ANOVA. Carboxylate exudation rates, the mean number of CR per plant and the individual weight of CR per development stage of *E. coccineum* were analysed for both treatments using a two-way ANOVA. Since the *O. myrtoidea* seedlings did not produce CR in the organic substrate, only the carboxylate exudation rates and the number and individual weight of CR per development stage of *O. myrtoidea* seedlings grown in volcanic substrate were compared with *E. coccineum*; for this we performed a one-way ANOVA. Additionally, we performed a one-way ANOVA to compare responses between both species when both grown in volcanic substrate, and the effects of the two substrates on *E. coccineum* CR responses such as total number of CR per plant, CR dry weight/ total root dry weight, CR dry weight/ total biomass dry weight, and acid phosphatase activity. Survival data and the percentage of plants with CR were analysed using a Chi-squared test (χ^2). The normality and homogeneity of the variances were analysed for all the data, and when appropriate, ln or square-root transformations were used. Tukey tests were used to identify significant differences ($P < 0.05$) between factors. All analyses were performed with Sigma Stat 11 (Systat Software, Inc., Chicago, IL).

Results

Plant survival, growth, and formation of CR

Only 27% of the *O. myrtoidea* plants survived when grown in the organic substrate but in the other treatments all the plants survived (Table 2) ($\chi^2_{0.1}$ calculated: 4.93 and $\chi^2_{0.1}$ observed: 4.61). The shoot/root ratio was higher in the organic than in the volcanic substrate ($F_{1,32} = 28.538$; $P < 0.001$) for both species (Table 2). RGRb of *E. coccineum* was significantly higher in the

organic than in the volcanic substrate, while in *O. myrtoidea* was similar between both substrates (RGRb: $F_{1,32} = 7.618$; $P = 0.010$) (Fig. 2a). Likewise, RGRh of *E. coccineum* was significantly higher in the organic substrate and *O. myrtoidea* did not present differences due to substrate type (RGRh: $F_{1,48} = 6.976$; $P = 0.011$) (Fig. 2b). The final height of *E. coccineum* seedlings grown in volcanic substrate was 15.35 ± 1.54 cm while it was 26.30 ± 2.44 cm for seedlings grown in organic substrate. Conversely, the final height of the *O. myrtoidea* seedlings grown in volcanic substrate was 3.58 ± 0.29 cm while those grown in organic substrate reached heights of 3.60 ± 0.63 cm.

All the *E. coccineum* seedlings formed CR regardless of the substrate in which they were grown. However, no *O. myrtoidea* seedlings grown in organic substrate had CR, and only 53% of the *O. myrtoidea* seedlings grown in volcanic substrate had CR (Table 2) ($\chi^2_{0.05}$ calculated: 6.62 and $\chi^2_{0.05}$ observed: 3.841). *E. coccineum* seedlings had more CR per plant ($F_{1,29} = 20.211$; $P < 0.001$) (Fig. 3a), greater total CR biomass per total root biomass ($F_{1,18} = 5.437$; $P = 0.032$) (Fig. 3b), and greater total CR biomass per total plant biomass ($F_{1,17} = 24.009$; $P < 0.001$) (Fig. 3c) when grown in volcanic substrate versus when grown in organic substrate. In the volcanic substrate, the *E. coccineum* seedlings had a greater total number of CR per plant ($F_{1,22} = 238.357$; $P < 0.001$), greater total CR biomass per total root biomass ($F_{1,16} = 7.894$; $P = 0.013$), and greater total CR biomass per total plant biomass ($F_{1,16} = 11.189$; $P = 0.004$) than *O. myrtoidea* seedlings.

