

**SOIL NITROGEN, AND NOT PHOSPHORUS, PROMOTES  
CLUSTER-ROOT FORMATION IN A SOUTH AMERICAN  
PROTEACEAE, *EMBOTHRIMUM COCCINEUM*<sup>1</sup>**

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- *Premise of the study:* Cluster roots are a characteristic root adaptation of Proteaceae species. In South African and Australian species, cluster roots promote phosphorus (P) acquisition from poor soils. In a South American Proteaceae species, where cluster roots have been scarcely studied and their function is unknown, we tested whether cluster-root formation is stimulated by low soil nutrition, in particular low P-availability.
- *Methods:* Small and large seedlings (< 6- and > 6-months old, respectively) of *Embothrium coccineum* and soil were collected across four different sites in Patagonia (Chile). We determined cluster-root number and relative mass, and leaf Pi concentration per mass ( $Pi_{mass}$ ) and per area ( $Pi_{area}$ ) for each seedling, and tested relationships with Olsen–P (OP), sorbed–P (sP) and total nitrogen (N) using generalized linear mixed-effects models and model selection to assess the relative strength of soil and plant drivers.
- *Key results:* Best-fit models showed a negative logarithmic relationship between cluster-root number and soil nitrogen (N), and between cluster-root relative mass and both leaf  $Pi_{area}$  and soil N, and a positive logarithmic relationship between cluster-root number and leaf  $Pi_{area}$ . Cluster-root relative mass was higher in small than in large seedlings.
- *Conclusions:* Contrary to that found in South African and Australian Proteaceae, cluster roots of *E. coccineum* do not appear to be driven by soil P, but rather by soil N and leaf  $Pi_{area}$ . We suggest that cluster roots are a constitutive and functional trait that allows plants to prevail in poor N soils.

**Key words:** *Embothrium coccineum*; establishment; intraspecific variation; leaf phosphate; Olsen–P; proteoid roots; succession; volcanic soils.

Phosphorus (P) is an essential nutrient for plant life and, along with nitrogen (N), one of the most limiting nutrients in wild plant communities (Richardson et al., 2004; Vitousek et al., 2010). Cluster roots (also called proteoid roots) are a major root adaptation to poor soil nutrition that is characteristic in the Proteaceae, a family predominantly distributed in the southern hemisphere (Purnell, 1960; Skene, 1998). Several studies have shown the importance of cluster roots for P-acquisition in Proteaceae species of South Africa and Australia, where the family is represented by more than 900 species in 30 genera (Lamont, 1982). In those species, low soil P availability and/or low leaf P content stimulate the formation and growth of cluster roots,

which in turn increase P-acquisition through the exudation of organic acids (e.g., malate, citrate) and phosphatases that mobilize unavailable forms of P in the soil (Lamont, 1972, 1982; Lamont et al., 1984; Watt and Evans, 1999; Lambers et al., 2006). Fewer studies have also examined the influence of N in the substrate (external N), finding that cluster roots are formed in response to low N concentrations instead of P availability, and that they likely participate in N-acquisition (Lamont, 1972; Schmidt et al., 2003; Paungfoo-Lonhienne et al., 2009). In southern South America (Chile and Argentina) there are only six Proteaceae species (*Embothrium coccineum* J.R. Forst. & G. Forst., *Gevuina avellana* (Molina) Gaertn., *Orites myrtoidea* (Poepp. & Endl.) Benth. & Hook. f. ex Sleumer, *Lomatia dentata* (Ruiz & Pav.) R.Br., *L. ferruginea* (Cav.) R.Br., and *L. hirsuta* Diels ex J.F. Macbr.) and the role of cluster roots in these species is unclear, i.e., the few published studies have brought equivocal evidence for what ultimately drives cluster-root formation (Zúñiga-Feest et al., 2010; Donoso-Ñanculao et al., 2011). For example, under controlled-environment conditions, seedlings of *E. coccineum* did not differ in the number of cluster roots when they were watered with solutions having different P levels, while seedlings watered with distilled water had higher cluster-root mass than seedlings watered with a Hoagland solution without P (Zúñiga-Feest et al., 2010). This finding suggests the existence of a factor other than P (e.g., N) influencing cluster-root growth. Working with the same species but under natural conditions, Donoso-Ñanculao et al. (2011) found that the cluster-root number was similar between two soils of different

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P-availability, and that relationships of cluster-root mass to soil P availability were season-dependent. Although these two studies set initial patterns relating cluster roots with soil P-availability, both examined a small portion of the full range of soil P-availability found at the field sites where the studied species, *E. coccineum*, actually occurs (Souto et al., 2009).

Proteaceae is a monophyletic group that originated in the ancient supercontinent of Gondwana (Barker et al., 2007). Because cluster roots are found in all genera of the family except *Persoonia* (Skene, 1998), they likely represent an ancestral trait that has been conserved during evolution. It is thought that cluster roots evolved to acquire P from weathered, impoverished, and P-depleted soils (Lambers et al., 2008; Lambers et al., 2012). Such soils are often found in South Africa and south-western Australia where cluster roots could explain the prevalence of Proteaceae species in these regions (Lambers et al., 2008). An unresolved question, however, is why cluster roots were kept in Proteaceae species of southern South America, where soils are generally young as a result of the high frequency of large-scale disturbances (e.g., volcanic activity, earthquakes, landslides, as well as glacial, fluvial, and wind erosion) (Veblen and Ashton, 1978; Grubb et al., 2013), and therefore, expected to have high P-availability (Walker and Syers, 1976; Richardson et al., 2004; Peltzer et al., 2010). A recent evolutionary explanation for cluster-root formation in southern South American Proteaceae species states that the volcanic nature of most soils of the region would have favored the presence of this trait (Lambers et al., 2012). The high volcanic activity in the Andes Cordillera is responsible for a large amount of allophane and sesquioxide in the soil, which have a high phosphate-fixing capacity, making P not readily available for plants (Escudéy et al., 2001; Redel et al., 2008; Vistoso et al., 2009). Cluster roots are thus posited to promote the establishment of Proteaceae species in these young volcanic soils through increased P-acquisition (Lambers et al., 2012).

In general, cluster roots are considered to be an adaptation to survive in extremely poor soils (Neumann and Martinoia, 2002). In pioneer species, poor soils may be more detrimental for the establishment of small, recently emerged seedlings than for larger seedlings, saplings, or mature trees. This is because small seedlings must be able to establish, often on very infertile and undeveloped soils, overcoming the critical and highly P-demanding phase of heterotrophic-autotrophic conversion (Reich and Schoettle, 1988; Thomson et al., 1992). For these seedlings to survive, biomass investment in cluster roots may be crucial, as it would warrant access to an essential nutrient (i.e., P), otherwise unavailable. This may be particularly true for some South American Proteaceae species which seem to account for lower P storage in the seeds—as suggested by the lower seed mass (Grubb et al., 2013) and seed P concentrations (e.g., 3–5 mg g<sup>-1</sup> for *E. coccineum*, M. Delgado, Universidad de la Frontera, unpublished data)—than their Australian and South African counterparts (10.2 mg g<sup>-1</sup> average for several species, Groom and Lamont, 2010). Due to low seed P storage, the heterotrophic-autotrophic conversion (and hence the highest demands for P by the seedling) should take place soon after seedling emergence. Thus, traits facilitating the access to soil nutrients (e.g., cluster roots) are expected to particularly benefit small seedlings. In contrast, larger seedlings would be less dependent on cluster roots for assuring their survival, as they have a larger root mass for exploring a greater volume of soil and likely benefit from their own leaf turnover. For large seedlings, biomass investment in aerial growth and leaf area

instead of cluster roots may be more important for light competition.

