Differences in investment and functioning of cluster roots account for different distributions of *Banksia attenuata* and *B. sessilis*, with contrasting life history

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Abstract

Aims *Banksia attenuata* is a resprouting species growing in deep sand, while *B. sessilis* is a fire-killed species occurring in shallow sand over laterite or limestone. We aimed to discover the ecophysiological basis for their different distributions by exploring their investment in deep non-cluster roots and shallow cluster roots, and their cluster-root functioning.

Methods Deep-pot (1 m), shallow-pot (400 mm), hydroponic experiments and phosphorus (P)-extraction experiment were carried out. Biomass allocation, cluster-root exudation, plant P and leaf manganese (Mn) concentrations were measured.

Results *Banksia attenuata* allocated more biomass to deep roots and less biomass to cluster roots than *B. sessilis* did in deep pots. The two *Banksias* released similar

carboxylates in all experiments, with similar carboxylate-exudation rates in hydroponics. The carboxylate amount per unit cluster root of *B. sessilis* grown in shallow pots was greater than that of *B. attenuata*, and *B. sessilis* acquired more P than *B. attenuata* did in limestone substrate.

Conclusions Greater investment in deep roots for water uptake accounts for the presence of *B. attenuata* in deep sand, and *vice versa* for the absence of *B. sessilis*. A larger investment in cluster roots, which released greater amounts of carboxylates, likely accounts for *B. sessilis* occurring over limestone. Trade-offs in investment and cluster-root functioning support the species' distribution patterns and life histories. Leaf Mn concentration was a good proxy for the plant capacity to acquire P.

Keywords: Biomass allocation, Carboxylates, Manganese, Phosphorus acquisition, Photosynthesis, Relative growth rate

Introduction

Edaphic factors are a key driving force for plant species distribution (Buri et al. 2017; Clark et al. 1998; 1999). An important physiological basis underpinning plants growing in a certain soil is their ability to acquire sufficient soil resources (water, nutrients) for growth (Rellán-Álvarez et al. 2016; and references therein). Therefore, ecophysiological traits linked with acquisition of soil water and nutrients have been explored to understand plant species distribution pattern (Poot and Lambers 2003, 2008; Ström et al. 1994; Sultan 1995). Investment in deep roots reflects a strategy to access soil water at depth, especially for plants growing in arid or semiarid environments (Canham and Froend 2015; Poot and Lambers 2008; Rellán-Álvarez et al. 2016). Poot and Lambers (2003) explored the relationship between species distribution and root functional traits for rare vs. widespread *Hakea* species in seasonally-dry environments. They found that endemics confined to ironstone habitats invest more biomass in deep roots, and less in cluster roots than widespread species do which provides them access to water in cracks in the ironstone in the dry season.

With respect to nutrients, differences among species in their ability to solubilise mineral nutrients will affect which particular soils plants can grow in (Ström et al. 1994). Limestone soil is a nutrient-poor substrate with sparingly available phosphorus (P), iron (Fe), manganese (Mn), copper and zinc (Lambers et al. 2002; Ström et al. 1994). Ström *et al.* (1994) argued that the inability of calcifuge species to grow in limestone soil is due to less root exudation of oxalic acid and citric acid, which calcicole species release in greater abundance, allowing them to solubilise P and Fe.

Cluster roots are a specialised structure to enhance P acquisition from soils with a low P availability (Shane and Lambers 2005; Lambers et al. 2018); they release carboxylates to replace P bound to soil particles (Lambers et al. 2011; Shane and Lambers 2005). Pearse *et al.* (2007) showed that *Lupinus cosentinii* can grow in soils in which *Brassica napus* cannot grow, due to its ability to access sparingly-available P via releasing carboxylates from its cluster roots. Previous studies showed that composition and amount of carboxylates exuded by cluster roots depend on soil properties, P source, P availability and plant species (Denton et al. 2007; Lambers et al. 2002, 2006; Suriyagoda et al. 2012; Veneklaas et al. 2003). For instance, calcifuge species did not release iso-citric acid which most acidifuge species exuded (Ström et al. 1994). Similarly, rates of carboxylate exudation differ dependent on plant species and P availability (Delgado et al. 2014; Kidd et al. 2018; Veneklaas et al. 2003). As to the P-mobilising efficiency, tricarboxylates and dicarboxylates are far more effective than monocarboxylates (Jones 1998). Moreover, because carboxylates mobilise Mn as well as P, leaf [Mn] can be used as a proxy for the effective release of carboxylates (Lambers et al. 2015; Pang et al. 2018; Shane and Lambers 2005). There is likely an investment trade-off between cluster roots and non-cluster roots for plants growing in P-impooverished soil to acquire both soil water and P. Such a trade-off in ecophysiological traits may underpin plant species distribution, but this remains to be further investigated.