CR of *E. coccineum* seedlings grown in volcanic substrate had small particles of the substrate adhered to the root structure (Fig. 4c), and some CR had grown through these small particles (Fig. 4f). The *O. myrtoidea* CR were more irregularly shaped and had longer lateral rootlets than those found in *E. coccineum* CR (Fig. 4a). The individual size of *E. coccineum* CR did not vary

Table 2. Effect of two substrate types with different P availability on survival (%), shoot/root ratio, and plants with CRs (%); values are shown for *Embothrium coccineum* and *Orites myrtoidea*

Species.	Survival (%)		Shoot/Root		Plants with CR (%)	
	Organic Substrate	Volcanic Substrate	Organic Substrate	Volcanic Substrate	Organic Substrate	Volcanic Substrate
E. coccineum	100	100	2.6 ± 0.3a	1.2 ± 0.1b	100	100
O. myrtoidea	27	100	4.0 ± 1.4a	1.6 ± 0.1b	0	53 ± 3.3

Each value is the mean of 15 independent samples ±1 standard error (with the exception of *O. myrtoidea* in organic substrate, $n = 4$). Lowercase letters indicate significant differences between substrates for each species ($P < 0.05$)
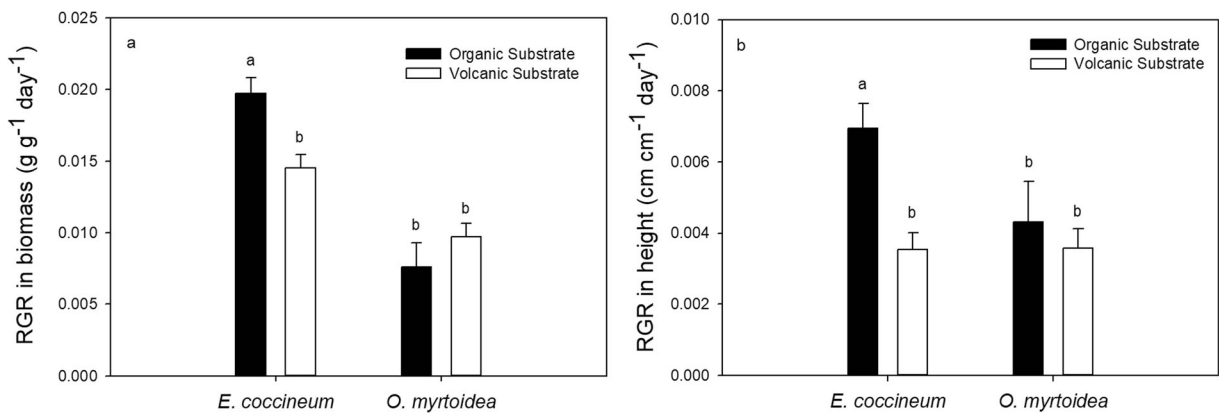
Fig. 2 Effect of two substrate types with different P availability on relative growth rates (RGR) in biomass (**a**) and height (**b**) of Embothrium coccineum and *Orites myrtoidea* seedlings. Each value is the mean of 15 independent samples +1 standard error (except for *O. myrtoidea* in organic substrate treatment, $n = 4$). Lower-case letters indicate significant differences ($P < 0.05$)

with substrate type but it varied according to development stage ($F_{2,71} = 37.986$; $P < 0.001$): mean dry mass per CR was 2.73 ± 0.50, 8.55 ± 1.79 and 0.81 ± 0.11 mg for each young, mature and senescent CR, respectively. In *O. myrtoidea*, mean dry mass per CR were 2.18 ± 0.66, 5.59 ± 0.48 and 1.27 ± 0.48 mg for each young, mature, and senescent CR ($F_{2,16} = 9.598$; $P = 0.002$).

Root exudates

Only malate and citrate were detected in the HPLC analysis of the collected exudates of the mature CR of both species. The mature CR of *E. coccineum* seedlings that grew in volcanic substrate had exudation rates of both citrate and malate approximately 50% greater than that found for seedlings grown in organic substrate (Table 3) ($F_{1,11} = 40.082$; $P < 0.001$). In both substrate types, the malate exudation of *E. coccineum* CR was higher than citrate exudation ($F_{1,11} = 23.853$; $P = 0.001$). When comparing both species growing in volcanic substrate, the malate exudation rate of *O. myrtoidea* CR was similar to that of *E. coccineum* CR ($0.52 \, \mu mol \, g^{-1} \, h^{-1}$); however, the citrate exudation rate of *O. myrtoidea* CR was approximately. 100% higher ($0.68 \, \mu mol \, g^{-1} \, h^{-1}$ volcanic substrate) than that of *E. coccineum* ($F_{1,11} = 10.817$; $P = 0.011$).