The main aim of this study was to assess whether cluster roots in a southern South American Proteaceae species are positively related to soil P availability. If this is the case, we hypothesize that: (1) low soil P availability and high P-sorption will promote a higher number and growth of cluster roots under natural conditions; and that (2) cluster roots compensate for potential decreases in the leaf P status associated with low soil P availability, i.e., low Pi concentration increases cluster-root number and relative mass, which in turn cause foliar P concentrations to increase. We also hypothesize that given that cluster roots are especially important during seedling establishment, (3) biomass investment in cluster roots should be higher in small than in large seedlings. In addition, we also examined the influence of other soil chemical properties that may be related to cluster-root formation (Lamont, 1972; Paungfoo-Lonhienne et al., 2009), thus we also hypothesize that (4) total soil N influences the formation of cluster roots, i.e., the lower the soil N concentrations, the higher the number of cluster roots. All these hypotheses combine different relationships between soil nutrition levels and plant traits. To test our hypotheses we investigated the relationship between intraspecific variation in cluster-root formation, soil P and N concentration, and leaf P concentration in the widespread pioneer species *E. coccineum* (Proteaceae) across four locations with contrasting precipitation and soil fertility in the Chilean Patagonia. We used a suite of a priori biological hypotheses along with model selection to assure maximal inference power.

## MATERIALS AND METHODS

**Study species**—*Embothrium coccineum* J.R. Forst. & G. Forst. (Proteaceae) is an endemic small evergreen tree of the temperate forests of southern South America that extends over a wide latitudinal range from 35° to 55° S. With such a long distribution there is also a high variation in mean annual temperature and precipitation, from 5 to 15°C and from 400 to >4000 mm, respectively (Donoso, 2006). *Embothrium coccineum* is a shade-intolerant species that colonizes highly disturbed areas where other tree species rarely occur, i.e., areas of frequent volcanic-ash depositions, lahars (layers of volcanic scoria subjected to high radiation and low night temperatures), glacial moraine depositions, and roadsides (Veblen and Ashton, 1978; Alberdi and Donoso, 2004; Grubb et al., 2013). Its wide distribution and prevalence in a diversity of habitats provides a unique opportunity to study intraspecific variation in cluster-root formation under contrasting soil conditions.

**Study sites**—The study was carried out in the Aysén Region, Patagonia, Chile. Four sites across a fertility and precipitation gradient were selected for seedling collection and soil analyses: (1) Exploradores, (2) Tranquilo, (3) Reserva Nacional Cerro Castillo (hereafter Cerro Castillo), and (4) Reserva Nacional Coyhaique (hereafter Coyhaique). Exploradores (46°29'51" S, 73°09'15" W, 137 m a.s.l.) is located in the Exploradores River valley, in an area covered by moraine deposits of glacial erosion origin from the Exploradores Glacier (Aniya et al., 2011). Seedlings of *E. coccineum* were collected from the bare soil at roadsides amid a second-growth temperate rainforest area composed by ca. 15 woody species, including *Nothofagus betuloides* (Mirb.) Oerst. (Nothofagaceae), *Drimys winteri* J.R. Forst. & G. Forst. (Winteraceae), *Podocarpus nubigena* Lindl. (Podocarpaceae) and *Lomatia ferruginea* (Proteaceae). Here, annual precipitation is very high, normally reaching more than 3000 mm (Table 1). Tranquilo (46°37'47" S, 72°46'49" W, 331 m a.s.l.) is located 40 km east of Exploradores. The soils are of volcanic origin but during the mid-20<sup>th</sup> Century were affected by massive fires used to burn forests to convert them into prairies. Forest recolonization has occurred patchily. Vegetation is dominated by *Nothofagus antarctica* (G. Forst.) Oerst., *N. betuloides*, *E. coccineum*, and *Raphithamnus spinosus* (Juss.) Moldenke (Verbenaceae). *Embothrium coccineum* regenerates on bare soil and decomposing logs (remnant from the large-scale fires) where competition from grasses is reduced. Cerro Castillo

TABLE 1. Climatic and mean ( $\pm 1$  SE) soil and seedling characteristics for the four locations considered in this study: Exploradores (46°30' S, 73°09' W, 137 m a.s.l.), Tranquilo (46°38' S, 72°47' W, 331 m a.s.l.), Cerro Castillo (46°07' S, 72°14' W, 451 m a.s.l.), and Coyhaique (45°32' S, 72°03' W, 466 m a.s.l.). Soil property comparisons among sites were conducted using linear mixed-effect models (LMM), and inference is presented as F-ratios, where significance levels are “\*\*\*” for  $P < 0.01$ , and “\*\*\*\*” for  $P < 0.001$ . Means with different letters in the same row are significantly different among sites [post hoc Tukey’s honestly significant difference (HSD) test].

|                                       | Exploradores               | Tranquilo                  | Cerro Castillo            | Coyhaique                  | F-ratio    |
|---------------------------------------|----------------------------|----------------------------|---------------------------|----------------------------|------------|
| Annual precipitation (mm)             | 3000 <sup>i</sup>          | 1000 <sup>iii</sup>        | 684 <sup>iii</sup>        | 1032 <sup>iv</sup>         |            |
| Mean temperature (°C)                 | 7.5 <sup>ii</sup>          | 6.5 <sup>iii</sup>         | 9.9 <sup>ii</sup>         | 7.24 <sup>ii</sup>         |            |
| <i>Soil characteristics</i>           |                            |                            |                           |                            |            |
| Olsen-P (mg kg <sup>-1</sup> )        | 2.55 (0.06) <sup>a</sup>   | 25.16 (2.10) <sup>b</sup>  | 2.22 (0.14) <sup>a</sup>  | 12.21 (1.64) <sup>c</sup>  | 203.30**** |
| sorbed-P (%)                          | 26.83 (1.58) <sup>a</sup>  | 31.83 (2.90) <sup>a</sup>  | 63.69 (0.59) <sup>b</sup> | 71.49 (1.31) <sup>b</sup>  | 10.61**    |
| organic matter (%)                    | 1.57 (0.28) <sup>a</sup>   | 45.64 (2.23) <sup>b</sup>  | 0.77 (0.09) <sup>c</sup>  | 5.07 (0.85) <sup>d</sup>   | 192.48**** |
| total nitrogen (N) (%)                | 0.05 (0.00) <sup>a</sup>   | 0.26 (0.02) <sup>b</sup>   | 0.04 (0.00) <sup>a</sup>  | 0.20 (0.04) <sup>b</sup>   | 47.56****  |
| pH                                    | 5.62 (0.04) <sup>a</sup>   | 4.28 (0.09) <sup>b</sup>   | 6.17 (0.02) <sup>c</sup>  | 6.42 (0.05) <sup>d</sup>   | 141.65**** |
| C/N                                   | 10.46 (1.28) <sup>a</sup>  | 280.1 (43.25) <sup>b</sup> | 9.68 (0.80) <sup>c</sup>  | 15.05 (0.40) <sup>c</sup>  | 96.81****  |
| <i>Small seedling characteristics</i> |                            |                            |                           |                            |            |
| biomass (g)                           | 0.09 (0.00) <sup>a</sup>   | 0.12 (0.01) <sup>b</sup>   | 0.07 (0.01) <sup>a</sup>  | 0.09 (0.01) <sup>ab</sup>  | 8.13****   |
| height (cm)                           | 3.52 (0.13) <sup>a</sup>   | 3.60 (0.16) <sup>a</sup>   | 2.83 (0.22) <sup>b</sup>  | 3.71 (0.26) <sup>ab</sup>  | 4.30**     |
| cluster-root number                   | 8.30 (0.58) <sup>a</sup>   | 4.70 (0.54) <sup>b</sup>   | 7.71 (0.79) <sup>a</sup>  | 5.21 (1.20) <sup>ab</sup>  | 7.40****   |
| cluster root: total root biomass      | 25.66 (1.56) <sup>a</sup>  | 9.38 (1.32) <sup>b</sup>   | 25.80 (2.51) <sup>a</sup> | 17.72 (3.53) <sup>ab</sup> | 20.44****  |
| cluster root: total plant biomass     | 9.25 (0.75) <sup>a</sup>   | 2.81 (0.39) <sup>b</sup>   | 9.28 (1.00) <sup>a</sup>  | 4.35 (0.71) <sup>b</sup>   | 23.10****  |
| <i>Large seedling characteristics</i> |                            |                            |                           |                            |            |
| biomass (g)                           | 0.55 (0.16) <sup>ab</sup>  | 0.27 (0.04) <sup>ab</sup>  | 0.23 (0.04) <sup>a</sup>  | 0.79 (0.26) <sup>b</sup>   | 3.15*      |
| height (cm)                           | 5.61 (0.44) <sup>a</sup>   | 5.51 (0.42) <sup>a</sup>   | 4.80 (0.47) <sup>a</sup>  | 8.52 (0.97) <sup>b</sup>   | 12.12****  |
| cluster-root number                   | 10.50 (1.29) <sup>ab</sup> | 12.36 (3.21) <sup>ab</sup> | 14.50 (2.29) <sup>a</sup> | 4.56 (1.02) <sup>b</sup>   | 4.39**     |
| cluster root: total root biomass      | 18.35 (3.18) <sup>a</sup>  | 9.08 (2.32) <sup>ab</sup>  | 13.93 (2.05) <sup>a</sup> | 3.89 (1.20) <sup>b</sup>   | 6.52****   |
| cluster root: total plant biomass     | 6.79 (1.32) <sup>a</sup>   | 4.35 (1.46) <sup>ab</sup>  | 6.10 (1.07) <sup>a</sup>  | 1.21 (0.33) <sup>b</sup>   | 4.76**     |