Ecophysiological traits affecting plant species distribution also affect a plant's life history (Bowen and Pate 2017; Clarke and Knox 2009; Clarke et al. 2013; Pate et al. 1990). In fire-prone Mediterranean vegetation, there are two contrasting life histories

with respect to fire: obligate seeders that are killed by fire, and resprouters that survive fire (Bell 2001; Lamont and Markey 1995; Pate et al. 1990, 1991; Pausas and Keeley 2014). Obligate seeders are short-lived and fire-sensitive, and regenerate from seeds, whereas resprouters are longer-lived, fire-resistant, and resprout from epicormic buds (Pate et al. 1991; Pausas and Keeley 2014). These life histories are associated with distinct ecophysiological traits. Seeders tend to grow faster, allocate resources predominantly above ground, and reproductively mature before the next catastrophic event, whereas resprouters grow more slowly and allocate a greater proportion of their resources below ground, where reserves are stored to sustain regrowth (Hansen et al. 1991; Knox and Clarke 2005; Paula and Pausas 2011; Pausas and Keeley 2014; Power et al. 2011). Compared with seeders, roots of resprouters grow deeper, and greater investment in roots allows resource storage and water acquisition from deep soil (Bell 2001; Canham and Froend 2015; Pate and Bell 1999; Paula and Pausas 2011). Although the above relationship between life history and ecophysiological traits is evident, we know little about the connection between life history traits and plant distribution. Lamont & Markey (1995) observed that resprouters tend to inhabit more fertile sites than seeders do, and Knox & Clarke (2005) argued more allocation to roots and starch reserves might enable seedlings of resprouters to be stronger competitors after fire on fertile sites. As yet, it is unknown how ecophysiological traits linked with life history determine plant distribution pattern.

In south-western Australia, an area with a Mediterranean climate, *Banksia* (Proteaceae) is one of the genera dominating in P-impooverished soils (Lambers et al. 2015; Lamont and Connell 1996; Lamont and Markey 1995). Resprouter *B. attenuata* and seeder *B. sessilis*, which both produce compound cluster roots (Shane and Lambers 2005), have different distribution pattern. *Banksia attenuata* is a calcifuge species restricted to deep sand and sometimes deep sand over laterite (FloraBase, <http://florabase.dpaw.wa.gov.au/>). In contrast, *B. sessilis* occur in shallow sand over laterite or limestone (Hayes et al. 2019; Pate and Bell 1999). However, the populations growing over limestone are likely different from those growing over

laterite, given that there are a number of different subspecies of *B. sessilis* (FloraBase, <http://florabase.dpaw.wa.gov.au/>). To elucidate the distinct distribution patterns of the two species with contrasting life history, we explored their root-investment strategies and cluster-root functioning, and tested the following hypotheses: (1) Resprouter *B. attenuata* invests more in deep roots for water uptake than seeder *B. sessilis* does to persist in deep sand during the dry season. (2) Both *B. attenuata* and *B. sessilis* change rhizosphere carboxylate composition in response to substrate type, and in limestone soil *B. sessilis* releases specific P-mobilising carboxylates, which *B. attenuata* does not exude. (3) To overcome the difficulty of P acquisition in limestone soil, *B. sessilis* allocates more to cluster roots and enhances cluster root function at mobilising P by releasing more carboxylates or/and exuding at a faster rate than *B. attenuata* does. (4) The traits supporting the distribution pattern of *B. attenuata* and *B. sessilis* match their life histories.

Materials and Methods

A deep pot experiment, a shallow pot experiment and a hydroponic experiment were carried out in the glasshouse at the University of Western Australia, Perth (31°59' S, 115°53' E) as randomised complete block designs. Glasshouse temperatures fluctuated between 13 and 33 °C over a whole year, and transmission of photosynthetically active radiation into the glasshouse was 60%. The seeds of *Banksia attenuata* R.Br. and *B. sessilis* (Knight) A. R. Mast & K. R. Thiele, purchased from Nindethana Seed Western Australia, were sown on filter paper, and one seedling was transferred into each experimental pot on 22nd May, 2016. The seeds of *B. sessilis* were collected from a coastal population, growing over limestone. The seedlings were watered with deionised water; details for each experiment are described below.

