

Cluster-root formation and carboxylate release in *Euplassa cantareirae* (Proteaceae) from a neotropical biodiversity hotspot

Patrícia de Britto Costa · Anna Abrahão · Ricardo Augusto Gorne Viani ·
Pedro Henrique Santin Brancalion · Hans Lambers ·
Alexandra Christine Helena Frankand Sawaya · Rafael S. Oliveira

Received: 30 April 2015 / Accepted: 6 August 2015 / Published online: 25 August 2015
© Springer International Publishing Switzerland 2015

Abstract

Background and Aims *Euplassa cantareirae* is a Neotropical Proteaceae, native to the Restinga forest in Brazil and it experiences a wide array of adverse environmental conditions, especially seasonal flooding and low nutrient availability. We aimed to investigate the effects of phosphorus (P) supply on plant growth and cluster root formation, as well as to characterize the

main carboxylates released by the cluster roots of this species.

Methods We conducted a greenhouse experiment where seedlings were grown in a sandy soil and watered three times a week with nutrient solution with the following P concentrations: 0, 10, 50 or 100 μM . Cluster-root production per plant, tissue P concentrations, growth parameters and carboxylate release were recorded and analyzed.

Results Remarkably, cluster-root formation, biomass production and leaf P concentration did not depend on P supply. These results differ from what have been found for almost all other investigated species, except for one other plant that inhabits a similar habitat in south-western Australia, *Viminaria juncea* (Fabaceae). The carboxylates that were found in the rhizosphere were similar to those reported before for other Proteaceae.

Conclusions *Euplassa cantareirae* is a Neotropical Proteaceae producing cluster roots, and contrary to what is known for other family members, it does not appear to regulate its cluster-root formation, growth and leaf P concentration by the soil P availability.

Responsible Editor: Antony Van der Ent.

P. d. B. Costa · A. Abrahão · A. C. H. F. Sawaya ·
R. S. Oliveira (✉)
Departamento de Biologia Vegetal, Instituto de Biologia,
Universidade Estadual de Campinas – UNICAMP, CP 6109,
13083-970 Campinas, SP, Brazil
e-mail: rafaelsooliv@gmail.com

R. S. Oliveira
e-mail: rafaelso@unicamp.br

R. A. G. Viani
Departamento de Biotecnologia e Produção Animal e Vegetal,
Centro de Ciências Agrárias, Universidade Federal de São Carlos
(UFSCar), Araras, SP, Brazil

P. H. S. Brancalion
Departamento de Ciências Florestais, Escola Superior de
Agricultura Luiz de Queiroz, Universidade de São Paulo (USP),
Piracicaba, SP, Brazil

H. Lambers · R. S. Oliveira
School of Plant Biology, University of Western Australia (UWA),
Crawley, WA, Australia

Keywords Atlantic forest · Root exudation ·
Phosphorus · Proteoid roots

Introduction

The Atlantic Forest features among the 25 global hotspots for biodiversity conservation priorities (Myers

et al. 2000). At the present, it retains less than 16 % of its original cover (Ribeiro et al. 2009) and about 2.7 % of all global plant species are endemic to this biome (Myers et al. 2000). The biodiversity is not equally distributed across the Atlantic Forest. Biodiversity patterns depend on land relief, climatic characteristics and soil properties (Oliveira-Filho and Fontes 2000). The most species-rich regions occur over two mountain ranges: Serra da Mantiqueira and Serra do Mar (Oliveira-Filho and Fontes 2000), which consist of Precambrian crystalline rocks formed 4.5 billion years ago (Almeida 1964). The mountain ranges represent an altitudinal gradient of ancient soils, characterized by a mosaic of soil types, most of them being shallow, acidic and very nutrient-poor (Rizzini 1997). This significant environmental heterogeneity is associated with a high biodiversity, which is often greatest in the mesic rainforest. However, other marginal ecosystems are also important in terms of biodiversity maintenance and generation (Scarano 2009).

The Restinga forest is a marginal ecosystem in the Atlantic Forest that occurs just above sea level and, although moderately species-rich, it is dominated by one or a few species (Scarano 2002). This habitat experiences a wide array of adverse environmental conditions, especially seasonal flooding, low soil pH (3.4), high salinity, low nutrient availability and high aluminum (Al) concentrations (Scarano 2002; Joly et al. 2012). Although lower than in mesic Atlantic Forest, species richness in Restinga forest is higher than in vegetation types with the same intensity and frequency of abiotic limitations (Scarano 2009), including species with mycorrhizal associations (Mardegan 2013) and with nitrogen-fixing bacteria (Silva and Tozzi 2009).

Phosphorus (P) is the most limiting macronutrient in the Restinga forests and the highest P concentrations are in the top 20 cm of the soil. Because of the extremely low pH (3.4) and high Al concentrations (15.6 mg kg^{-1}), P strongly binds to Al(III), which is bound to humic substances, by ligand exchange, replacing OH^- and H_2O . This process render the P unavailable for plant uptake in the soil solution (Gerke 1992). In P-impooverished habitats, plants exhibit a variety of mechanisms that allow survival and growth, such as long leaf life span, high degree of scleromorphy and high P-use efficiency (Lambers et al. 2010). Plants can also present mechanisms that enhance P acquisition, such as mycorrhizal associations and non-symbiotic root specializations (Lambers et al. 2010). Cluster roots are non-

symbiotic root specializations characterized by a dense set of lateral roots with abundant rootlets of determinate growth and high density of root hairs (Purnell 1960), which increases their root surface area. This large root surface area is associated with exudative bursts of carboxylates that solubilize forms of P unavailable to most plant species (Lambers et al. 2015).

