REGULAR ARTICLE

Contrasting responses of cluster roots formation induced by phosphorus and nitrogen supply in *Embothrium coccineum* populations from different geographical origin

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Abstract

Aims Embothrium coccineum is a pioneer tree that produces cluster roots (CR) induced by phosphorus (P) or nitrogen (N) deficiency, but the role which both N and P play in CR formation for different *E. coccineum* populations is still unknown. We hypothesized that in *E. coccineum*, N influences the CR formation response, primarily induced by P limitation. However, this response depends on local adaptations to their specific conditions of geographic origin.

Methods Seedlings from three contrasting edaphoclimatic conditions (Northern, Central and Southern populations) were grown in hydroponic culture under high and low N supply and at five different P

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supply. Morphophysiological responses, including CR number, growth, biomass, P and N plant concentration, were studied.

Results Our results showed that at high N supply, CR formation was stimulated with increased P supply, but just until it reaches a maximum of 5 μM, above this concentration CR formation decreases. However, at low N supply, a constitutive response was observed regardless of P supply. Moreover, each of these responses depends on the populations.

Conclusions Our study suggests that N drives CR formation induced by P supply and that a differential response exists among *E. coccineum* populations, that could be related to their origin edaphoclimatic conditions.

Keywords Cluster root · Proteaceae · Nutrition · N/P · Geographic-origin · Ecotype · Local-adaptation

Abbreviations

Ca	Calcium
CR	Cluster Root
CR formation	CR number and CR/Total dry mass ratio
E. Suppl. Mat	Electronic Supplementary Material
GAM	Generalized additive models
HN	High Nitrogen
K	Potassium
LN	Low Nitrogen
Mg	Magnesium
N	Nitrogen
Na	Sodium
P	Phosphorous
RGRh	Relative growth rates for height

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Introduction

In environments with low P bioavailability, some plant species modify their root architecture by increasing lateral and root hair production and elongation, in order to improve soil exploration (Abel et al. 2002; Lambers et al. 2006; Richardson et al. 2011). Some species also form the so-called cluster roots (CR), which are ephemeral clusters of fine rootlets around a central axis (Purnell 1960; Lamont 2003). This morphological adaptation enhances the access to soil nutrients, specifically P and N, through the considerably higher exudation (than non-CR roots) (Roelofs et al. 2001; Delgado et al. 2013) of a range of compounds. These including inorganic and organic compounds, as carboxylates and phosphatases (Neumann and Martinoia 2002; Shane and Lambers 2005; Lambers et al. 2006; Shane et al. 2013) and proteases enzymes (Pate and Jeschke 1993; Schmidt et al. 2003). As a result, plants with CR can grow in low nutrients soils.

Except for the genus *Persoonia*, CR occurs in all species belonging to Proteaceae and similar root structures occur in species of other families (e.g. Fabaceae, Betulaceae, Casuarinaceae, among others) (Louis et al. 1990; Lamont 2003; Lambers et al. 2006; Maistry et al. 2015). Proteaceae family is a group of plants which predominantly inhabit the Southern hemisphere, and most species are found in South Western Australia and South Africa, being present on a variety of extreme oligotrophic habitats (Skene 1998; Lamont 2003; Lambers et al. 2008, 2012). In contrast, Proteaceae species from southern South America often grow on disturbed and more fertile landscapes that are rich in soil total P, but low in soil P availability, due to the volcanic origin of most of the soils (Lambers et al. 2012).

In recent years, it has been reported that CR of *Embotrium coccineum* (J.R. et G. Forster), a Proteaceae from southern South America, are induced by P deficiency when they grow under hydroponic P availability limited conditions (Delgado et al. 2013, 2014). An increase in soil P-Olsen and more labile forms of P in

the surrounding rhizosphere soil of *Orites myrtoidea* (Poepp. & Endl.) Benth. & Hook. F. ex B.D. Jacks and *E. coccineum* seedlings have been reported when CR is formed (Delgado et al. 2015; Ávila-Valdés et al. 2019). This suggests that CR probably allow solubilizing P from soils with a high-P sorption capacity in P limited environments. Controversially, it has also been reported that CR formation of *E. coccineum* may be induced by N and not by P limitation in natural conditions in Chilean Patagonia (Piper et al. 2013). To date, there is no clear understanding on how N and P interact and affect CR formation in southern South American Proteaceae, even though *E. coccineum* is one of the most studied Proteaceae in this zone (Zúñiga-Feest et al. 2010; Piper et al. 2013; Delgado et al. 2014). In general, this specie colonize extremely disturbed environments (e.g. young volcanic substrates) and can grow in a wide geographic distribution across southern latitudes (35–56° S) with variations in both soil chemical and climatic conditions. *E. coccineum* has a naturally patchy distribution in woodland scrub, typically occurring in gaps, open areas or along roadsides and it is rarely dominant wherever it occurs. (Rodríguez et al. 1983; Alberdi and Donoso 2004; Souto et al. 2009; Delgado et al. 2018).

Several studies have focused on analysing intraspecific variations of *E. coccineum* at both morphologic and genetic level. Morphological leaves comparisons and genetic-marker loci have demonstrated variations among populations distributed in a latitudinal gradient and some controversies have raised about how many genetic groups has this species (Souto and Premoli 2007; Souto et al. 2009; Vidal-Russell et al. 2011). Likewise, Zúñiga-Feest et al. (2015) reported a different morphological response to P limitation or P excess in CR formation of *E. coccineum* seedlings from different edaphoclimatic conditions. These authors found that CR formation in seedlings from Northern population (Curacautín, 38° S) suppressed by increasing P supply, whereas seedlings from Southern populations (Coyhaique, 45° S) not affected. These results strongly suggest an intraspecific variation among seedling of *E. coccineum* from different populations in terms of root adaptation related to origin edaphoclimatic conditions (Zúñiga-Feest et al. 2010, 2015). Until now, it is unclear if N supply and its interaction with P may differentially affect the morphophysiological responses of different *E. coccineum* populations.