Experiment 1: Deep-pot experiment

This experiment aimed to explore why *B. attenuata* occurs in deep weathered sand, where *B. sessilis* is absent, exploring an allocation trade-off between non-cluster

150 roots and cluster roots of plants growing in a pot experiment mimicking the beginning
151 of the Mediterranean dry season (summer). There were seven replicates for both *B.*
152 *attenuata* and *B. sessilis*, each grown in sand media in free-draining PVC cylinders
153 with an inner diameter of 100 mm and a height of 1000 mm. One PVC tube
154 measuring 20 mm in diameter and 1200 mm in height was inserted into each pot,
155 which allowed water to feed directly to the bottom. Approximately 8.4 kg washed
156 river sand with a total P concentration of $6 \mu\text{g g}^{-1}$, was added inside plastic bags lining
157 the deep pots, so roots could be removed readily at harvest. To simulate what happens
158 in their natural habitat after winter rains stop, plants were initially watered three times
159 per week (Mondays, Wednesdays, and Fridays) from the soil surface for four weeks.
160 After that, they were watered from the bottom only, via small PVC pipes. In either
161 case, the watering was stopped when water started leaching from the drainage holes in
162 the bottom 100 mm of the pots. No additional nutrients were supplied to plants during
163 the course of the experiment.

164 After 22 weeks of growing in pots, plants were harvested. The soil bags lining the
165 pots were removed and cut open. The root systems were carefully removed from bulk
166 soil and the loose soil was shaken off gently. Roots and rhizosheath (i.e. the soil that
167 adhered to the roots) were placed into 500 ml beaker, and rinsed with 50 ml of 0.2
168 mM CaCl_2 solution until all visible sand had been washed into solution. Then, one
169 milliliter of solution was extracted and filtered through a $0.20 \mu\text{m}$ Acrodisc® syringe
170 filter into a 1 ml high-performance liquid chromatography (HPLC) vial. The HPLC
171 samples were acidified with 25 μl of orthophosphoric acid (H_3PO_4), and stored at -
172 20 °C for carboxylate analyses. Tricarboxylates and dicarboxylates were considered
173 effective carboxylates to mobilise soil P, and monocarboxylates and minor
174 carboxylates were classified as ‘others’ (the same classification for carboxylates as in
175 experiment 2 and 3). After collecting root exudates, roots were washed and classified
176 into two parts: cluster roots and non-cluster roots. Fresh mass (FM) was separately

measured for leaves, stems, cluster roots and non-cluster roots. Relative growth rate (RGR) for the two species was calculated by the formula: $(\ln W_2 - \ln W_1) / (T_2 - T_1)$, where W_1 and W_2 are seed weight and plant fresh weight at harvest; T_1 and T_2 are the dates for sowing and harvesting, respectively; $T_2 - T_1$ is expressed as weeks.

Experiment 2: Shallow-Pot experiment

To simulate the Mediterranean winter-wet season, a shallow-pot experiment was designed to identify ecophysiological mechanism explain why *B. sessilis* is able to grow across a wide range of P-impoverished soil types by comparing the investment strategy, resource allocation pattern and cluster-root functioning of *B. attenuata* and *B. sessilis*. Three soil treatments were imposed, based on washed river sand as per the deep pot experiment: sand, sand plus laterite (SLAT) and sand plus limestone (SLIM). Total P concentration of the substrates used in the experiment was $6 \mu\text{g g}^{-1}$ for washed river sand, $66 \mu\text{g g}^{-1}$ for laterite gravel and $203 \mu\text{g g}^{-1}$ for limestone gravel, respectively. The shallow pots (PVC cylinder with 100 mm inner diameter and 400 mm height) were lined with plastic bags. For each soil treatment, 3.0 kg soil substrate was used to fill the pots. A 100 mm layer at 50 mm below the soil surface was filled with laterite or limestone gravel for the SLAT and SLIM treatment, respectively, and other layers were filled with sand. There were ten replicates for each treatment and each species. Field capacity of each treatment was determined first, and the pots were watered to a constant weight at 80% of field capacity three times a week. A basal nutrient solution, except for phosphorus, containing (per kilogram of soil): 217.5 mg KNO_3 ; 74 mg CaCl_2 ; 140 mg K_2SO_4 ; 80 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 28.9 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 10 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 5 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.7 mg H_3BO_3 ; 0.5mg $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 20 mg FeNaEDTA , was applied to each pot once every second week.