Cluster roots were first studied in Proteaceae, mainly Australian and South African species growing in extremely nutrient-impooverished soils. In species from these habitats, like in *Hakea prostrata*, *Grevillea crithmifolia* and *Bankisia* species, the investment in cluster-root biomass is large when grown at low-P supply. Depending on the species, the allocation ranges from 25 to 75 % of the total biomass (Delgado et al. 2014). In contrast, cluster-root formation is suppressed at high P availabilities (Lamont 1972). In *Hakea prostrata* (Proteaceae), for example, shoot P concentrations increase with increasing P supply and this is associated with suppression of cluster-root formation (Shane et al. 2003). Likewise, in a southern South American Proteaceae, *Embothrium coccineum*, inhabiting volcanic ash soils with a large amount of total P, but with low P availability, cluster-root formation is also suppressed at high P supplies (Delgado et al. 2014). However, in *E. coccineum*, the investment in cluster root biomass is much lower than that in south-western Australian Proteaceae (less than 5 % of total biomass), and this is compensated by a faster rate of exudate release (Delgado et al. 2014). Notably, *Viminaria juncea* (Fabaceae), an Australian native species that occurs in heathlands near swamps with low P availability (although not quite as low as sites dominated by Proteaceae species), produces cluster roots even at high P supply with no increase in shoot P concentration, showing an ability to tightly down-regulate its P acquisition (de Campos et al. 2013). In addition to cluster roots, this species can also produce mycorrhizal associations and nodules for nitrogen fixation. These studies provide evidence that cluster-root formation and functioning depend not only on P availability (Zúñiga-Feest et al. 2015). The functioning of cluster roots in species from contrasting habitats is still under debate, and we do not know to what extent nutrient status is the only factor that influence cluster-root production..

Euplassa cantareirae Sleumer is a Proteaceae native to the Atlantic Forest, which, like *V. juncea* in south-western Australia, also inhabits seasonally-flooded habitats in the Restinga forest. There is no record of its

capacity to produce cluster roots and for their role in P nutrition. The aim of this study was to investigate if this species produce cluster roots and if so, whether high P availability would suppress cluster-root formation in a Neotropical Proteaceae endemic to global biodiversity hotspot. We further hypothesize that as P supply increases, *Euplassa cantareirae* would decrease cluster-root formation until total suppression, such as observed for species that inhabit soils with higher total P, but low P availability (e.g., *Embothrium coccineum*).

Material and methods

Species studied

The genus *Euplassa* is endemic to the Neotropics, and belongs to the subfamily Grevilleoideae and the subtribe Macadamieae, which includes the largest American endemic genera *Euplassa*, *Panopsis* and *Roupala* (Prance and Plana 1998). *Euplassa cantareirae* is a canopy tree from the Atlantic Rainforest that occurs in wet premontane forest along the Coastal range of Serra do Mar in south-eastern and southern Brazil (Prance et al. 1997; Prance and Amorim 2015), but also in lowland Restinga forests, where it is a moderately abundant species, with 22 individuals per hectare (Assis et al. 2011). In these environments we find 84 species per hectare from 32 families, the most abundant are *Pera glabrata* (Euphorbiaceae), *Euterpe edulis* (Arecaceae), *Myrcia racemosa* (Myrtaceae) and *Jacaranda puberula* (Bignoniaceae) (Assis et al. 2011).

Site description, seed collection and maintenance of plant material

Seeds of *E. cantareirae* were collected in July 2010, in a lowland Atlantic Forest (Restinga forest), at Serra do Mar State Park - Picinguaba nucleus (23° 27' 50" and 23° 15' 00" S and 45° 15' 00" and 44° 43' 30" W), located at Ubatuba - SP, in the north of the state of São Paulo, south-eastern Brazil. Local mean annual precipitation is 2,100 mm, and the mean annual temperature is about 22 °C (Bencke and Morellato 2002).

The Restinga forest, where *E. cantareirae* naturally occurs, is located on young Pleistocenic coastal Atlantic sand plains. The soil is characterized as Quartzipsamment (Alves et al. 2010), acidic and with high Al availability (15.6 mg Kg⁻¹) (Martins 2010). The

highest P concentrations are in the top 20 cm; resin P concentrations decreases from 11.5 for the top 5 cm soil layer to about 4.0 mg Kg⁻¹ of dry soil in 20 cm depth (Martins 2010). The soil presents seasonal fluctuations of the water table, being waterlogged during the rainy season, which occurs from December to March (Saenz 2013), and exhibits a relatively low plant-available P concentration, partly due to the low pH (Table 1) (Martins 2010).

Seeds were germinated in native soil, collected at the same site where seeds were collected. Seedlings were maintained in pots containing native soil in a greenhouse and watered with tap water three times a day, from July to October 2010.