Acid phosphatase activity

E. coccineum acid phosphatase activity was approximately. 50% higher when grown in volcanic versus organic substrate ($F_{1,7} = 10.321$; $P = 0.018$) (Fig. 5).

Due to the high mortality of seedlings in the organic substrate, the acid phosphatase activity of *O. myrtoidea* was only assessed for the seedlings grown in the volcanic substrate; from this, values were similar to those found for *E. coccineum* ($F_{1,8} = 0.149$; $P = 0.711$) (Fig. 5).

Availability of phosphorus in both substrates

At the end of the experiment, the concentration of Olsen P in the rhizospheric soil of both species changed depending on the substrate treatment ($F_{1,30} = 4.648$; $P = 0.04$). In volcanic substrate, the Olsen P concentration in the rhizospheric soil was 40% and 120% higher for *E. coccineum* and *O. myrtoidea*, respectively, at the end of the experiment than at the beginning (Fig. 6). In organic substrate, in contrast, the Olsen P concentration in the rhizospheric soil decreased by 30% in *E. coccineum* seedlings and increased by 14% in *O. myrtoidea* seedlings. However, this last result was obtained for the few *O. myrtoidea* seedlings that survived in the organic substrate (27%, $n = 4$ seedlings), and hence must be considered with caution.

Leaf P concentration

In both species, P concentration in leaves formed during the experiment was similar between substrates type (Fig. 7). *E. coccineum* grown in organic substrate showed approximately 32% higher leaf P concentration than *O. myrtoidea* grown in the same substrate ($F_{1,14} = 13.839$; $P = 0.003$).
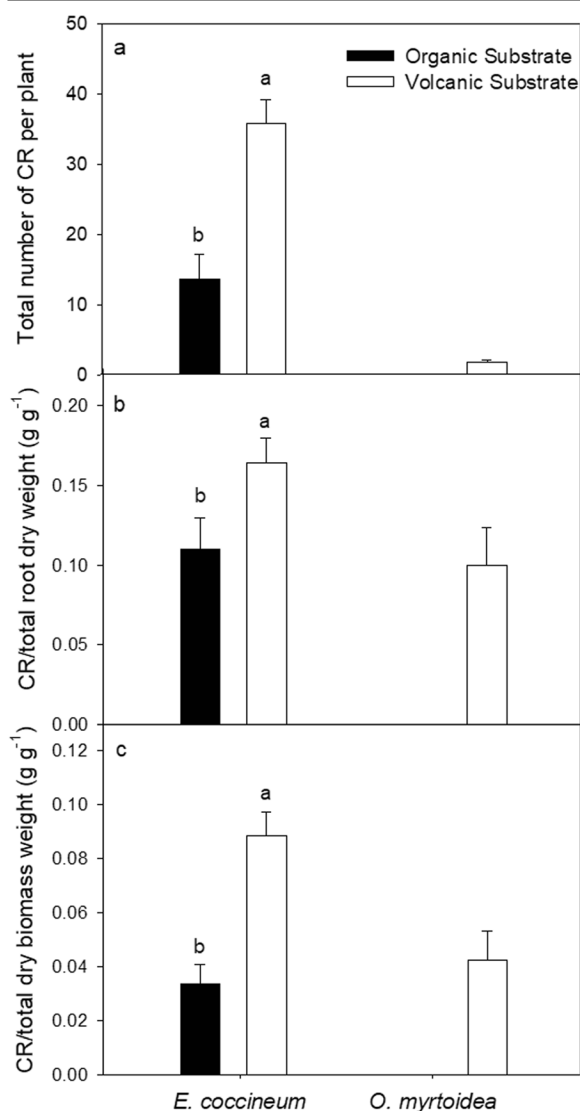
Fig. 3 Effect of two substrate types with different P availability on the CR production of Embothrium coccineum and *Orites myrtoidea* seedlings. **a** Total mean number of CR per plant. **b** CR dry weight / total root dry weight ratio. **c** CR dry weight / total dry biomass weight ratio. Each value is the mean of 15 independent samples +1 standard error (except for *O. myrtoidea* in organic substrate treatment, n = 4). Lower-case letters indicate significant differences between treatments ($P < 0.05$)

Discussion

CR formation and function are stimulated in a volcanic substrate (hypothesis 1)