Our study evaluated the importance of N and P in CR formation as well as the intraspecific variation of *E. coccineum* seedlings from different populations of contrasting latitudinal and soil-site origins. *E. coccineum* seedlings from three different populations were grown under different levels of N and P supply in a hydroponic system. Morphological (e.g. relative growth rate, shoot/root, CR number) and chemical (N and P plant concentration) responses were evaluated. Based on the evidence that seedlings from Southern population did not suppress CR formation when they were supplemented with P (Zúñiga-Feest et al. 2015) and that CR are induced by low N and not by low P (Piper et al. 2013) we expected that CR of seedlings from the Southern population would present higher levels of CR formation under low N supply compared to seedlings from Northern population. Similarly, in seedlings from Central population, P and N co-limitation would induce higher CR formation. Finally, we hypothesized that in *E. coccineum* seedlings, N strongly influences the morphological response induced by P limitation, but this response depends on the local adaptation of each population to their specific edaphoclimatic conditions.

Materials and methods

Sites of seeds collection and edaphoclimatic description

Embothrium coccineum seeds were collected from January to April 2016 from three populations with diverse edaphoclimatic conditions: *1)* the Northern population from Fundo Niagara, Curacautín, Araucanía Region (38°27′19.62"S - 71°44′12.84"W), *2)* the Central population from Katalapi park, Puerto Montt, Los Lagos Region (41°31′17.60"S - 72°45′15.30"W), and *3)* the Southern population from Torres del Paine, Magallanes and Antártica Chilena Region (51°22′42.86"S - 72°47′52.56"W). To characterize the seeds collection sites we considered seven bioclimatic variables: annual rainfall (mm), annual temperature (°C), minimum temperature (°C), maximum temperature (°C), elevation (m a. s. l), solar radiation (kJ m^{-2} day^{-1}), and wind speed (m s^{-1}) with a spatial resolution of 30 s (~1 km^2) and a representative of the period 1970–2000 for the analysis. All data was downloaded from current bioclimatic variables available at WorldClim Version2 (Fick and Hijmans 2017) (Table 1).

For each seed collection site, a minimum of three soil samples was collected at 0–20 cm depth and transported to the laboratory for further chemical analysis. Soil

samples were air-dried and homogenized for chemical measurements. Soil pH was determined in a 1:2.5 soil to solution ratio in water (Sadzawka et al. 2006). Soil N and P availability were determined by the Kjeldalh and the Olsen method, respectively (Kjeldahl 1883; Olsen and Sommers 1982). Potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) were measured by atomic absorption and emission AAS Spectrophotometer (A Analyst 400, PerkinElmer) after an ammonium acetate extraction as described in Sadzawka et al. (2006).

Experimental design and plant material

In June 2017, seeds were germinated with a pre-treatment in gibberellic acid, at 250 mg L^{-1} for 24 h and transferred to Petri dishes in a temperature-controlled growth chamber at 20–23 °C. After emergence and cotyledons development, seedlings were moved to 250 mL pots containing vermiculite, and grown for eight weeks watered with distilled water. Seedlings were grown at 20–23 °C and 16/8 h light/dark photoperiod (at 710 μmol m^{-2} s^{-1} of active photosynthetic radiation) in a temperature-controlled growth chamber.

Sixty seedlings per population with similar height (4 cm mean) were transferred from vermiculite to black plastic pots of 2 L (six plants per pot) in a hydroponic system with the following nutrient Hoagland solution (μM): 200 K_2SO_4; 54 $MgSO_4*7H_2O$; 20 KCl; 0.24 $MnSO_4$; 0.1 $ZnSO_4*7H_2O$; 0.018 $CuSO_4*5H_2O$; 2.4 H_3BO_3; 0.3 $Na_2MoO_4*2H_2O$; 10 Fe-EDTA (pH 5.6). Ca $(NO_3)_2*4H_2O$ and KH_2PO_4 were used as an N and P source, respectively. The seedlings were separated into two N supply levels: High N (HN: 200 μM) and low N (LN: 10 μM) and for each N level, five P supply concentrations were used: 0.5, 1, 5, 10 and 20 μM, in order to produce ten different nutritional conditions. The nutrient solutions were continuously aerated and replaced once a week. The pots were randomly distributed to avoid bordering effect. Plants were grown for seven weeks with the same temperature and light conditions described above.