Greenhouse experiment

When the seedlings were about 10 cm tall, they were transplanted to 3 l pots containing a mixture of two thirds of washed river sand and one third of vermiculite. Pots were then watered three times a week with 200 ml of a nutrient solution containing all nutrients, except P. In µM, the nutrient solution contained 400 NO₃⁻, 200 Ca²⁺, 200 K⁺, 154 SO₄²⁻, 54 Mg²⁺, 20 Cl⁻, 2 Fe-EDTA, 0.24 Mn²⁺, 0.1 Zn²⁺, 0.02 Cu²⁺, 2.4 H₃BO₃ and 0.3 Mo⁴⁺. The nutrient solution was prepared with deionized water with pH buffered to 5.8 (Shane et al. 2004).

The experiment was organized into four treatments with 15 plants each. Each treatment received 40 ml of P (KH₂PO₄) three times a week, in the following concentrations: 0, 10, 50 and 100 µM. The experiment was conducted in a closed greenhouse, located at the University of Campinas, Campinas-SP, south-eastern

Table 1 Soil pH and resin P concentration in mg kg⁻¹ of dry soil at different soil depth at the Restinga Forest in at Serra do Mar State Park - Picinguaba nucleus. Values extracted from Martins 2010

Soil depth (cm)	pH	Resin P concentration (mg kg ⁻¹)
0-5	3.39	11.5
5-10	3.46	7.0
10-20	3.73	4.0
20-30	3.97	2.0
30-40	4.09	2.0
40-50	4.14	2.0
50-75	4.17	2.0
75-100	4.16	2.0

Brazil. During the experiment the temperature ranged from 15 to 37 °C with a mean of 28 °C and relative humidity varied between 50 and 80 %.

Data collection

Plants were grown for 4 months, from November 2010 to February 2011. During the first 2 months, three growth parameters were recorded: stem diameter, plant height and leaf area. With nail polish, we made a trace in the stem base and measured the stem diameter with calipers at the beginning and at the end of the experiment. Plant height was measured with a ruler from the nail polish trace to the end of the apical meristem both at the beginning and at the end of the experiment. Finally, the total leaf area was determined by measuring the length and width of each leaf blade both at the beginning and at the end of the experiment.

Two months after transplanting the seedlings to the experimental pots, we performed a test to evaluate *E. cantareirae* root acid exudation by placing root samples of both fresh and senescing cluster roots in Petri dishes containing agar (12.5 g l⁻¹) with 1 % (w/v) bromocresol purple, a pH indicator with range shift at pH 5.2 (Neumann et al. 2000). After confirming the acid exudation, we proceeded with the identification of the main carboxylates released by the roots. Firstly, the root systems were gently shaken to remove sand excess. Then, the roots and rhizosphere soil were transferred to a beaker containing 0.2 μM CaCl₂ solution. The volume was just enough to allow complete immersion of the cluster roots; the use of CaCl₂ ensured cell integrity (Ryan et al. 2012). A 1 ml subsample of the rhizosphere extract was filtered through a 0.22 μm syringe filter and then analyzed in an ultra-performance liquid chromatography (UPLC-MS) (see method in Abrahão et al. 2014), using standard organic acids commonly released by cluster roots (Roelofs et al. 2001). Seven plants, randomly chosen, and one cluster of each individual were used to collect the exudates.

After 4 months of treatment, the plants were carefully removed from the pots. Leaves, stems and roots were dried in an oven at 60 °C for 48 h and weighed on an analytical balance to determine plant dry mass. We ground the plant dry material in a knife grinder, and analyzed P concentration through acid digestion with perchloric and nitric acid followed by colorimetric analysis with the malachite green method (Motomizu et al. 1983).

We also made field observations and collections. In March 2011, we dug up and exposed the roots of five seedlings of *E. cantareirae* to observe the root architecture and cluster-roots. In April 2013, we collected, in the same Restinga forest, 10 soil samples for soil pH and P availability analyses. Soil pH was determined by potentiometry in a CaCl₂ solution and soil P availability by the resin extraction method (Turner and Romero 2009) in the Soil Science Laboratory of the Luiz de Queiroz College of Agriculture, Piracicaba-SP.

Data analyses

Growth data (stem width, plant height and leaf expansion) as well as plant dry mass and tissue P concentration were compared among treatments by an ANOVA test followed by a *post-hoc* Tukey test comparison, both performed using R (R Core Team 2012). We considered a *p* critical value of 0.05.

Results

Soil analyses of field-collected material

Soil analyses from the Restinga forest where *E. cantareirae* naturally occurs showed P available values between 4 and 15 mg P kg⁻¹ of dry soil, with an average of 8.7 mg P kg⁻¹ of dry soil in the top 20 cm layer.