Note: C/N stands for carbon–nitrogen ratio. Mean temperature refers to annual average. i: Aniya et al. (2011); ii: records from 25 November 2010 to 4 October 2012 (F. Piper, unpublished data); iii: Dirección General de Aguas (Servicio Meteorológico Nacional); iv: Luebert and Plissock (2006).

(46°06'31" S, 72°13'48" W, 451 m a.s.l.) is located in the Rio Ibáñez basin, within the Reserva Nacional Cerro Castillo conservation area. The forest is mainly composed by *Nothofagus pumilio* (Poepp. & Endl.) Krasser, *N. antarctica*, and *N. dombeyi* (Mirb.) Oerst. Small patches of *E. coccineum* occupy the most open and steep areas. The soil here is derived from Aeolian volcanic–ash deposits. This area has been recurrently impacted by eruptions of the Hudson volcano, including the latest eruption of 1991, considered one of the two largest eruptions in the Andes during the 20th century (Naranjo and Stern, 1998). At this study site, several layers of ash depositions constitute the superficial soil horizon down to > 2 m deep. Seedlings of *E. coccineum* establish abundantly on bare soil but most die during the first year. The fourth site, Coyhaique, is located 2 km north of Coyhaique city within the Reserva Nacional Coyhaique conservation area (45°32'50" S, 72°02'53" W, 466 m a.s.l.). The soil here is derived from Aeolian volcanic–ash deposits, although, in contrast to Cerro Castillo, no recent deposition has occurred for approximately the past 150 yr. A second–growth, post–fire forest of *N. pumilio* dominates the stand (Fajardo and McIntire, 2010). Like in Cerro Castillo, there are some isolated patches of *E. coccineum* at this location.

**Sampling**—Sampling was carried out between mid–January and mid–March (late southern hemisphere summer) from 2010 to 2013. Fourteen to twenty-five 1–m<sup>2</sup> plots were randomly assigned along four east–west 100 m long transects at each site. Transects were laid out under full open conditions, except in Exploradores, where canopy openness was ca. 50%. At each plot, all seedlings found were carefully excavated with shovels, producing minimal root damage, then transferred into plastic bags, and sealed to avoid dehydration. They were transported during the same day to the laboratory. Both small and large seedlings were collected. We defined *small seedlings* as those seedlings that germinated during the spring immediately previous to sampling, i.e., < 6–months old. *Large seedlings* were all seedlings > 6–months old and < 40 cm tall. We distinguished between *small* and *large* seedlings by observing the presence of annual bud scars formed when buds break, and woody shoots (i.e., secondary growth), in the latter. Scars are very noticeable in *E. coccineum*. A total of 47, 54, 38, and 14 small seedlings, and 32, 11, 23, and 16 large seedlings were sampled in Exploradores, Tranquilo, Cerro Castillo, and Coyhaique, respectively. In addition, from every plot we collected ca. 2 kg of the upper 20 cm layer of soil for chemical analyses. For this, we removed vegetation, gravels, and litter from the ground surface and then collected soil with shovels and transferred the samples to plastic bags that were immediately sealed and maintained in

fresh conditions (shade, < 15°C air temperature) for circa 2 hours until they were placed in a refrigerator (3°C).

**Biomass analyses**—Seedlings were refrigerated one night and then they were processed in the laboratory (Centro de Investigación en Ecosistemas de la Patagonia, Coyhaique, Chile). For small seedlings, growth was evaluated as the seedling height and biomass at harvest time. For large seedlings we computed a height relative growth rate (RGRh) for the growing season, which was defined as

$$\text{RGRh} = \ln(\text{height}_t) - \ln(\text{height}_{t-1})$$

where  $\text{height}_t$  is the total height of large seedlings at harvest time and  $\text{height}_{t-1}$  is the seedling height of previous year. Seedlings were separated into stems, leaves, cluster roots, and non–cluster roots. For leaf area determination, all leaves of each seedling were displayed separately on a white sheet along with a reference square of known size (4 cm<sup>2</sup>), covered with a transparent glass, and photographed with a Sony DSC–S750 digital camera (Sony Corporation, Tokyo, Japan). Images were later processed for total projected leaf area calculation using the image–processing software SIGMAPROC 5.0 (Systat Software, Richmond, California, USA). Roots were gently washed and rinsed and the total number of cluster roots was recorded for each seedling. Cluster roots are easily recognizable in *E. coccineum* as they appear as fine, short, closely–spaced lateral roots along main root axes (Zúñiga-Feest et al., 2010) (Fig. 1). To quantify cluster roots, only the portion of main root axis that forms the cluster was included. All cluster roots were counted, irrespective of their developmental stage, as we were interested in the total number of cluster roots. Seedling tissues were dried at 70°C until constant weight and the dry mass was recorded. Cluster-root relative mass was calculated both as the proportion (in percentage) of total root dry-mass and total whole–plant dry mass.

**Chemical analyses**—Soil samples were kept at 3°C until shipped for chemical analyses for the determination of organic matter content, total N, pH, Olsen–P (as a surrogate of readily available P), and C/N ratio (Laboratorio de Suelos, Universidad de Concepción, Chillán, Chile). Olsen–P (g Kg<sup>-1</sup>) represents a measure of soil P availability. Olsen–P is a widely used method appropriate to quantify P availability in soils with neutral to basic pH, although in acidic volcanic soils it shows a good correlation with other methods (e.g., Bray–P) (Araos, 1969, 1971; Rojas, 1976). We additionally quantified sorbed–P (%),