Plant growth measurements and parameters analysis

Both height and biomass of *E. coccineum* seedlings was measured at the beginning and the end

Table 1 Edaphoclimatic conditions at natural habitats of *E. coccineum* locations. One-way ANOVA was applied with *post-hoc* Newman–Keuls test (level of significance *P value* < 0.05) of chemicals analysis. Each value corresponds to a mean of 3 samples

	Curacautín (Northern)	Puerto Montt (Central)	Torres del Paine (Southern)
Climatic parameters[a]			
Annual rainfall (mm)	2236	2032	529
Annual Temperature (°C)	9.76	11.06	6.91
Min. Temperature (°C)	−2.94	1.97	−3.02
Max. Temperature (°C)	21.56	19.43	16.16
Elevation (m a.s.l)	749	67	58
Solar radiation (kJ m^{-2} day^{-1})	2147	1562	1444
Wind speed (m s^{-1})	2.99	3.90	6.57
Soil chemical analysis			
N (mg kg^{-1})	79 a	91 a	25 b
P-Olsen (mg kg^{-1})	1.5 b	10.3 a	2.9 b
pH (H_2O)	5.3 b	5.3 b	5.8 a
K (mg kg^{-1})	156 a	122 a	63 b
Na (mg kg^{-1})	67 a	41 b	11 c
Ca (mg kg^{-1})	1073 a	1002 a	555 b
Mg (mg kg^{-1})	76 b	171 a	46 b

[a] Source: WorldClim Version2

of the experiment. Relative growth rate for height (RGRh) and weight (RGRw) were calculated as described previously (Barrow 1977): RGR = (ln X Final - ln X Initial) / (t Final - t Initial); where X = height (cm) or dry weight (mg) and t = time (days). At the end of the hydroponic experiment (seventh week) seedlings were dried at 65 °C for 48 h and weighed on an analytical balance (Biobase, Shandong, China) in order to determine the total dry weight. CR of each plant was categorized according to phenological stages (juvenile, mature and senescent) following criteria described by Delgado et al. (2013) and were counted to register the total CR number. The shoot/root ratio, CR/total plant dry biomass ratio, and dry weight of individual CR (mg) were also calculated. The concentration of N, P and C were analysed for seed collections and complete seedlings (leaf, stem and root) at the end of the experiments (Sadzawka et al. 2007) as described above.

Statistical analysis

Generalized additive models (GAMs, Hastie 2017) using a cubic spline were fitted in order to evaluate whether there is an effect of the P supply over different parameters related to seedling development from three different populations. GAMs were performed independently for each of the sites, but also combined for all the parameters analysed. Gaussian distribution with identity function was applied based on AIC criteria. All statistical tests and models were performed with a significance level of 0.05, using the R environment v.3.6.1. GAMs were fitted using the mgcv package in R.

A three-way ANOVA was conducted to determine the effects of populations (Northern, Central and Southern), N supply (HN, LN) and P supply (0.5, 1, 5, 10 and 20 μM) on morphological and physiological parameters. A Newman–Keuls test (level of significance *P value* < 0.05) was used as a *post-hoc* test in order to reveal significant differences among groups. Assumptions of normality and homogeneity of variance were tested using a Kolmogorov-Smirnov and Lilliefors test. A one-way ANOVA was applied with a *post-hoc* Newman–Keuls test (level of significance *P value* < 0.05) for soil and seeds chemical analysis. Analyses were performed using STATISTICA 6.0 software (Statsoft Inc.). Differences in the responses among populations for N and P treatments and the influence of edaphoclimatic conditions were visualized using a principal component analysis (PCA) with R 3.6.1 Statistics software ('devtools' and 'ggbiplot' packages).

Results

Edaphoclimatic characterization of the origin sites

Embothrium coccineum seeds were collected from three different sites with particular climatic conditions. (i) A Northern population (Curacautín), which is the site with the highest altitude (749 m.a.s.l.) and is exposed to higher solar radiation and rainfall rates in comparison with the other two populations (Table 1). (ii) A Central population (Puerto Montt), which is the site with the highest annual air temperature (11 °C) and (iii) a Southern population (Torres del Paine), the location with the lowest annual minimum and maximum temperature (−3 and 16 °C, respectively) and with the lowest rainfall rates, in comparison with the other sites, and that is also

regularly exposed to the highest wind velocity (6.57 m s^{-1}) (Table 1).

The soil samples from the studied sites showed significant differences in nutrient content (Newman-Keuls test, *P < 0.05* by population). Thus, soil samples from the Central population presented the highest values for both N and P soil concentration (91 and 10.3 mg kg^{-1}, respectively), while the samples from the Southern population showed the lowest values for N soil concentration (25 mg kg^{-1}) and low values for P soil concentration (2.9 mg kg^{-1}), similar to those obtained for the samples from the Northern population (1.5 mg kg-1). Regarding the concentrations of K, Na, Ca and Mg in soil, samples from the Northern and Central populations showed very similar values, in contrast to the samples from the Southern population that had significantly lower values for each of the ions analysed. Finally, the soil from the Southern population showed the highest pH values among the studied sites (Table 1).

Effect of N and P supply in growth parameters in seedlings from *Embothrium coccineum* populations

Seedlings from the three studied populations exhibited contrasting phenotypes under different N and P supply (Fig. 1a-l). Seedlings from the Central population showed a higher RGRh than Northern or Southern populations, independent of the N supply (Fig. 2a, b). However, only in the HN supply the P supply significantly favoured the growth of the seedlings from the Central population (0.2 cm cm^{-1} day^{-1} at 20 μM of P), but not in seedlings from both Northern and Southern populations (0.07 and 0.03 cm cm^{-1} day^{-1} at 20 μM of P, respectively) (Fig. 2a, Newman-Keuls test, *P < 0.05*, E. Suppl. Mat, Tables S1, S2). The statistical analysis reveals that the RGRh depends on the interaction with the N-P supply and the population (E. Suppl. Mat, Tables S1, S2).