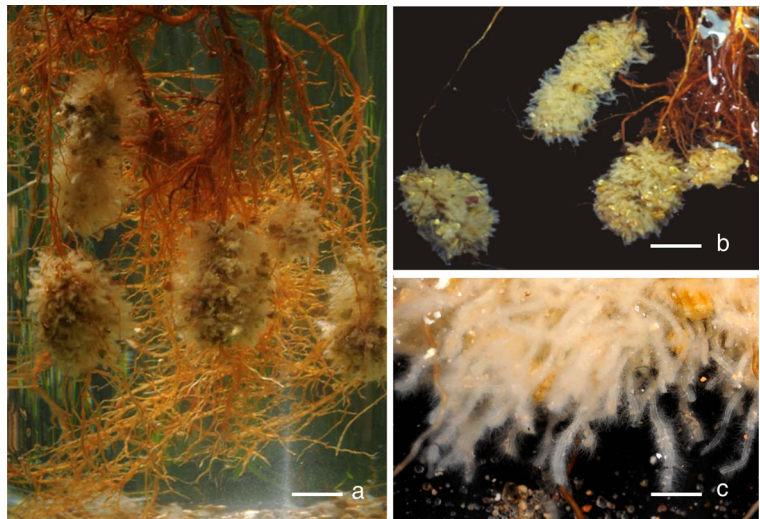
Cluster-root formation and functioning

Seedlings of *E. cantareirae* produced cluster roots in all treatments. The increase in P supply did not decrease cluster root production, neither the number of cluster roots (df=3, *F*=0.279, *p*=0.84) nor cluster root to total biomass (Fig. 4d). These structures were abundant, simple (with only first-order rootlets emerging from a lateral root axis) and bottlebrush-shaped (Fig. 1). The size of the cluster roots ranged from 10 to 40 mm. They were also observed in the field, at the seedling stage, where they clearly did not form a mat at the soil surface, but were found only along the entire root length, down 10 to 20 cm deep.

Mature cluster roots of *E. cantareirae* presented acid exudation (pH<5.2) as shown by the yellow aura in the bromocresol purple assay (Fig. 2a). However, we did not observe the yellow outline around senescing cluster roots, indicating they did not release acid exudates (Fig. 2b). We identified, in a descending concentration,

Fig. 1 Root system of *Euplassa cantareirae* seedlings cultivated for 4 months in a greenhouse.

a–b. *E. cantareirae* root system with cluster roots. **c.** Details of the cluster roots hairs (on secondary lateral roots). Seedlings of *E. cantareirae* produced cluster roots equally in all treatments



oxalic, malic, isocitric, citric, malonic, maleic, lactic, fumaric, succinic and tartaric acids in the *E. cantareirae* rhizosphere. The first four acids were released in greater proportions than the others, with concentrations of 593, 167, 165 and 22 $\mu\text{mol l}^{-1}$ respectively, representing almost 80 % of the total amount of organic acids that were exuded by cluster roots of *E. cantareirae* (Fig. 3).

Growth response to P supply

Plant growth did not differ among P treatments. Total biomass, cluster roots to total biomass ratio, leaf expansion rate and height increment did not differ among treatments. Likewise, root dry weight did not vary significantly among treatments ($df=3$, $F=1.062$, $p=0.376$); values of root dry weight varied from 0.25 to 2.95 g plant^{-1} , and represented between 10 and 37 % of the total plant dry weight (Fig. 4 a–d).

Tissue P concentration

Plants showed remarkably little variation in tissue P concentration among treatments, despite the very wide

range of P supply. The average leaf [P] was similar in the four treatments (0.77, 0.74, 0.82 and 1.03 mg P g^{-1} of DW, for 0, 10, 50 and 100 $\mu\text{mol l}^{-1}$ P, respectively) and ranged from 0.37 to 1.45 mg P g^{-1} of dry weight (DW) (Fig. 5a). Leaf [P] of *E. cantareirae* seedlings collected in the field was 0.61 mg P g^{-1} of DW. In addition, the individuals of the treatments with higher P availability did not store the extra available P in other tissues such as stems or roots (Fig. 5b). Root [P] and stem [P] were the same among treatments ($df=3$, $F=0.317$, $p=0.813$ and $df=3$, $F=0.312$, $p=0.816$, respectively). Root [P] varied from 0.18 to 0.47 mg P g^{-1} of DW and stem [P] from 0.13 to 0.90 mg P g^{-1} of DW.

Discussion

Euplassa cantareirae occurs in a stressful habitat (Scarano 2002), which experiences seasonal flooding and also is dependent on nutrients from litter decomposition (Bonilha et al. 2012). Nutrient concentrations in the soil are low, but constant throughout the year, since leaves are shed during the entire year in the Restinga

Fig. 2 Cluster roots of *Euplassa cantareirae* in the bromocresol purple assay. **a** The yellow color indicates release of acid exudates ($\text{pH}\leq 5.2$) by young cluster roots. **b** The senescent cluster roots did not release acid exudates and therefore showed no yellow outline