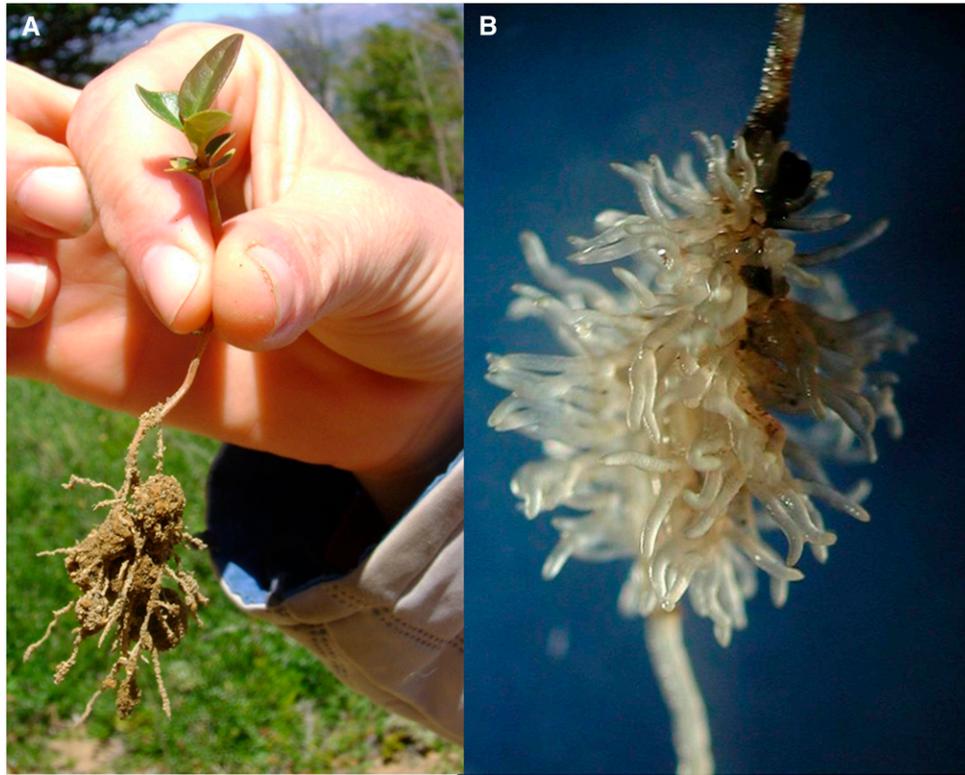


Fig. 1. Cluster roots in *Embotrium coccineum* (Proteaceae). (A) Small seedling (ca. 5 mo-old) collected in Reserva Nacional Cerro Castillo, Chile, with its noticeable cluster roots holding soil. (B) One young cluster root; notice that they are simple cluster roots (i.e., bottle-brush-like structures). Photographs: (A) by Frida Piper, (B) by Mabel Delgado.

which is thought to drive cluster-root formation and growth in volcanic soils (Lambers et al., 2012). The analysis of sorbed-P is based on the quantification of the P fraction that cannot be extracted with Olsen extractant (0.5 M NaHCO<sub>3</sub> pH 8.5) after the addition of 100 mg P kg<sup>-1</sup> (Vistoso et al., 2009). Both Olsen-P and sorbed-P are not necessarily correlated; low Olsen-P is found at different values of sorbed-P (e.g., Exploradores vs. Cerro Castillo, Table 1). Total N was analyzed by the direct nesslerization semi micro-Kjeldahl method (Middleton, 2007) and organic matter content was estimated by the rapid dichromate oxidation method (Walkley and Black, 1934).

Leaf inorganic phosphate (Pi) concentration was determined for all seedlings collected. This was done by combining all the leaves of a seedling, homogenizing 20 mg of leaf dry material in 1 ml 2% acetic acid, and digesting for 30 min before centrifuging at 6000 rpm (Fredeen et al., 1989). Leaf Pi concentrations were determined following the procedure of Murphy and Riley (1962) which, briefly, consists in the formation of an antimony-phosphomolybdate complex and its subsequent reduction to phosphomolybdenum blue, a bluish-purple color complex. The absorbance of this complex is read at 880 nm and converted into phosphate concentration using a calibration curve of potassium dihydrogen phosphate. Phosphate concentrations were expressed both in a leaf dry mass (leaf Pi<sub>mass</sub>) and area basis (leaf Pi<sub>area</sub>).

**Inference and statistical analyses**—Although many factors may influence cluster-root formation and growth in *E. coccineum*, based on previous studies and our own observations we identified four dominant factors that are biologically meaningful, i.e., soil P (in the form of Olsen-P and sorbed-P), soil N, leaf Pi, and seedling age. Since we require very precise predictions to maximize inference in this observational study (McIntire and Fajardo, 2009), we present a list of a priori biological hypotheses and expectations of the aforementioned factors influencing number of cluster roots per seedling (*CR*), cluster roots:total root biomass ratio per seedling (*CR/R*), and cluster roots:total plant biomass ratio per seedling (*CR/P*) (Table 2). The four main hypotheses are: (1) low soil Olsen-P and high sorbed-P increase the formation and growth of cluster roots (cluster roots are a direct functional response to soil P availability); (2) low leaf Pi, both expressed in a mass and an area basis, may either increase or decrease *CR*, *CR/R*, and *CR/P*, as it can be a cause/effect stimulus for cluster-root formation

and growth through up- and down-regulation mechanisms (Shane et al., 2003); (3) *CR/R* and *CR/P* are higher for small than for large seedlings given that they are the most likely to die because of poor soil-nutrient conditions, whereas *CR* is higher in large seedlings as it is a direct function of seedling size; and (4) low soil N increases *CR*, *CR/R*, and *CR/P* (cluster roots are a direct functional response of the plant to the low availability of N). As these hypotheses may not act in isolation, we combined them into more specific hypotheses that merge two or all factors under scrutiny, e.g., low soil P and seedling age but not low soil N may be the factors influencing the formation of cluster roots (Table 2). In the translation from biological hypotheses to statistical models we also tested the possibility of having linear (monotonic) and logarithmic (existence of a plateau) relationships between the number of cluster roots (and biomass ratios) and the factors hypothesized to affect their formation. To test these hypotheses, we consider the plot as our sampling unit, thus we averaged seedling attribute values and contrasted them with the soil datum at each plot.

When we analyzed the number of cluster roots per seedling (*CR*) (hypotheses 1, 3, and 4), we used generalized linear mixed-effect model regressions (GLMM) with a Poisson family and a log link function. We considered soil Olsen-P, soil sorbed-P, soil N, leaf Pi, and seedling age as fixed effects with site and plots nested within a site as random effects. For *CR/P* and *CR/R*, we similarly used GLMM but with a binomial family and logit link function because they represent ratios. We fitted GLMMs using lme4 (Bates and Maechler, 2009) in R version 2.15.3 (R Development Core Team, 2009). Thus, we fitted a suit of models in R, from which the most complex and synthetic model (Table 2) had the form of:

$$\text{full model, } \log(CR) = OP + sP + N + fP + A + s_k + \alpha_p,$$

where  $\log(CR)$  is the log link used for number of cluster roots found in a seedling; *OP* and *N* are soil Olsen-P and soil N concentration, respectively, which are expected to decrease *CR*; *fP* stands for leaf-Pi concentration, for which we do not have clear expectations (see above); *sP* is soil sorbed-P which is expected to increase *CR*; *A* is a categorical factor representing seedling ontogeny (small vs. large seedlings), where we expect *CR* will be lower in small than in large seedlings; *s<sub>k</sub>* and  $\alpha_p$  are random effects to control for site (*k* = 4 sites) and plots

TABLE 2. Description and word representation of a priori models concerning the effects of soil P (Olsen-P, *OP* and sorbed-P, *sP*), soil total N (*N*), leaf  $P_{\text{area}}$  (*fP*) concentrations, and seedling ontogeny (*A*, small or large) on the number of cluster roots per plant (*CR*) of seedlings of *Embothrium coccineum* (Proteaceae) at several forest sites in Patagonia, Chile. We used the same list of models for cluster roots:total root biomass per plant (*CR/R*), and cluster roots:total plant biomass per plant (*CR/P*) as response variables.