We found that two southern South American Proteaceae species with contrasting geographic and edaphic distributions exhibited higher CR formation and function in a

recent volcanic than in an organic substrate. The stimulation of CR formation in *E. coccineum* when grown in a recent volcanic substrate is consistent with previous findings reporting CR formation in volcanic soils in natural conditions (Piper et al. 2013); it is also in line with studies reporting increased CR formation in response to hydroponic treatments with low concentrations of Olsen P (Delgado et al. 2013, 2014) and sand grown seedlings watered with low P nutrient solutions (Zúñiga-Feest et al. 2010). However, our study shows for the first time that the young volcanic substrate stimulated CR function (carboxylate exudation) in *E. coccineum*, a fact that thus far has only been reported under hydroponic treatments with low concentration in P availability (Delgado et al. 2013, 2014). Our study additionally reports for the first time that these responses are not exclusive of the most studied southern South American Proteaceae species, *E. coccineum*, but also occurred in the species with the narrowest distribution (*O. myrtoidea*). Additionally, both species maintained their leaf P concentrations similar between substrates, despite the much lower P availability of the volcanic substrate. These results support the role of cluster roots in making P available to plants growing in volcanic substrates (Lambers et al. 2012), which would allow both species to maintain their P concentration in leaves. Thus, our results are consistent with the hypothesis that the CR in southern South American Proteaceae play a fundamental role in P mining and acquisition in soils of volcanic origin (rich in total and adsorbed P, poor in Olsen P), and help to explain the colonization capacity of these species, including *E. coccineum* and *O. myrtoidea* (Delgado et al. 2014; Lambers et al. 2012).

The phosphatase activity of *E. coccineum* differed greatly between substrates; specifically, it was two times higher in plants grown in recent volcanic substrate compared to those grown in organic substrate. Previous studies have reported increased phosphatase activity in response to watering with low P concentration (Delgado et al. 2013, 2014). Soils found in southern Chile are composed of around 50% organic forms of P, and high phosphatase activity has been shown to increase a plant's ability to take up organic forms of P (Borie and Rubio 2003). Several authors have proposed that plants bearing CR can simultaneously exude carboxylates and have phosphatase activity; thus, they are thought to benefit from micro-scale nutrient rich patches (for example high Inositol-P) to obtain P (Delgado et al. 2013). Our study confirms that both mechanisms to obtain P occurred indeed in *E. coccineum* in volcanic soils.
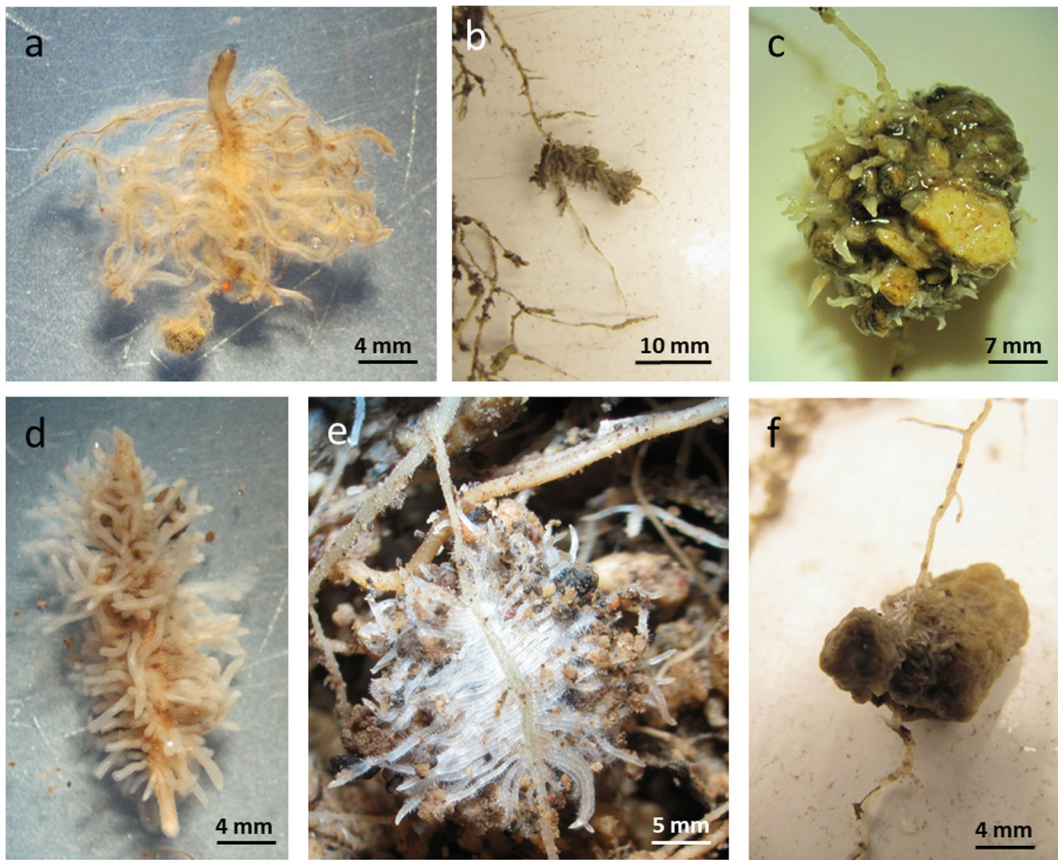
Fig. 4 Cluster root morphology formed during the experiment. **a** and **b** *Orites myrtoidea* CR formed in volcanic substrate. **c** to (**f**) Embothrium coccineum CR formed in volcanic substrate, substrate particles attached on CR surface (**e**) and aggregation around each of the CRs (**c**). CR formed inside a volcanic rock (**f**). Each individual picture includes the corresponding scale