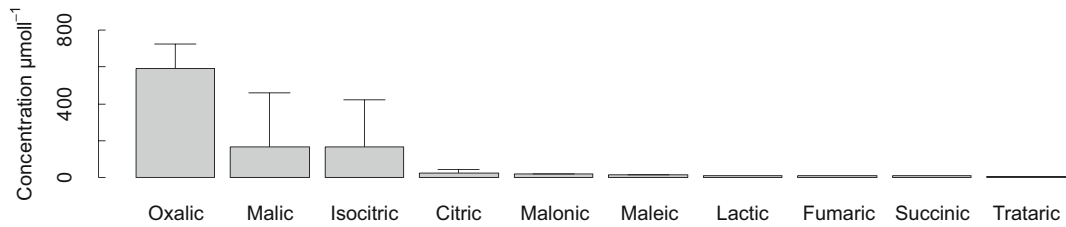


Fig. 3 Organic acid concentration ($\mu\text{mol l}^{-1}$) released in the solution by cluster roots of *Euplassa cantareirae* plants cultivated in a greenhouse. The bars represent the mean carboxylate concentration

in the rhizosphere ($n=7$) and the lines in each bar represent the standard error

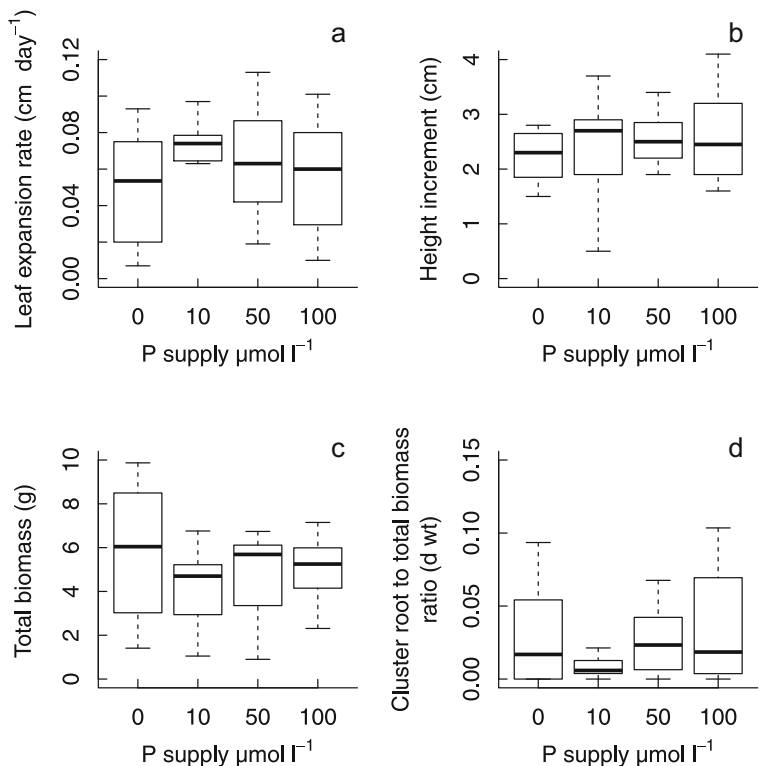
(Bizuti 2011). Total soil [P] at the study site was about 200 mg kg^{-1} soil (Bizuti 2011), while available (resin) [P] where seeds were collected was 8.7 mg kg^{-1} soil. The available soil P levels at the *E. cantareirae* site were low, but not as low as typically found for sites inhabited by south-western Australian Proteaceae species (25 and 1.7 mg kg^{-1} soil, for total and available P respectively, de Campos et al. 2013). Restinga forests also presented much lower total [P], but similar P availability to sites occupied by Chilean species (1600 and 0.1 to 13 mg kg^{-1} soil, for total and available [P], respectively, Donoso-Nanculao et al. 2010; Delgado et al. 2014).

Restinga forests experience a seasonal rise of the water table. Plants from seasonally-flooded habitats are usually

flood-tolerant and during flooding maintain, although at lower rates, transpiration, photosynthesis and growth rates (de Oliveira and Joly 2010), and also show lower nutrient-absorption rates (Steffens et al. 2005). The lower nutrient-absorption rate is a consequence of hypoxia. Under hypoxia the ATP production decreases, which affects the H^+ -pumping ATPase activity in root tissues, thus lowering absorption rates (Steffens et al. 2005). An efficient mechanism for nutrient acquisition, such as cluster roots, could benefit the maintenance of *E. cantareirae* in Restinga forests by increasing P acquisition after flooding, during which there is little nutrient uptake.

The simple bottlebrush-like cluster roots developed by *E. cantareirae* can be up to 40 mm long and are

Fig. 4 Comparison of growth parameters measured in seedlings of *Euplassa cantareirae* after 2 months of exposure to different concentrations of phosphorus (P0; P10; P50; P100, are respectively, 0, 10, 50 and $100 \mu\text{mol l}^{-1}$ of P in the added nutrient solution) in the soil in a greenhouse experiment. **a.** Leaf-expansion rate ($\text{df}=3$, $F=1.409$, $p=0.263$). **b.** Height increment ($\text{df}=3$, $F=0.179$, $p=0.91$). **c.** Total biomass dry weight ($\text{df}=3$, $F=0.987$, $p=0.409$). **d.** Cluster root to total biomass ratio (dry weight) ($\text{df}=3$, $F=0.765$, $p=0.521$). The boxes represent the quartiles, the central line the median and vertical bars represent the maximum and minimum values. All treatments showed no difference for the four parameters investigated ($p<0.05$ one-way ANOVA test)