| Biological hypotheses   | Models   |
|---|--|
| ~1  | 1, random  |
| soil P  |  |
| <i>OP</i>   | 2, the lower the <i>OP</i> the higher the <i>CR</i> (linear relationship)  |
| log ( <i>OP</i> )   | 3, the lower the <i>OP</i> the higher the <i>CR</i> (logarithmic relationship)   |
| <i>sP</i>   | 4, the higher the <i>sP</i> the higher the <i>CR</i> (linear relationship)   |
| log ( <i>sP</i> )   | 5, the higher the <i>sP</i> the higher the <i>CR</i> (logarithmic relationship)  |
| soil N  |  |
| <i>N</i>  | 6, the lower the <i>N</i> the higher the <i>CR</i> (linear relationship)   |
| log ( <i>N</i> )  | 7, the lower the <i>N</i> the higher the <i>CR</i> (logarithmic relationship)  |
| soil P & N  |  |
| <i>OP</i> + <i>sP</i> + <i>N</i>  | 8, <i>CR</i> decreases linearly with soil <i>OP</i> and <i>N</i> , and increases linearly with <i>sP</i>   |
| log ( <i>OP</i> + <i>sP</i> + <i>N</i> )                                | 9, <i>CR</i> decreases logarithmically with <i>OP</i> , and increases logarithmically with <i>sP</i>   |
| foliar P  |  |
| <i>fP</i>   | 10, <i>CR</i> decreases or increases linearly with foliar <i>P</i>   |
| log ( <i>fP</i> )   | 11, <i>CR</i> decreases or increases logarithmically with foliar <i>P</i>  |
| soil & foliar P   |  |
| log ( <i>OP</i> + <i>sP</i> + <i>fP</i> )                               | 12, <i>CR</i> decreases logarithmically with <i>OP</i> , increases logarithmically with <i>sP</i> , and decreases or increases logarithmically with foliar <i>P</i>  |
| log ( <i>N</i> + <i>fP</i> )  | 13, <i>CR</i> decreases logarithmically with soil <i>N</i> , and decreases or increases logarithmically foliar <i>P</i>  |
| log ( <i>OP</i> + <i>sP</i> + <i>N</i> + <i>fP</i> )                    | 14, <i>CR</i> decreases logarithmically with <i>OP</i> and soil <i>N</i> , increases logarithmically with <i>sP</i> , and decreases or increases logarithmically with foliar <i>P</i>                                |
| ontogeny  |  |
| <i>A</i>  | 15, <i>CR</i> is higher in large than in small seedlings   |
| soil & ontogeny   |  |
| log ( <i>OP</i> + <i>sP</i> ) + <i>A</i>                                | 16, <i>CR</i> decreases logarithmically with <i>OP</i> and increases logarithmically with <i>sP</i> and linearly with seedling age   |
| log ( <i>N</i> ) + <i>A</i>   | 17, <i>CR</i> decreases logarithmically with soil <i>N</i> and increases linearly with seedling age  |
| log ( <i>OP</i> + <i>sP</i> + <i>N</i> ) + <i>A</i>                     | 18, <i>CR</i> decreases logarithmically with <i>OP</i> and soil <i>N</i> , and increases logarithmically with <i>sP</i> and linearly with seedling age   |
| foliar P & ontogeny   |  |
| log ( <i>fP</i> ) + <i>A</i>  | 19, <i>CR</i> increases linearly with seedling age, and decreases or increases logarithmically with foliar <i>P</i>  |
| soil & foliar P & ontogeny  |  |
| log ( <i>OP</i> + <i>sP</i> ) + log ( <i>fP</i> ) + <i>A</i>            | 20, <i>CR</i> decreases logarithmically with <i>OP</i> , increases logarithmically with <i>sP</i> and linearly with seedling age, and decreases or increases with foliar <i>P</i>                                    |
| log ( <i>N</i> ) + log ( <i>fP</i> ) + <i>A</i>                         | 21, <i>CR</i> decreases logarithmically with soil <i>N</i> , increases linearly with seedling age, and decreases or increases logarithmically with foliar <i>P</i>   |
| log ( <i>OP</i> + <i>sP</i> + <i>N</i> ) + log ( <i>fP</i> ) + <i>A</i> | 22, <i>CR</i> decreases logarithmically with <i>OP</i> and soil <i>N</i> , increases linearly with seedling age and logarithmically with <i>sP</i> , and decreases or increases logarithmically with foliar <i>P</i> |

(nested within sites, *p*) differences, respectively. For *CR/R* and *CR/P* the models were similar, except that *A* is predicted to have the opposite effect (higher *CR/R* and *CR/P* in small than in large seedlings). Finally, the suite of models was compared and assessed using an information-theoretic approach for model selection based on Akaike's Information Criterion corrected for small sample size ( $AIC_c$ ); the model with the lowest  $AIC_c$  was considered the best-fit model (Burnham and Anderson, 2002; Murtaugh, 2009). As there is commonly model selection uncertainty (i.e., more than one model is deemed equivalent), we also computed differences between individual models and the best model's  $AIC_c$  ( $\Delta AIC_c$ ), Akaike weights ( $w_i$ ), and the explained deviance of each model ( $R^2$ ).

To test if cluster roots compensate for potential decreases in leaf Pi driven by low soil P availability (hypothesis 2), we fitted a linear mixed-effects model (LMM) regression where we assessed the relationship between leaf-Pi concentration and soil Olsen-P, and if this relationship is mediated by seedling age. We assume that a lack of relationship between leaf-Pi concentration and soil Olsen-P (i.e., a decoupling of leaf Pi from soil P conditions) may be mediated by cluster roots. On the contrary, if the relationship is positive, i.e., the lower the Olsen-P the lower the leaf-Pi concentration, leaf and soil P coupling would suggest cluster roots have little to do with plant P status. In this case, Olsen-P and seedling age were the fixed factors and site and plots nested within sites were the random factors. We assessed *p*-values for main factors and interaction effects (ANOVA function on lme object in R). Finally, relative height growth was compared between small and large seedlings using LMM, with site and plot (nested into the site) as random factors; likewise, soil chemical properties and seedling growth were also compared among sites using LMM, with site as a fixed factor and plots as the random factor.

## RESULTS

Sites effectively differed in soil chemical properties, thus representing a natural fertility gradient (Table 1). A highly significant variation was found in Olsen-P concentration, which was ten times higher at Tranquilo than at Exploradores and Cerro Castillo, showing intermediate values at Coyhaique (Table 1). Sorbed-P concentration was in general very high at all sites ( $\geq 25\%$ ), although Cerro Castillo and Coyhaique were two-fold higher than Exploradores and Tranquilo. Similar to Olsen-P, organic matter content, total N, and C/N ratio were higher at Tranquilo and very low at Cerro Castillo and Exploradores. Cerro Castillo, however, had lower organic matter content and C/N ratio than Exploradores, while Tranquilo and Coyhaique had similar values of total N (Table 1). All sites showed relatively low pH values, with Tranquilo exhibiting the lowest. While seedling biomass was highest in Tranquilo and Coyhaique for small and large seedlings, respectively (Table 1), it was lowest in Cerro Castillo for both small and large seedlings (Table 1). The lowest seedling height was found in Cerro Castillo. Finally, seedlings of Exploradores and Cerro Castillo showed the highest cluster-root number and relative mass (Table 1).

All the proposed statistical models were significantly different from the random-only model (model 1), meaning that stochastic processes are not the main determinants of the number and mass of cluster roots in *E. coccineum*. We found that models with leaf  $Pi_{area}$  showed a much better fit than models with leaf  $Pi_{mass}$ , thus we continued working with the former in the subsequent models. According to the lowest  $AIC_c$  and  $\Delta AIC_c$  ( $\leq 2$ ), we found support for model 21 to be the best predictor for  $CR$  (Table 3). Model 21, which describes the variability in the data quite well ( $R^2 = 0.91$ ), is essentially the combination of model 13 ( $N, fP$ ) and 15 ( $A$ ), and it does not contain any soil  $P$  component. In more detail, it indicates that the number of cluster roots decreases significantly and logarithmically with soil  $N$  concentration, increases logarithmically with  $fP$ , and is significantly lower ( $b = 0.574$ , Table 4) for small (mean = 6.6) than for large seedlings (mean = 10.71) (Figs. 2, 3). For  $CR/R$ , we found strong support for model 22, which is the full model containing all components, with a high deviance explained ( $R^2 = 0.81$ , Table 3). In this case, soil  $N$  and foliar  $P$  ( $fP$ ) showed a significantly negative and logarithmic relationship with  $CR/R$ . Also,  $CR/R$  was found to decrease significantly with  $A$  ( $b = -0.289$ , Table 4), being higher for small seedlings than for large seedlings (Fig. 3). Although  $OP$  and  $sP$  are essential components of the model and  $CR/R$  decreases logarithmically with  $OP$  and increases logarithmically with  $sP$  (hypothesis 1), these relationships were not significant ( $p$ -value  $> 0.05$ ). Finally, when fitting models for  $CR/P$ , we found support for model 14 and model 22 ( $\Delta AIC_c \leq 1$ ); basically, model 14 differs from model 22 in that it does not include the seedling age ( $A$ ) component; the model robustness did not improve much when  $A$  was added (Table 2). The model fairly described the variation in the data ( $R^2 = 0.47$ , Table 3). Again,  $N$  and  $fP$  correlated significantly with  $CR/P$  in a negative and logarithmic way (Table 4), while neither  $OP$  or  $sP$  significantly influenced  $CR/P$ . Overall, small seedlings grew significantly faster than large seedlings (1.18 vs. 0.64 cm.cm<sup>-1</sup>, for small and large seedlings, respectively;  $F$ -value = 76.5;  $p$ -value  $< 0.001$ ).