According to biomass distribution (Figs. 2c–d, S1a-d), similarly to what was found in RGRh, only in the HN condition the P supply significantly favoured the shoot/root biomass ratio of the seedlings from the Central population (3.6 at 20 μM of P), but not in seedlings from both Northern and Southern populations (2 at 20 μM of P) (Fig. 2c, Newman-Keuls test, *P < 0.05*, E. Suppl. Mat, Tables S1, S2). There were no significant differences in the shoot/root biomass ratio among populations in the range of P supply under LN supply (Fig. 2d, Newman-Keuls test, *P < 0.05*, E. Suppl. Mat,
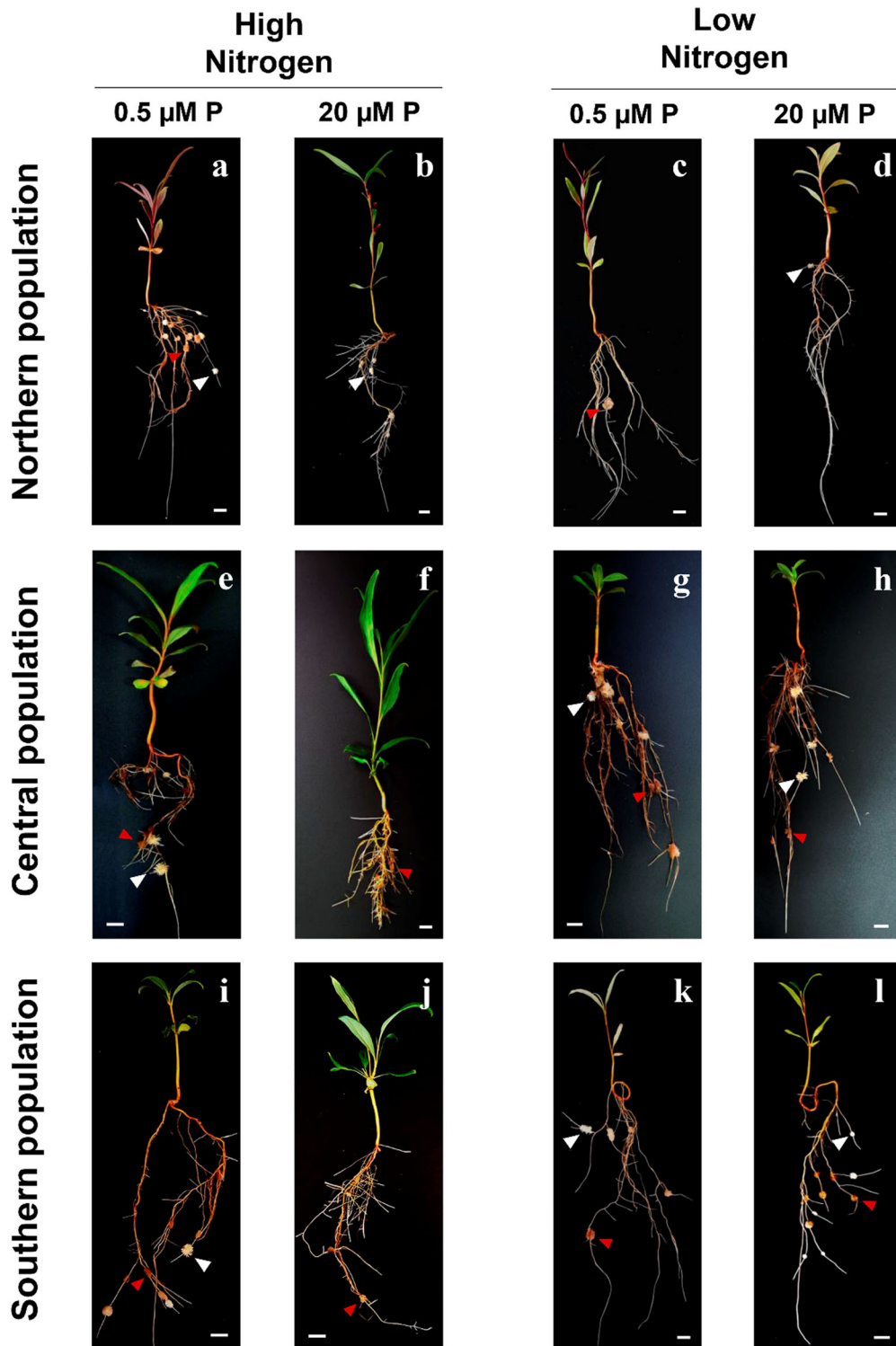
Fig. 1 *Embothrium coccineum* seedling phenotypes from three populations subjected to different nutritional treatments. Seedlings from Northern (**a**–**d**), Central (**e**–**h**) and Southern (**i**–**l**) populations were grown in hydroponic conditions and supplemented with 0.5 μM of P (**a**, **c**, **e**, **g**, **i** and **k**) and 20 μM of P (**b**, **d**, **f**, **h**, **j** and **l**) in high and low N (10 μM and 200 μM N, respectively). The white arrows indicate mature CR, whereas the red arrows indicate senescent CR
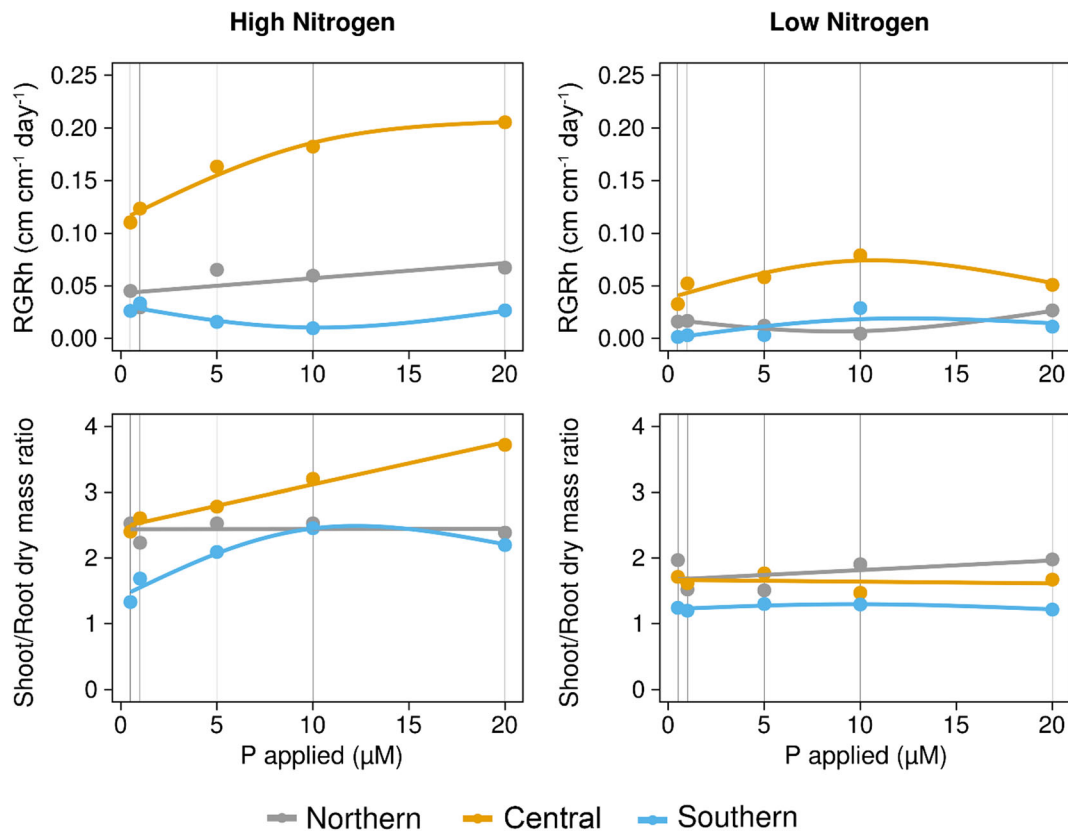
Fig. 2 **Effects of N and P supply on growth rate and biomass distribution of *Embothrium coccineum* seedlings from three populations. a, b** The relative growth rate in height (RGRh) and **c, d** Shoot/Root ratio in *E. coccineum* grown in hydroponic conditions in a factorial design, with 0.5, 1, 5, 10 and 20 μM of P supply, in a high N supply of 200 μM (**a–c**) and a low N supply of 10 μM (**b–d**). Each value corresponds to the mean of 6 samples. A generalized additive model using a cubic spline was fitted using the mgcv package in R environment. Each population is displayed as a different colour, where the Northern population is grey, Central population is orange, and the Southern population is blue. Significant interactions and regression parameters are indicated in E. Suppl. Mat, Table S1 and S2