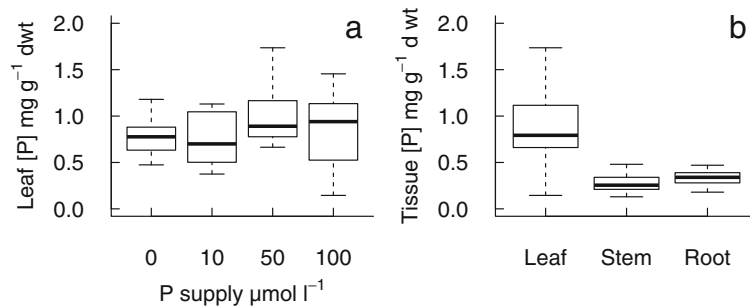


Fig. 5 Phosphorus (P) concentration in *Euplassa cantareirae* seedlings after 4 months of cultivation in a greenhouse ($n=43$). **a.** Foliar concentration at different P supply (P0; P10; P50; P100, are respectively, 0, 10, 50 and 100 μM of P in the added nutrient solution). All treatments showed no difference to each other ($\text{df}=\text{$

3, $F=2.338$, $p=0.088$). **b.** P Concentration in different parts of plants of all treatments. The *boxes* represent the quartiles, the *central line* represents the median and the *vertical bars* represent maximum and minimum values

morphologically very similar to those described for species from nutrient-poor soils in Australia such as *Hakea* spp. and *Telopea* spp. (Shane and Lambers 2005), and also to species from young volcanic soils in Chile such as *Gevuina avellana* (Ramirez et al. 2004). *Gevuina avellana* is phylogenetically close to *Euplassa* (Weston 2014), but despite the morphological similarity, cluster-root formation responded differently to what is known for the above-mentioned species.

Irrespective of P supply, *E. cantareirae* developed cluster roots, presenting the same proportion of non-cluster roots to cluster roots. The signaling for cluster-root production appears to involve the low leaf [P] (Shane and Lambers 2005), and since *E. cantareirae* maintained low P concentration in its leaves at all P supplies, we surmise that the seedlings received the same signal for cluster-root production in all treatments. In addition, the constant shoot P concentration in all treatments (concentration ranged from 0.74 to 1.03 mg g^{-1} DW among treatments) indicates the plants have the capacity to strongly down-regulate their P uptake, as observed before in *V. juncea* (in which the concentration ranged from 1.19 to 1.68 mg g^{-1} DW) (de Campos et al. 2013). In contrast, other Proteaceae species that develop cluster roots only at low P supply (Shane and Lambers 2005), increase their leaf P concentration with increasing P supply. For example, *Hakea prostrata*, which shows a limited capacity to down-regulate its P-uptake system, increases its leaf [P] until toxicity levels (from 0.02 to 13.6 mg g^{-1} DW). Conversely, *Grevillea crithmifolia* and *Embothrium coccineum*, which do show down-regulation of their P-uptake capacity, marginally control their leaf [P], and show some increase (from 0.15 to 2.05 mg g^{-1} DW, and from 0.49 to 3.16 mg g^{-1} DW, respectively) (Delgado et al. 2014).

The present results on *E. cantareirae* show both a fine adjustment of plant growth when P supply is scarce, and a strong down-regulation of P-uptake capacity at the highest levels of P supply. This species invests heavily in cluster-root growth, forming large structures that release carboxylates, irrespective of soil P availability. A species that also maintains tissue P levels and cluster-root formation under a large range of P supplies is *V. juncea*, which controls its P uptake very tightly, and occupies slightly P-richer habitats than south-western Proteaceae (de Campos et al. 2013). We suggest that the small increase in available P in the soils where *E. cantareirae* and *V. juncea* occur selects the ability to down-regulate P uptake capacity to avoid P toxicity (de Campos et al. 2013).

Along with cluster-root morphology and formation, root organic acids play an important functional role in nutrient acquisition at sites with low P availability (Jones 1998). Phosphorus is poorly mobile in soil and forms complexes with oxides and hydroxides of iron and aluminum in acidic soils, rendering it unavailable for plant uptake. Carboxylates compete for binding sites with complexes that sorb P, thus mobilizing both inorganic and organic P (Gerke 2015). As has been reported for Proteaceae species native from Australia, the acids commonly released are citric and malic (Roelofs et al. 2001). A similar pattern was found for *E. cantareirae*; oxalic, malic, isocitric and citric acid were the major acids in cluster-roots rhizosphere of *E. cantareirae*. Citrate and malate are usually the most abundant carboxylates in the rhizosphere and effective in mobilizing P (Roelofs et al. 2001). However, in the *E. cantareirae* rhizosphere, we found oxalate in higher concentrations, similar to those of citrate in some Australian species

(Dessureault-Rompré et al. 2007). This feature might be related to the P form present in the soil, because oxalate can have a similar effect to that of citrate in Al-rich soils (Gerke et al. 2000), which is the type of soil of the Restinga forest. Although less efficient, other carboxylates also play a role in P mobilization, e.g., malate and isocitrate.