In general, we found a positive and significant relationship between leaf  $Pi_{mass}$  and soil Olsen-P ( $F = 5.57$ ;  $P = 0.021$ ). Small seedlings had, in average, higher leaf  $Pi_{mass}$  than large seedlings ( $F = 4.11$ ;  $P = 0.044$ ). The interaction term between Olsen-P and seedling age was also significant ( $F = 7.70$ ;  $P = 0.006$ ). We therefore run the analysis for each seedling age

separately and found out that the relationship between leaf  $Pi_{mass}$  and soil Olsen-P was significant only in large seedlings (Fig. 4). Leaf  $Pi_{area}$ , in contrast, was not related to soil Olsen-P ( $F = 0.84$ ;  $P = 0.41$ ) and was similar between small and large seedling age ( $F = 1.39$ ;  $P = 0.24$ ). There was no significant interaction between Olsen-P and seedling age on leaf  $Pi_{area}$  either ( $F = 0.46$ ;  $P = 0.53$ ).

## DISCUSSION

**Cluster roots and soil P availability**—Cluster-root formation in *E. coccineum* seedlings did not relate to soil P availability across contrasting sites in Patagonia, Chile. Our statement is based on two facts: first, the best-fit model accounting for cluster-root number per seedling did not include Olsen-P or sorbed-P as explanatory variables, but did include soil total N, leaf-Pi concentration and seedling age (Table 3); and second, although models accounting for allocation of mass to cluster roots ( $CR/R$ ,  $CR/P$ ) did improve their predictive power when Olsen-P and sorbed-P were considered, these variables did not have significant effects on the mass proportion of cluster roots (Tables 3, 4). In general, these findings were quite unexpected, knowing that low soil P availability is a major driver for cluster-root formation and growth in Proteaceae species of other regions, i.e., Australia and South Africa (Lamont, 1972; Lamont et al., 1984; Shane et al., 2003). Although our findings are in agreement with previous studies in *E. coccineum* that found no relationship between cluster-root number and substrate P availability (Zúñiga-Feest et al., 2010; Donoso-Nanculao et al., 2011), ours is more general because we did consider a wider range of P availability and quantified the total cluster-root number in seedlings that germinated and emerged in situ. For recent germinated seedlings, growth is highly dependent on soil P because of the need for rapid cell-division and expansion during the heterotrophic-autotrophic transition, along with a low root-exploratory volume (Reich and Schoettle, 1988; Thomson et al., 1992). This necessity should be especially important in medium-seeded species like *E. coccineum* (Grubb et al., 2013), where nutrient provision from seed storage can be expected to last a few days (Swanborough and Westoby, 1996). By including early seedling growth, therefore, we probably included the ontogenetic stage where cluster-root formation should be highest. In addition, our study considered a wide range of soil P availability (10-fold in Olsen-P across sites, Table 1), similar to the range of the species distribution (Souto et al., 2009), hence reflecting the maximal intraspecific variation in cluster-root number and mass under natural conditions. Thus, we suggest that soil P availability does not influence the formation or growth of cluster roots in *E. coccineum*.

Soil total N concentration was the only soil factor accounting for cluster-root number and relative mass. In particular, seedlings of *Embothrium coccineum* tended to have more cluster roots and invest more biomass in cluster roots (relative to root mass and to whole-plant mass) when soil N concentration decreased (Table 4, Fig. 2). Overall, these results are consistent with the hypothesized role of cluster roots as an adaptation to survive under extremely poor soil conditions (Neumann and Martinoia, 2002). Increased cluster-root formation in response to low soil N availability has been observed in species of the genus *Hakea* (Proteaceae) (Lamont, 1972), where cluster roots could be facilitating N mobilization and acquisition from organic sources through exudation of proteolytic enzymes

TABLE 3. Top three best-fit models predicting number of cluster roots per seedling ( $CR$ ), cluster root:total root biomass ratio per seedling ( $CR/R$ ), and cluster root:total biomass per seedling ( $CR/P$ ) of *Embothrium coccineum* across several sites in Patagonia, Chile.

|        | model | Parameters         | $AIC_c$ | $\Delta AIC_c$ | $w_i$ | $R^2$ |
|--------|-------|--------------------|---------|----------------|-------|-------|
| $CR$   | 21    | $N, fP, A$         | 671.57  | 0              | 1     | 0.91  |
|        | 22    | $OP, sP, N, fP, A$ | 676.79  | 5.21           | 0.07  | 0.07  |
|        | 20    | $OP, sP, fP, A$    | 679.48  | 7.89           | 0.02  | 0.02  |
| $CR/R$ | 22    | $OP, sP, N, fP, A$ | 1342.39 | 0              | 1     | 0.81  |
|        | 20    | $OP, sP, fP, A$    | 1345.28 | 2.89           | 0.24  | 0.19  |
|        | 21    | $N, fP, A$         | 1353.19 | 10.79          | 0.01  | 0.01  |
| $CR/P$ | 14    | $OP, sP, N, fP$    | 601.68  | 0              | 1     | 0.47  |
|        | 22    | $OP, sP, N, fP, A$ | 602.19  | 0.52           | 0.77  | 0.36  |
|        | 12    | $OP, sP, fP$       | 604.89  | 3.21           | 0.20  | 0.09  |

Notes:  $AIC_c$  = Akaike Information Criterion corrected for small sample size;  $\Delta AIC_c$  =  $AIC_c$  differences between individual models and the best model;  $w_i$  = Akaike weights;  $R^2$  = explained deviance.

TABLE 4. Estimated slope parameters (1 SE) for the best-fit models predicting number of cluster roots per seedling ( $CR$ ), cluster root:total root biomass ratio per seedling ( $CR/R$ ), and cluster root:total biomass ratio per seedling ( $CR/P$ ) of *Embothrium coccineum* across several sites in Patagonia, Chile. \*, \*\*, \*\*\* indicate significant differences at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively.

| parameters | $CR$              |  | $CR/R$            |  | $CR/P$           |                  |
|------------|-------------------|--|-------------------|--|------------------|------------------|
|            | model 21          |  | model 22          |  | model 14         | model 22         |
| $OP$       |                   |  | -0.127 (0.223)    |  | -0.200 (0.182)   | -0.194 (0.173)   |
| $sP$       |                   |  | -0.111 (0.132)    |  | -0.079 (0.110)   | -0.077 (0.106)   |
| $N$        | -0.451 (0.103)*** |  | -0.594 (0.238)*   |  | -0.518 (0.201)** | -0.528 (0.192)** |
| $jP$       | 0.037 (0.064)     |  | -0.254 (0.048)*** |  | -0.191 (0.075)*  | -0.190 (0.075)*  |
| $A$        | 0.574 (0.063)***  |  | -0.289 (0.054)*** |  |                  | -0.106 (0.081)   |

(Schmidt et al., 2003; Paungfoo-Lonhienne et al., 2008, 2009). Our study is the first one showing that N is a major external factor controlling both the formation and growth of cluster roots in a South American Proteaceae species. The effect of N appeared to be strong and consistent across sites, irrespective of variation in climate or other soil-chemical properties. For example, precipitation and sorbed-P differed two and four times, respectively, between Exploradores and Cerro Castillo sites (Table 1), however both sites had low soil N concentrations and thus a high cluster-root number was predicted (and observed, Table 1) for both sites. Whether cluster roots of *E. coccineum* or other species of South American Proteaceae increase N-acquisition, as observed for Australian species, is unknown, although previous studies seem to be consistent with this idea. For example, in an examination of the distribution of *E. coccineum* from 37° to 55° SL across sites of contrasting fertility, soils were found to have < 0.15% of total N in 7 out of 26 populations (Souto et al., 2009), suggesting that the species occurrence in soils with low N concentration is quite common. Noteworthy, a multispecies field study found that estimated annual N losses in leaf litter of *E. coccineum* are much higher than any other of the 10 tree species examined (Lusk and Contreras, 1999). Similarly, *Lomatia hirsuta*, another South American Proteaceae species, occupies soils with the lowest total soil N concentrations when compared to other tree species of the region, and yet, it notably produces the richest N leaf-litter (Diehl et al., 2003; Satti et al., 2003). We acknowledge, however, that

*E. coccineum* distributes as well in sites accounting for higher soil N concentrations than those found in our sites (Souto et al., 2009; Zúñiga-Feest et al., 2010). Thus, the predictive capacity of our results may be restricted to overall N-poor soils.