Tables S1, S2). Overall, it seems that plants reallocated more resources to root at LN than in HN supply.

The CR formation of *Embothrium coccineum* populations under different N and P supply

A maximum of CR formation (CR number and CR / Total dry mass ratio) was observed when 5 μM of P was applied at the HN supply in seedlings from both Northern and Central populations (9 and 20 CR number, respectively). While, in the seedlings from the Southern population the increment in P supply had a negative impact in the CR formation (10 and 5 CR number at 5 and 20 μM of P, respectively) (Fig. 3a, c, Newman-Keuls test, *P < 0.05*, E. Suppl. Mat, Tables S1, S2). Under LN supply, no evident P-dependent changes were observed in seedling from both the Northern and

Southern populations (Fig. 3b, d, Newman-Keuls test, *P < 0.05*, E. Suppl. Mat, Tables S1, S2). As observed in RGRh, the CR formation was higher in Central population (15 CR number and 0.075 CR / Total dry mass ratio, on average) compared to both Northern (5 CR number and 0.025 CR / Total dry mass ratio, on average) and Southern (10 CR number and 0.05 CR / Total dry mass ratio, on average) populations in both N supplies (Fig. 3a–d). Overall, the CR formation depended on the interaction among populations, N and P supply (Fig. 3a–d; Newman-Keuls test, *P < 0.05*, E. Suppl. Mat, Tables S1, S2).