Concluding remarks

Our study represents the first description of cluster-root formation in a native tree species from a Neotropical seasonally flooded ecosystem belonging to the Atlantic Forest domain, a global biodiversity hotspot. The results presented for *E. cantareirae* are very similar to those for the Australian *V. juncea* (de Campos et al. 2013), which naturally occurs in very similar seasonally-flooded soil conditions. *E. cantareirae* exhibits similar shoot P concentrations at all P supplies, indicating control of its P acquisition. We also observed the release of acid exudates with the bromocresol assay, and identified oxalic, malic, isocitric, and citric carboxylates as the main exudates of *E. cantareirae* cluster roots. These carboxylates participate in P acquisition, especially during the period of a low water table, compensating for the low nutrient acquisition during flooding (Steffens et al. 2005). Taken together, cluster roots and carboxylate exudation are an effective mechanism of P acquisition in P-poor soils of Restinga forests.

Acknowledgments We thank FAPESP (Fundo de Apoio à Pesquisa do Estado de São Paulo) for the grant to RS Oliveira (FAPESP 2010/17204-0) and for the scholarship granted to Patricia de B. Costa (2011/17037-9), for the use of the UHPLC-MS equipment (FAPESP/BIOEN grant 2008/58035-6) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant CNPq 474670/2008-2) to Rafael S. Oliveira. We also thank Instituto Florestal de São Paulo for the research permit to work at Parque Estadual da Serra do Mar. We are grateful to Caio G Pereira, Felipe H Crivelari and all the colleagues that contributed to the development of this study.

References

- Abrahão A, Lambers H, Sawaya ACHF et al (2014) Convergence of a specialized root trait in plants from nutrient-impooverished soils: phosphorus-acquisition strategy in a nonmycorrhizal cactus. *Oecologia* 176:345–355
- Almeida F (1964) Fundamentos geológicos do relevo paulista. *Bol Inst Geogr Geol* 41:169–263
- Alves LF, Vieira S, Scaranello M et al (2010) Forest structure and live aboveground biomass variation along an elevational gradient of tropical Atlantic moist forest (Brazil). *For Ecol Manag* 260:679–691
- Assis MA, Prata EMB, Pedroni F et al (2011) Florestas de restinga e de terras baixas na planície costeira do sudeste do Brasil: vegetação e heterogeneidade ambiental. *Biota Neotrop* 11: 103–121
- Bencke CSC, Morellato LPC (2002) Estudo comparativo da fenologia de nove espécies arbóreas em três tipos de floresta atlântica no sudeste do Brasil 1. *Rev Bras Bot* 25:237–248
- Bizuti DTG (2011) Ciclagem do fósforo em Floresta Densa dos Núcleos de Picinguaba e Santa Virgínia – SP. Escola Superior de Agricultura “Luiz de Queiroz”
- Bonilha RM, Casagrande JC, Soares MR, Reis-Duarte RM (2012) Characterization of the soil fertility and root system of restinga forests. *Rev Bras Ciênc Solo* 36:1804–1813
- De Campos MCR, Pearse SJ, Oliveira RS, Lambers H (2013) *Viminaria juncea* does not vary its shoot phosphorus concentration and only marginally decreases its mycorrhizal colonization and cluster-root dry weight under a wide range of phosphorus supplies. *Ann Bot* 111:801–9
- De Oliveira VC, Joly CA (2010) Flooding tolerance of *Calophyllum brasiliense* Camb. (Clusiaceae): Morphological, physiological and growth responses. *Trees Struct Funct* 24:185–193
- Delgado M, Suriyagoda L, Zúñiga-Feest A et al (2014) Divergent functioning of Proteaceae species: the South American *Embothrium coccineum* displays a combination of adaptive traits to survive in high-phosphorus soils. *Funct Ecol* 28: 1356–1366
- Dessureault-Rompré J, Nowack B, Schulin R, Luster J (2007) Spatial and temporal variation in organic acid anion exudation and nutrient anion uptake in the rhizosphere of *Lupinus albus* L. *Plant Soil* 301:123–134
- Donoso-Ñanculao G, Castro M, Navarrete D et al (2010) Seasonal induction of Cluster Roots in *Embothrium coccineum* development. *Chil J Agric Res* 70:559–566
- Gerke J (1992) Orthophosphate and organic phosphate in the soil solution of four sandy soils in relation to pH-evidence for humic-FE-(AL-) phosphate complexes. *Commun Soil Sci Plant Anal* 23:601–612
- Gerke J (2015) The acquisition of phosphate by higher plants: effect of carboxylate release by the roots. A critical review. *J Plant Nutr Soil Sci* 178:351–364
- Gerke J, Beiüner L, Römer W (2000) The quantitative effect of chemical phosphate mobilization by carboxylate anions on P uptake by a single root. I. The basic concept and determination of soil parameters. *J Plant Nutr Soil Sci* 163:207–212
- Joly C, Assis M, Bernacci L et al (2012) Florística e fitossociologia em parcelas permanentes da Mata Atlântica do sudeste do Brasil ao longo de um gradiente altitudinal. *Biota Neotrop* 12:125–145
- Jones DL (1998) Organic acids in the rhizosphere – a critical review. *Plant Soil* 205:25–44
- Lambers H, Brundrett MC, Raven JA, Hopper SD (2010) Plant mineral nutrition in ancient landscapes: high plant species diversity on infertile soils is linked to functional diversity for nutritional strategies. *Plant Soil* 334:11–31