Allocation to growth and CR varied with the species geographic range (hypothesis 2)

We found that the substrate type affected differently CR formation and function of the two southern South American Proteaceae species. The widely-distributed species *E. coccineum* had high survival rates in both substrates but showed a significant variation in growth and CR formation and function in response to the substrate type. Specifically, it grew less, and it formed more

Table 3 Carboxylate exudation rate by mature *Embothrium coccineum* CRs grown in organic and volcanic substrates

		Organic acid anions exudation rate	
		(μmol g FW^{-1} h^{-1})	
		Malate	Citrate
E. coccineum	Organic Substrate	0.243 ± 0.01 Ab	0.127 ± 0.02 Bc
	Volcanic Substrate	0.540 ± 0.05 Aa	0.297 ± 0.05 Bb
O. myrtoidea	Organic Substrate	NE	NE
	Volcanic Substrate	0.517 ± 0.04 Aa	0.680 ± 0.09 Aa

Each value is the mean of three independent determinations ± 1 standard error (except for *Orytes myrtoidea* in organic substrate, not evaluated due to low percentage of survival, NE). Lowercase letters show significant differences between treatments and capital letters indicate significant differences between each type of carboxylate exuded ($P < 0.05$)

Fig. 5 Acid phosphatase activity of entire root systems of Embothrium coccineum and *Orites myrtoidea* seedlings. Each value is the mean of 5 independent samples +1 standard error (except for *O. myrtoidea* in organic substrate, not evaluated). Lower-case letters indicate significant differences between treatments ($P < 0.05$)
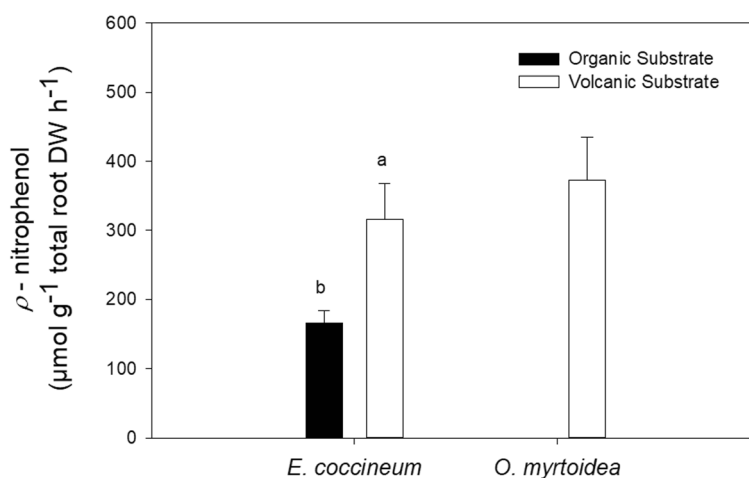
CR which were in turn more active in terms of exudation in the recent volcanic than in the organic substrate. In contrast, the narrowly-distributed species *O. myrtoidea* showed unexpectedly low survival and null CR formation in the organic substrate, at the time that it grew similarly in both substrates. The lack of plasticity in growth of *O. myrtoidea* in response to different substrates is likely to reflect local adaptation to limiting soil nutrient availability, which characterizes the whole geographic distribution of this species. *O. myrtoidea* is exclusively distributed in areas with limited soil development (Fig. 1) like high elevation sites with lahars and