The negative logarithmic relationship between cluster-root number (and mass) and soil N found in our study could have some implications at the community-level. The decrease in the number of cluster roots per seedling with soil N concentration was pronounced (multiplicative or geometric but not additive) at low values of N (between 0.02% and 0.20%, Fig. 2) but then tended to be constant with higher N concentrations, reaching a minimum plateau at higher values of N (0.4%, Fig. 2). This pattern illustrates that the formation and growth of cluster roots is highly sensitive to very low levels of soil N (small changes in N triggers large changes in cluster-root number and mass), but as long as the soil becomes richer in N, the trait's expression falls quickly. We interpreted this trend in an economic context. Cluster roots occur at the expense of aerial growth because they necessarily entail energy costs for the plant (Lambers et al., 2008). When soil fertility limits plant performance, cluster roots become very advantageous, as increased nutrient acquisition pays back any cost involved in their formation. However, when soil N concentration increases, cluster roots may not be essential as this nutrient could be taken by non-cluster roots, which are thought to be cheaper (Lambers et al., 2008). In Cerro Castillo, for example, which was the site with the lowest soil N concentration, seedlings had a high cluster-root number and relative mass, but a low biomass and height when compared to seedlings from other sites (Table 1). Finally, the fact that cluster roots do not totally disappear at high soil N concentrations may suggest that they represent a constitutive (i.e., always present) trait. This constitutive quality may in part account for the general tradeoff between surviving in extremely limited conditions and growing fast. Most Proteaceae species are pioneers in disturbed areas characterized by poor soil nutrition and high exposure (e.g., young volcanic soils), and yet they become less abundant species in late-successional mature forests where traits maximizing light acquisition are crucial.

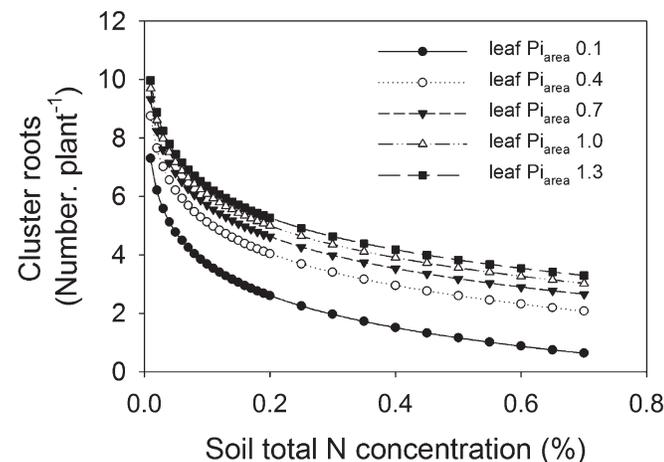


Fig. 2. Predicted number of cluster roots in small seedlings of *Embothrium coccineum* based on variation in soil total N concentration and leaf  $Pi_{area}$ . Predictions were made using a logarithmic regression model (model 21, Table 4). Only model for small seedling is shown.

**Leaf and soil P are decoupled**—In plant communities under natural conditions it is expected that both leaf P content and soil P availability are coupled (Austin and Vitousek, 1998; Ordoñez et al., 2009). Our study suggests that cluster roots may mediate a decoupling between leaf  $Pi_{area}$  concentration and Olsen-P, particularly in small seedlings. This is indicated by three facts: 1) the strong relationships between number (positive) and relative mass (negative) of cluster roots and leaf  $Pi_{area}$  (Table 4); 2) the higher mass proportion (at root and seedling level) of cluster roots in small than in large seedlings (Fig. 3); and 3) the absence of any relationship of leaf  $Pi_{mass}$  and leaf

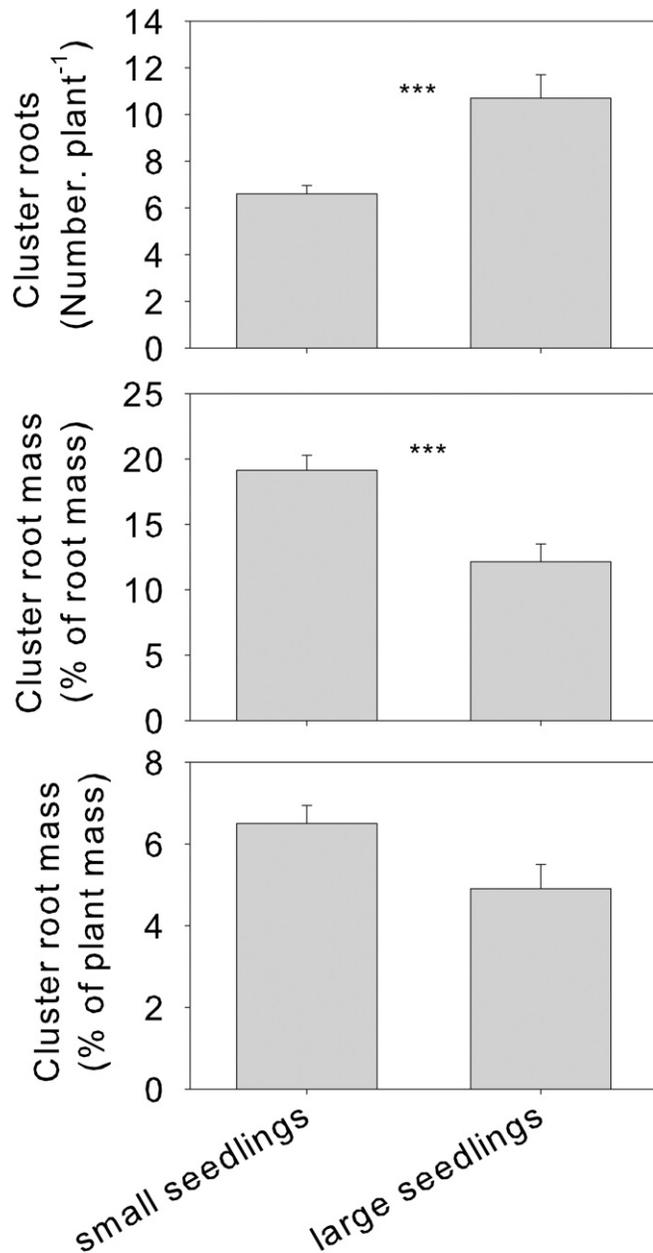


Fig. 3. Number and relative mass of cluster roots in small (< 6-mo old) and large seedlings (> 6-mo old) of *Embotrium coccineum* in Patagonia, Chile. Mean values and standard errors are indicated. Asterisks indicate significant ( $P < 0.05$ ) differences between age classes (Table 4).