N and P plant concentration

A significant increase in plant N concentration was observed in seedling from both the Northern and Central
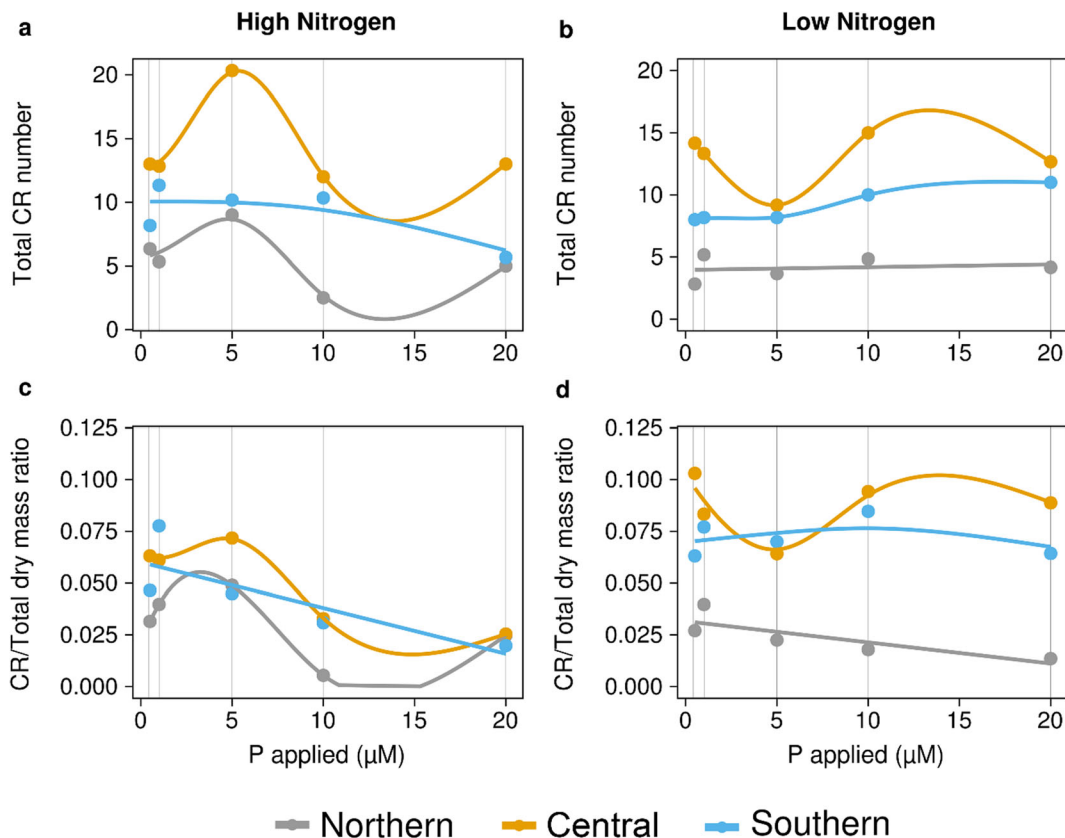
Fig. 3 Effects of N and P supply on CR parameters of *Embothrium coccineum* seedlings from three populations. **a**, **b** Total CR number and **c**, **d** CR/Total plant dry biomass ratio in *E. coccineum* grown in hydroponic conditions in a factorial design with 0.5, 1, 5, 10 and 20 μM of P supply, in a high N supply of 200 μM (**a–c**) and a low N supply of 10 μM (**b–d**). Each value corresponds to the mean of 6 samples. A generalized additive model using a cubic spline was fitted using the mgcv package in R environment. Each population is displayed as a different colour, where the Northern population is grey, Central population is orange, and the Southern population is blue. Significant interactions and regression parameters are indicated in E. Suppl. Mat, Table S1 and S2

populations when increasing P supply in the HN supply (8 to 18 mg g^{-1} at 0.5 to 20 μM of P) (Fig. 4a). Under LN supply, the P supply did not affect the plant N concentration in all seedlings analysed (8 mg g^{-1} on overage) (Fig. 4b). No significant differences in plant N concentration were observed among populations across the different P supply, except for the high P supply (9 mg g^{-1} for seedling from Northern population and 5 mg g^{-1} for seedling from both Central and Southern populations at 20 μM of P) (Fig. 4a, b, Newman-Keuls test, $P < 0.05$, E. Suppl. Mat, Tables S1, S2). At HN supply, a significant increase in plant P concentration was observed in seedlings from the three populations (0.5 to 1.8 mg g^{-1} at 0.5 to 20 μM of P) (Fig. 4c). However, under LN supply, only seedlings from the Southern population displayed a P-dependent increase in plant P concentration (0.5 to 2.5 mg g^{-1} at 0.5 to