rocky volcanic soils, where low but constant growth rates during spring and summer are enough to warrant its establishment (Pfanzelt et al. 2008; Hechenleitner et al. 2005). The investment in CR formation and exudation rather than in fast growth (as we found in this study) should be much more rewarding for this species in terms of fitness, as the soils where *O. myrtoidea* is distributed are extremely poor in P availability (Fig. 1). Conversely, *E. coccineum* is distributed in areas of highly contrasting soil nutrient availability (Souto et al. 2009; Piper et al. 2013; Fajardo and Piper 2015), where therefore the degree of interspecific competition favoring a fast growth
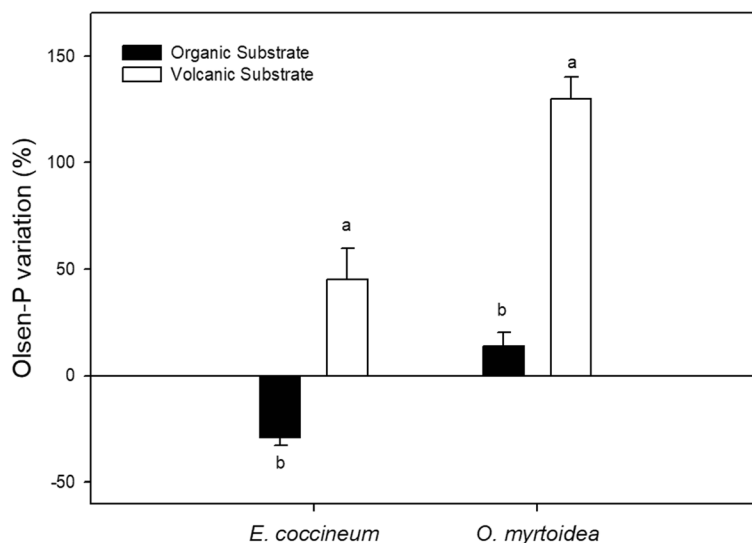

Fig. 6 Olsen P variation in rhizospheric soil of *Embothrium coccineum* and *Orites myrtoidea* seedlings at the end of the experiment. Initial value of Olsen P in each substrate is shown as zero in the graph, values higher than zero represent increases in Olsen P (available P) (see initial Olsen *P* values in Table 1 and details of the calculations in Materials and methods). Each bar is the mean of 10 independent samples +1 standard error (except for *O. myrtoidea* inorganic substrate treatment, $n = 4$). Lower-case letters indicate significant differences between treatments for each species ($P < 0.05$)

Fig. 7 Concentration of phosphorus in leaves of Embothrium coccineum and *Orites myrtoidea* seedlings, formed during the experiment. Each value is the mean of 6 independent samples +1 standard error (except for *O. myrtoidea* inorganic substrate treatment, $n = 4$). Lower-case letters indicate significant differences ($P < 0.05$)
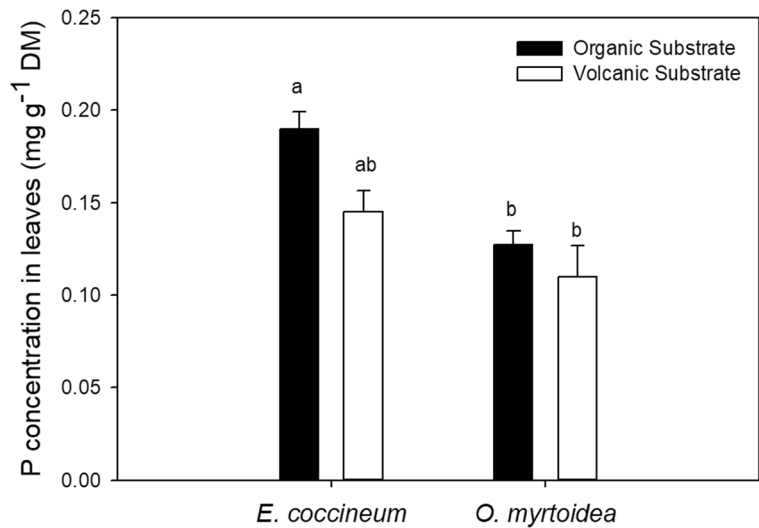
may be also very variable. In the temperate rainforest, for example, faster growth is advantageous for *E. coccineum* which allows it to quickly colonize forest gaps (Lusk and Corcuera 2011). In contrast, in exposed and bare ground areas of Patagonia a higher investment in CRs rather than in fast growth is likely to be more rewarding (Piper et al. 2013). Accordingly, *E. coccineum* seedlings from contrasting provenances exhibit different aboveground growth when grown under common conditions (Zúñiga-Feest et al. 2015).