$Pi_{area}$  with Olsen-P concentrations in small seedlings (Fig. 4). In contrast, large seedlings with relatively lower biomass investment in cluster roots than small seedlings (Fig. 3) had leaf  $Pi_{mass}$  concentrations closely linked to soil P availability (i.e., significant relationship). The compensation for low soil P availability in small seedlings of *E. coccineum* could be explained by an increase in P-acquisition mediated by cluster roots or by supplements provided from P stored in the seeds. Seed storage is important during early stages of seedling establishment as it can provide the resources to plant growth when they are not available in the environment (Muller-Landau, 2010). We

believe, however, that seed storage unlikely explained the leaf Pi compensation in small seedlings of *E. coccineum* because this is not a particularly large-seeded species (Grubb et al., 2013), and because their seed P concentrations are overall low (0.3–0.5%, M. Delgado, Universidad de la Frontera, unpublished data). Furthermore, as *E. coccineum* germinates in early spring, the small seedlings we sampled in late summer were older than 4 months. Therefore, irrespective of how much P was provided from the seeds, the period for small seedlings to depend on seed storage was small regarding the autotrophic period, and so any initial nutrient supply from the seed should have been “diluted” during the following months. On the other hand, the thesis of cluster roots increasing P-acquisition seems more plausible. This cluster-root function has been documented in Australian and South African Proteaceae species (Lamont et al., 1984; Shane et al., 2003; Lambers et al., 2006), and recently it has also been hypothesized to occur in the South American Proteaceae (Lambers et al., 2012). We found that the cluster-root relative mass decreased with leaf  $Pi_{area}$ , suggesting that low P status stimulated cluster-root growth (Shane et al., 2003). Cluster-root number, however, related positively to leaf  $Pi_{area}$ . The apparent inconsistency between these two results can be explained by the way as we quantified cluster-root number (i.e., total number). A decrease of cluster-root number with leaf Pi implies an increase of the total cluster-root number—a surrogate of accumulated cluster-root formation. In addition, it is possible that the up-regulation of leaf Pi mediated by cluster-root number occurred earlier than that mediated by cluster-root mass (e.g., Shane et al., 2003). In large seedlings, the similar leaf  $Pi_{area}$  (but not leaf  $Pi_{mass}$ ) across a range of Olsen-P values suggests that cluster roots alone were not sufficient to compensate for leaf  $Pi_{mass}$  concentrations, but that cluster roots combined with adjustments in leaf structure were effective to compensate for potential reductions in leaf  $Pi_{area}$  concentration.

**Seedling ontogeny**—We found support for the hypothesis that biomass investment in cluster roots is higher in small, < 6-mo old seedlings, than in large seedlings (Fig. 3). This result illustrates a key ecological role of cluster roots during seedling establishment (e.g., the first six months of life) of *Embotrium coccineum* that can be associated with its successional status and seed size. *Embotrium coccineum* is a medium-seeded, early successional species (Grubb et al., 2013), thus, small seedlings with their short period of nutrient provision from seeds and their small root exploratory capacity, must be able to meet P-demands of survival and growth in poor soils. In addition, we found that small seedlings grew faster than large ones, suggesting that the rate of P-acquisition was higher in small than in large seedlings. We hypothesized that cluster roots increased nutrient acquisition of small seedlings and hence paid back the costs involved in their formation and growth. Larger seedlings, in contrast, have larger root mass that allows a higher soil exploration for nutrients. They also might benefit from the litter produced from their leaves, upon decomposition and mineralization (Lambers et al., 2012). Large seedlings, on the other hand, with their relatively lower biomass investment in cluster roots (Table 2) could account for greater allocation to aerial growth, which may be advantageous for light competition at later stages of life.

**Conclusions**—Under the field conditions examined in our study, we found that cluster-root formation and growth in

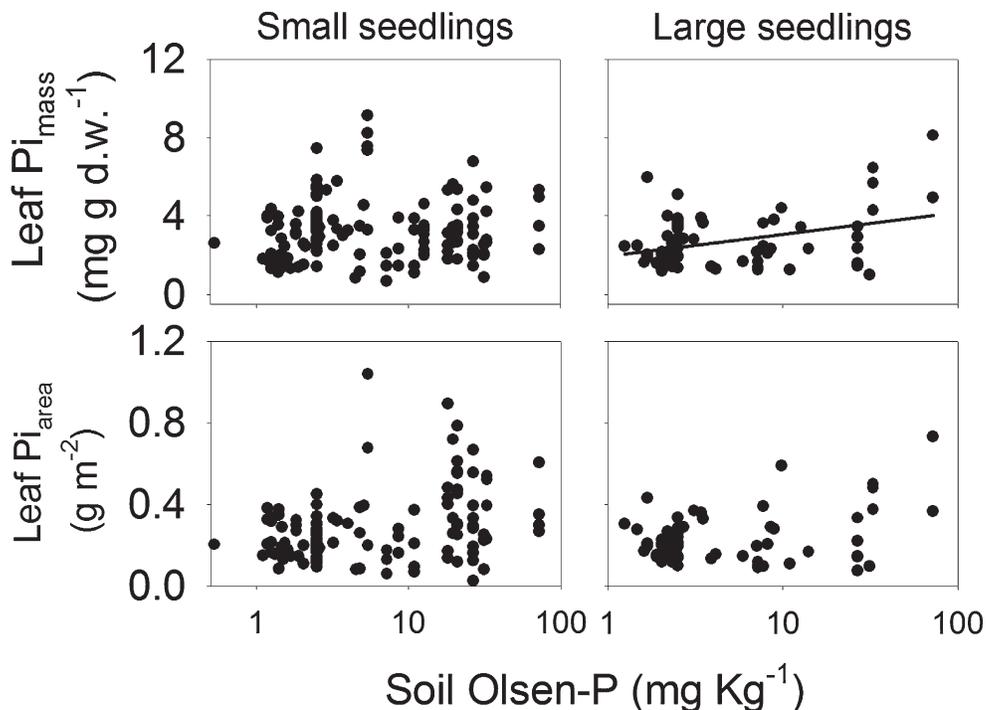


Fig. 4. Leaf Pi concentration on leaf mass and leaf area basis vs. soil Olsen-P concentration in small (< 6-mo old) and large (> 6-mo old) seedlings of *Embothrium coccineum* in Patagonia, Chile. The regression between leaf  $Pi_{mass}$  and Olsen-P was significant for large seedlings ( $R^2 = 0.56$ ;  $P = 0.012$ ) but not for small ones ( $R^2 = 0.13$ ;  $P = 0.600$ ).

*E. coccineum* were not stimulated by low soil P-availability but by low soil N concentrations. We also found that small seedlings invested more biomass in cluster roots than large seedlings did, and decoupled their leaf-Pi concentrations from soil P concentrations, suggesting that cluster roots prevented these seedlings from experiencing decreases in leaf P driven by low soil P-availability. Thus, our findings support a key role of cluster roots for seedling establishment through an increased P-acquisition, and suggest that cluster roots could also promote N-acquisition. Increased access to P and N in soils with poor nutritional status could have favored the persistence of cluster roots in Proteaceae in southern South America, since these types of soils are common in this region.

Although our study is a piece of evidence on the most probable soil factors promoting cluster-root formation in a southern South American Proteaceae species, further research is needed to explore the generality of our results and the potential influence of other factors. The very strong effect of soil N concentration on both the formation and growth of cluster roots found in this study suggests that this nutrient has a central role in the function of cluster roots. However, it is worth determining if low soil N concentration is also a driver of cluster-root formation in soils different to those examined here, where the species also occurs (e.g., with less or no volcanic material, like southern Patagonia or the Chilean Coastal Range). It cannot be discarded that the nil effect of soil P availability on cluster-root formation and growth that we found here was a consequence of N limitations occurring all over the soils we studied. Does low soil P availability trigger cluster-root formation under conditions of soil N concentration richer than those examined here? Finally, our results show that cluster roots mediate P-acquisition in small seedlings, and strongly suggest that cluster roots could be promoting

N-acquisition, as found in Australian Proteaceae species (Schmidt et al., 2003). This needs to be tested, and also whether leaf N and Pi concentrations (e.g., leaf N: P ratio) interact in the regulation of cluster-root formation.

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