20 μM of P) (Fig. 4d). Overall, the N and P plant concentrations in *E. coccineum* seedlings showed statistically significant interaction among populations, N and P supply (Fig. 4a–d, Newman-Keuls test, $P < 0.05$, E. Suppl. Mat, Tables S1, S2). To relate the morphological responses of *E. coccineum* seedlings from three populations grown under different N and P supplies with plant and seed nutrient concentrations and edaphoclimatic conditions from each geographical origin, we carried out a PCA analysis. The total variation explained by the first principal component (PC1) and the second principal component (PC2) was 78% (41.0% for PC1 and 36.7% for PC2). In this analysis, PC1 clearly separated the three populations, while PC2 separated the Northern population from both the Central and Southern populations (E. Suppl. Mat, Fig. S2, Table S4).
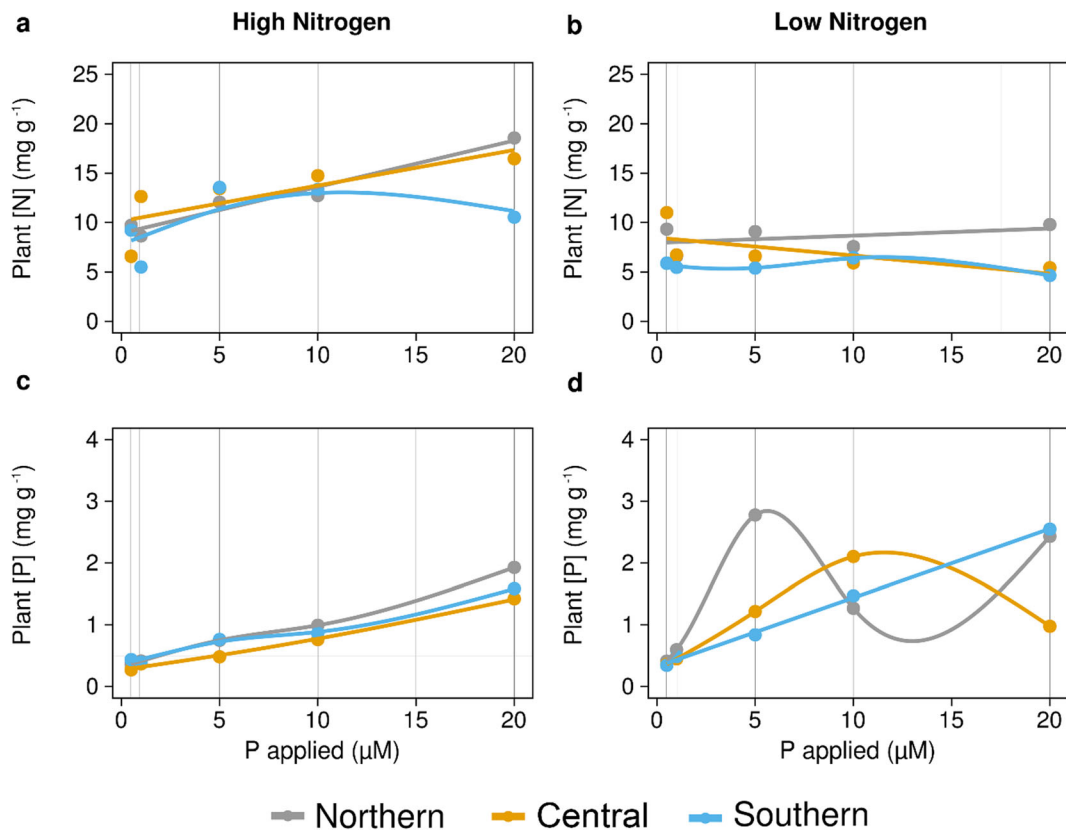
Fig. 4 Effects of N and P supply on both plant N and P concentration in *Embothrium coccineum* seedlings from three populations. **a**, **b** Plant N concentration and **c**, **d** plant P concentration in *E. coccineum* grown in hydroponic conditions in a factorial design with 0.5, 1, 5, 10 and 20 μM of P supply, in a high N supply of 200 μM (**a**–**c**) and a low N supply of 10 μM (**b**–**d**). Each value corresponds to the mean of 6 samples. A generalized additive model using a cubic spline was fitted using the mgcv package in R environment. Each population is displayed as a different colour, where the Northern population is grey, Central population is orange, and the Southern population is blue. Significant interactions and regression parameters are indicated in E. Suppl. Mat, Table S1 and S2

Discussion

Previous studies have reported that CR formation in *E. coccineum* is induced by N and P limitation, even though these studies have only evaluated each nutrient separately (Delgado et al. 2013; Piper et al. 2013). Our results confirmed the essential effect of N, not only on growth but also on CR formation and P acquisition. However, we observed that these responses differed among seedling from different populations, responses that could be related to the edaphoclimatic conditions from the regions of their origin.

N orchestrate the CR formation induced by P limitation

Considering the phenotypic outcomes, we found that N supply is an essential nutrient for the growth and CR

formation of *E. coccineum,* even more than P supply (Figs. 1, 2, 3). When we compared a similar P supply, significant differences between HN and LN supply were observed in seedlings from the three populations (Figs. 2, 3, E. Suppl. Mat, Tables S1, S2). Under LN supply, the tendency was to produce smaller plants with larger roots and bigger CR, especially in both Central and Southern populations (Fig. 3b-d, E. Suppl. Mat, Fig. S1a-d). On the contrary, it seems that in species of the Proteaceae family, N supply does not have a stimulating effect on CR formation. For example, increasing N supply (200 to 5000 μM) did not induce a response in root formation of *Hakea prostrata* (Prodhan et al. 2016).

Additionally, Piper et al. (2013) reported that under field conditions, the growth and CR formation in *E. coccineum* were correlated with low N soil concentrations. In general, under HN supply, an

increased CR formation was observed as they were supplied with intermediate P supply (5 μM of P). This result agrees with those findings by Shane et al. (2013), who reported that at both a deficient P supply (<1 μM of P) and at a high P supply (25 or 75 μM P) the CR formation was reduced or entirely suppressed, respectively (Shane et al. 2013). Our results showed that the optimal P supply for CR formation in *E. coccineum* is 5 μM of P, at least, when grown under HN supply in hydroponic conditions. Thus, we suggest that CR formation induced by low P supply in *E. coccineum* is a facultative response controlled by N availability.

As expected, P supply increased plant P concentration at HN supply but not at LN supply. Recently, it was reported the discovery of active control of P starvation responses by N signalling pathways in *Arabidopsis thaliana*, a phenomenon also conserved in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Medici et al. 2019). The mechanism involves the nitrate transceptor CHLORINA 1/NITRATE TRANSPORTER 1.1 (CHL1/NRT1.1) and the PHOSPHATE STARVATION RESPONSE 1/PHR1 transcription factor, serving as master regulators of crosstalk signalling for P sense. There is no knowledge about the expression of either N or P transporters in CR of *E. coccineum*, which therefore possess an interesting aspect for future research. In contrast, seedlings grown under low P supply and both LN and HN supply showed similar levels of N plant concentration (Fig. 4a–b). Similar results were reported for southwestern Australian Proteaceae, *Hakea prostrata*, in which low N plant concentration under low P supply was demonstrated. This study showed that *H. prostrata* tightly controls N acquisition and assimilation independently of N supply to the roots and that this tight control is an adaptive mechanism which plays a pivotal role in enabling this species to function in its low-P environment (Prodhan et al. 2016). Unlike the soils where the other species of Proteaceae family naturally occur (South Africa and Australia), the soils of Southern Chile are characterized by having high levels of P, but low P bioavailability (Borie and Rubio 2003) and in some areas low N level (Souto et al. 2009; Piper et al. 2013). Thus, our result suggests that *E. coccineum* developed a strategy where N supply could be orchestrating CR formation in response to low P supply, but additional studies are required to confirm this.