- Lambers H, Clode P, Hawkins H et al (2015) Metabolic adaptations of the non-mycotrophic Proteaceae with low phosphorus availability. *Annu Plant Rev* 48:289–336
- Lamont B (1972) The effect of soil nutrients on the production of proteoid roots by *Hakea* species. *Aust J Bot* 20:27
- Mardegan SF (2013) Variação na dinâmica do nitrogênio e nos atributos foliares em fisionomias de restinga da região Sudeste do Brasil. Dissertation Universidade de São Paulo
- Martins SC (2010) Caracterização dos solos e serapilheira ao longo do gradiente altitudinal da Mata Atlântica, estado de São Paulo. Dissertation Universidade de São Paulo
- Motomizu S, Wakimoto T, Tgei K (1983) Spectrophotometric determination of phosphate in river waters with Molybdate and Malachite Green. *Analyst* 108:361–367
- Myers N, Mittermeier RA, Mittermeier CG et al (2000) Biodiversity hotspots for conservation priorities. *Nature* 403:853–8
- Neumann G, Massonneau A, Langlade N et al (2000) Physiological aspects of Cluster Root function and development in phosphorus-deficient white Lupin (*Lupinus albus* L.). *Ann Bot* 85:909–919
- Oliveira-Filho AT, Fontes MA (2000) Patterns of floristic differentiation among Atlantic forests in Southeastern Brazil and the influence of climate. 32:793–810
- Prance GT, Amorim AMA (2015) Proteaceae. In: List. Espécies da Flora do Bras. Jard. Botânico do Rio Janeiro. <http://www.floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB13785>. Accessed 25 Jan 2015
- Prance GT, Plana V (1998) The American Proteaceae. *Aust Syst Bot* 11:287–299
- Prance GT, Plana V, Edwards KS, Pennington RT (1997) Monograph of Neotropical Proteaceae. *Flora Neotrop* 100: 1–220
- Purnell M (1960) Studies of the family proteaceae. I. Anatomy and morphology of the roots of some Victorian species. *Aust J Bot* 8:38–50
- R Core Team (2012) R: a language and environment for statistical computing
- Ramirez GC, Valenzuela FE, San Martin PC (2004) Nuevos antecedentes sobre desarrollo temprano, morfología y anatomía de las raíces proteiformes de *Gevuina avellana*. *Agro Sur* 32:33–44
- Ribeiro MC, Metzger JP, Martensen AC et al (2009) The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biol Conserv* 142:1141–1153
- Rizzini C (1997) Tratado de Fitogeografia do Brasil: aspectos ecológicos, sociológicos e florísticos. Âmbito Cultural Edições Ltda, Rio de Janeiro
- Roelofs RFR, Rengel Z, Cawthray GR et al (2001) Exudation of carboxylates in Australian Proteaceae: chemical composition. *Plant Cell Environ* 24:891–903
- Ryan MH, Tibbett M, Edmonds-Tibbett T et al (2012) Carbon trading for phosphorus gain: the balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition. *Plant Cell Environ* 35:2170–2180
- Saenz GDCV (2013) Uso de água de três espécies arbóreas de uma restinga florestal em Ubatuba, SP. Dissertation Universidade Estadual de Campinas
- Scarano FR (2002) Structure, function and floristic relationships of plant communities in stressful habitats marginal to the Brazilian Atlantic Rainforest. *Ann Bot* 90:517–524
- Scarano FR (2009) Plant communities at the periphery of the Atlantic rain forest: Rare-species bias and its risks for conservation. *Biol Conserv* 142:1201–1208
- Shane MW, Lambers H (2005) Cluster roots: a curiosity in context. *Plant Soil* 274:101–125
- Shane MW, De Vos M, De Roock S et al (2003) Effects of external phosphorus supply on internal phosphorus concentration and the initiation, growth and exudation of cluster roots in *Hakea prostrata* R. Br. *Plant Soil* 248:209–219
- Shane MW, McCully ME, Lambers H, Sciences A (2004) Tissue and cellular phosphorus storage during development of phosphorus toxicity in *Hakea prostrata* (Proteaceae). *J Exp Bot* 55:1033–1044
- Silva ED, Tozzi AMGA (2009) Leguminosae in Picinguaba, Serra Do Mar State Park, São Paulo. *Braz Anais Congr Lat Am Ecol* 1:1–3
- Steffens D, Hütsch BW, Eschholz T et al (2005) Water logging may inhibit plant growth primarily by nutrient deficiency rather than nutrient toxicity. *Plant Soil Environ* 51:545–552
- Turner BL, Romero TE (2009) Short-term changes in extractable inorganic nutrients during storage of tropical rain forest soils. *Soil Sci Soc Am J* 73:1972
- Weston PH (2014) What has molecular systematics contributed to our Knowledge of the plant family Proteaceae? *Mol Plant Taxon Methods Protoc Methods Mol Biol* 1115:233–255
- Zúñiga-Feest A, Delgado M, Bustos-Salazar A, Ochoa V (2015) The southern South American Proteaceae, *Embothrium coccineum* exhibits intraspecific variation in growth and cluster-root formation depending on climatic and edaphic origins. *Plant Soil*