E. coccineum had higher CR number than *O. myrtoidea* when both grew in recent volcanic substrate, but *O. myrtoidea* exuded more carboxylate. Specifically, both species had similar phosphatase activity and CR malate exudation in the volcanic substrate, while the citrate exudation rate of *O. myrtoidea* was significantly higher than that of *E. coccineum*. Malate and citrate have been previously reported as carboxylates commonly exuded by *E. coccineum* seedlings exposed to low P availability in hydroponic treatments (Delgado et al. 2013, 2014); however, this study is the first to show that *O. myrtoidea* also exudes these two carboxylates and that indeed the exudation rate of citrate is even higher than that found for *E. coccineum*. In general, carboxylate composition and exudation rates can differ among species, soils types, plant developmental stages, and stress factors (Veneklaas et al. 2003; Uren 2000). Indeed, it is interesting that CR of *E. coccineum* seedlings have been found to exude significantly much more citrate than malate after one month growing under hydroponic treatments of low P availability (Delgado et al. 2013), whereas here we found that malate

exudation was significantly higher. This suggests that the type of carboxylate exuded by *E. coccineum* depends on the growth conditions and substrate type. The chemical composition of each carboxylate influences the capacity to release P from different sources (Randall et al. 2001). For example, the effectiveness of citrate in making P available to plants is higher than that of malate and oxalate (Ryan et al. 2001). It is possible that in the volcanic substrate of our study, where *E. coccineum* seedlings had on average approximately 35 CR per plant, malate was sufficiently effective for P acquisition. In contrast, in the hydroponic media used by Delgado et al. (2013), *E. coccineum* seedlings had on average approximately 5 CR: a smaller number of roots might have provided a relatively small amount of P to shoots, which could have provided a signal for enhanced exudation of citrate as opposed to malate. In the same line, *O. myrtoidea* exuded citrate faster than *E. coccineum,* but had fewer CR, and thus probably was more effective in terms of exudation rate than *E. coccineum* at mobilizing P; CR function compensated for a lower CR formation. This conclusion is supported by the higher Olsen P concentrations in the rhizospheric soil at the end of the experiment, indeed much higher in *O. myrtoidea* than in *E. coccineum*. On the other hand, our measurement of acid phosphatase included the whole roots (with CR), although the acid phosphatase activity is likely highest for CR; since *O. myrtoidea* had very few CR but both species had similar acid phosphatase activity, the phosphatase acid activity per CR was probably much higher in *O. myrtoidea* than in *E. coccineum*.

We found that the volcanic substrate promoted CR formation and exudation in both species, and, in turn, increased the P availability in the rhizosphere of the seedlings grown in volcanic substrate. Specifically, the pots containing volcanic substrates had higher relative Olsen P concentrations at the end of the experiment compared to at the beginning and this was true for both species; this suggests that increased soil P availability was driven by CR formation and activity, although the phosphatase activity of the whole root system could have contributed. Additionally, solubilizing microorganisms could have, in part, increased P availability in the rhizospheres of these seedlings. It is well known that microorganisms and other biological features of soil can affect P availability and phosphatase activity (Borie and Zunino 1983; Dakora and Phillips 2002). Specifically, acid phosphatase of microbial origin can hydrolyze a range of P compounds, and the release of these compounds is typically high when P is low (Tarafdar et al. 2001). Recently, we have found that the metabolic diversity of the microbial community in the rhizosphere of *E. coccineum* differs depending on CR development stage (Renderos 2017). Overall, it seems that these increases in P availability could be produced by the combination of root carboxylate exudation, plant phosphatase activity from the root surface, and microbial phosphatase activity. Further research is needed to elucidate the specific mechanism by which P availability is increased in the rhizosphere of these species.