The geographical origin influences the growth, CR formation and N/P acquisition

Although the growth conditions for *E. coccineum* seedlings from the three populations was the same, we found differences in the response, in terms of relative growth rate in height (RGRh) and CR formation. Interestingly, the Southern population showed the lowest RGRh (Fig. 2a, b) and the higher seed P concentration (E. Suppl. Mat, Table S3), which could be related with their higher capacity of acquisition of P (through an increased P availability) in a site where the soil P availability is reduced (Table 1). In contrast, seedlings from the Central population showed the highest RGRh (Fig. 2a, b), but the lowest seed P concentration (E. Suppl. Mat, Table S2), despite the seeds came from the site with the higher P availability in the soil. Thus, considering the higher or lower capacity of P uptake from the soil and the seed P concentration, these plants can have a further capacity of P translocation to seeds to ensure germination of the next generation (Zúñiga-Feest et al. 2015; Delgado et al. 2018). More investigations about P use efficiency are necessary to understand this differential performance among populations.

Significant differences in the capacity to produce CR in function of P and N supply were founded among *E. coccineum* seedlings from the different populations (Fig. 3). As previously detailed, at HN supply seedling from both the Northern and Central populations showed a maximum of CR formation at 5 μM, while in the seedlings from the Southern population the CR formation remained constant, independent of the amount of P supplied. Interestingly, under LN supply, the CR formation of seedlings from both the Central and Northern populations have a differential response when compared to the Southern population, that maintains the trend shown at HN supply. In cold environments, such as those from the Southern population, low soil temperatures limit the mineralization of the organic matter, thus, affecting the nutrient availability, especially N (Table 1). Under these harsh conditions, plants should be very efficient in nutrient acquisition and utilization. Indeed, Delgado et al. (2018) reported that *E. coccineum* plants from the southernmost population (55.22 °S, the same Southern population used in our study) had the highest P and N resorption efficiency compared with Northern populations. A similar response was reported before by Zúñiga-Feest et al. 2015 who showed that in *E. coccineum* seedlings from Curacautín (the Northern

population), the CR formation was suppressed when increasing P supply, whereas, the seedlings from Coyhaique (another Southern population) were not. Thus, it is proposed that plants from regions subject to frequent disturbances due to volcanic activity (e.g. Northern and Central populations, with 2 to 36 and 10 volcanic eruptions in one hundred years, respectively) (Dzierma and Wehrmann 2010, 2012), could respond faster to changes in nutrient availability than plants from soil buffered conditions (i.e. the Southern population, with lower volcanic influence).

Empirical evidence suggests that in peripheral populations (e.g. Northern and Southern populations), the phenotypic responses differed from central populations, and these differences were usually related to growth (Rehfeldt et al. 2002). The peripheral populations are under higher selective pressure and may be better adapted to extreme or fluctuating conditions (Borovics and Mátyás 2013). For example, the Northern population was characterized by having a more significant volcanic influence with the ability to stabilize organic matter, storing water and retaining P (Mazzarino et al. 1998; Dzierma and Wehrmann 2010). On the other hand, the Southern population has a freezing climate with lower annual rainfall rates distributed throughout the year and very windy conditions defining a sub-Antarctic climate (Donoso et al. 2004).

It has been proposed that *E. coccineum* can face severe environmental changes through modification of their phenotype which is related to the precipitation gradient that occurs throughout its distribution (Alberdi and Donoso 2004; Ezcurra and Brion 2005). The PCA results confirmed the separation of the three populations of *E. coccineum*. Both axis PC1 and PC2 strongly correlated with both edaphoclimatic variables (E. Suppl. Mat, Fig. S2, Table S4) which could explain differential responses of *E. coccineum* seedlings under the different P and N supplies. We postulate that the Central population occupies the optimal ecological niche for *E. coccineum*, because it has the highest growth and CR production (Figs. 2 and 3), along with the fact that this locality has less extreme edaphoclimatic conditions (Table 1). While there is no clarity regarding the population genetic structure of *E. coccineum* (Souto and Premoli 2007; Vidal-Russell et al. 2011), our results suggest that in *E. coccineum*, the growth and CR production could be related to hereditary genetic identity. That would indicate some degree of local adaptation to

edaphoclimatic conditions from their geographical origin (Souto and Smouse 2013; Zúñiga-Feest et al. 2015).

Conclusions

To date, this is the first study showing a novel interaction between N and P for the processes involved in the CR formation in *E. coccineum*. Our findings allow us to conclude: *1)* N modulates the ability of *E. coccineum* to produce CR at different P supplies. At HN, a differential response depended on P supply, while at LN, a constitutive response was observed, suggesting a possible role in N uptake. *2)* CR production responses and the following repercussions on plant growth are dependent on the adaptation to the geographical origin. Overall, we conclude that N orchestrates the CR formation induced by P limitation, and we confirmed the differential responses of *E. coccineum* from different populations subjected to variable edaphoclimatic conditions.

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