The magnitude of the increase in Olsen P concentration of the rhizosphere in the volcanic substrate differed by species. The concentration of P in the *O. myrtoidea* rhizosphere increased by 120% while the concentration of P in the *E. coccineum* rhizosphere increased by only 40%. Since *E. coccineum* grew more than *O. myrtoidea* in the recent volcanic substrate, it is possible that both species were similarly efficient at mining P, but that *E. coccineum* demanded more P for its own growth (i.e. mined and acquired). By contrast, *O. myrtoidea* might have had a much lower P demand for growth, which would account for the very high Olsen P concentrations of the rhizosphere. Olsen P also increased in the rhizosphere of *O. myrtoidea* growing in the organic substrate, although the magnitude of the increase was much lower than in the volcanic substrate. In contrast, for *E. coccineum* in the organic substrate the Olsen P concentration seemed to decrease throughout the experiment. This response could have been driven by the high growth rate of this species when grown in the organic substrate, as growth would result in high nutrient demand. In fact, a previous study has shown that the growth rate of *E. coccineum* is high when nutrient conditions are non-limiting (Zúñiga-Feest et al. 2010).

The survival rate of *O. myrtoidea* grown in organic substrate was unexpectedly low. One of the possible explanations for this low survival could be low tolerance to high concentrations of nutrients. Toxicity by excess nutrients has been reported in several Proteaceae species, including *Hakea prostrata* from South Western Australia (Shane et al. 2004b). Considering the edaphic conditions where *O. myrtoidea* naturally grows, its nutritional requirements could be low and additionally its capacity to downregulate P-acquisition under conditions of high nutrient availability could be rather limited. Although, we did not measure leaf P concentration in the plants that died in organic substrate, it is possible that these plants were not able to downregulate while surviving seedlings were able to, as suggest by the similar leaf P concentrations showed by *O. myrtoidea* in organic and volcanic substrates. Delgado et al. (2014) reported that *E. coccineum* seedlings have a high capacity to downregulate P acquisition when grown in high nutrient conditions, but this capacity has not been evaluated in other southern South American Proteaceae. Another explanation for the low survival of the *O. myrtoidea* seedlings in the organic substrate could be toxicity to increasing local Al solubilization. It is well known that many plant species are killed by high Al concentrations (Ma 2000; Zheng et al. 1998). When the pH of the soil solution is lower than approximately 5, as was the case for the organic substrate in our study, Al is solubilized and absorbed by the roots, thus producing negative effects in the plant (Matsumoto 2000). Indeed, the concentration of exchangeable Al was around 5 fold higher in the organic than in the volcanic substrate. Finally, the fact that the organic substrate was a commercial product rather than collected from natural conditions could have inhibited CR formation in *O. myrtoidea*. Commercial substrates are usually sterilized; and specific soil microbiota that could help CR initiation are probably absent.

Conclusions

We found that a volcanic substrate type stimulated cluster root (CR) formation and function of two southern South American Proteaceae species. Although previous studies have already reported CR formation in volcanic substrates in some southern South American Proteaceae (Piper et al.

2013; Ramírez et al. 1990), we demonstrated here that CR formation and functioning (exudation) increases in a recent volcanic substrate (pumice). We also found that allocation to CR formation and function and to growth were more plastic in a widely distributed southern South American Proteaceae species than in a narrowly-distributed counterpart which exclusively inhabits undeveloped, young volcanic soils, suggesting local adaptation in the latter. Thus, our study adds to the evidence that the role of CR in southern South American Proteaceae is to mediate P mining and acquisition in young originated volcanic soils that are rich in total P concentration but poor in available P concentration, as has been suggested by other authors (Lambers et al. 2012; Delgado et al. 2014).

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