Harvesting and exudate collection

After 50 weeks of growing in pots, plants were harvested. Soil bags were carefully pulled out of the pots and cut open. All leaves on a plant were sampled, and the bulk soil was gently shaken off the roots before cutting off the stem. The cluster roots with

rhizosheath soil were detached from the rest of the root system, and rinsed with a recorded amount of 0.2 mM CaSO₄ solution (150-850 ml, depending on plant size) in a beaker. Using a syringe, the rhizosphere soil was flushed clear of as much soil as possible. Then, a subsample of the elution liquid was sampled for subsequent carboxylate analysis using the methods described for experiment 1. Cluster roots were collected by washing and filtering elution liquid with no. 1 Whatman filter paper. The stem and non-cluster roots were separated, and soils on non-cluster roots were carefully washed off. Leaves of each plant were scanned at 200 dpi (Epson 1680) to calculate leaf area using ImageJ 1.4. Thereafter, samples of leaves, stems, cluster roots and non-cluster roots were dried at 70 °C for 72 h, and DM was determined. Specific leaf area (SLA) for each plant was calculated as the ratio of total leaf area to total leaf DM. The RGR was calculated by the formula described for experiment 1, and using dry weight instead of fresh weight for W_2 .

Determination of phosphorus and manganese concentrations

Dried leaf samples were ground to a fine powder using Geno/Grinder 2010 (Spex SamplePrep, Metuchen, New Jersey, USA), and dried stems, cluster roots and non-cluster roots were ground by a conventional grinder. All ground samples were digested using a hot concentrated nitric-perchloric (3:1) acid mixture. Using inductively-coupled plasma optical-emission spectrometry (ICP-OES, UWA, Perth, WA, Australia), P and Mn concentrations of leaf samples were determined. The P concentration of the cluster roots and non-cluster roots was determined by the malachite green method (Motomizu et al. 1983) using a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The amount of P acquired per plant was calculated by total P content minus seed P content (Hayes et al. 2019).

Experiment 3: Hydroponic experiment

To assess carboxylate-exudation rates of *B. attenuata* and *B. sessilis*, a hydroponic experiment was carried out. Fourteen seedlings (seven replicates for each species) of uniform size were selected and transplanted in seven 4.5 l pots, with their shoots supported by a grey foam disc that made a light-tight seal. Each pot contained two

plants (one *B. attenuata* and one *B. sessilis*) and 4 l of continuously-aerated nutrient solution, maintained at 18 °C in a root-cooling tank. The composition of basal nutrient solution in deionised water was (µM): KNO₃, 150; MgSO₄, 27; MnSO₄, 0.18; ZnSO₄, 0.075; H₃BO₃, 1.8; Na₂MoO₄, 0.0225; Fe-NA.EDTA, 7.5; CaCl₂, 20; KH₂PO₄, 1; CuSO₄, 0.018. To the 4 l nutrient solution in each pot, 5 ml 50 µM Na₂O₃Si was added. All nutrient solutions were replenished three times per week. From April to June, 2017, 28 mature and active cluster roots were intermittently sampled for each species to collect exudates. Sixteen hours before exudate collection, a P-free nutrient solution was supplied to the plants. At 9:00 am, the targeted cluster roots were excised and quickly submerged in a tube with CaCl₂ (10 mM) in the dark. Then, the excised cluster roots were taken to the lab as soon as possible, and transferred to a tube submerged by 4 ml CaCl₂ (10 mM) solution for 1.5 hours with continuous aeration in the dark. One milliliter of the collecting fluid was sampled for the subsequent carboxylate analysis using the methods described in experiment 1, and the cluster roots were dried at 70 °C for 72 h to determine DM.

Experiment 4: Phosphorus-extraction experiment

To evaluate the P-mobilising capacity of carboxylates on different soil substrates, 3 g soil substrate (sand, laterite gravel and limestone gravel) with five replicates was extracted with 30 ml of a solution of 0.5 mM citrate, iso-citrate or deionised water. The solutions were prepared in 5 mM KCl and adjusted to pH 5.5 using KOH or HCl. We added 75 µl of chloroform (0.25%, v/v) to each vial to inhibit microbial breakdown of carboxylates. The vials were shaken for 16 hours on an end-over-end shaker, centrifuged at 1000 rpm (200 g) for 30 min and the supernatant was filtered through a 0.45 µm syringe filter (Pal Gelman Acrodisc®). The P concentration was determined with the malachite green method (Motomizu et al. 1983), using a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Carboxylate analysis

Working standards of acetate, *cis*-aconitate, citrate, fumarate, *iso*-citrate, lactate, maleate, malate, malonate, shikimate, succinate and *trans*-aconitate (ICN Biomedicals

Inc., Aurora, OH, USA) were used to identify carboxylates by liquid chromatography (Waters 600E, Milford, MA, USA). The detailed analysis method and procedures were described in Cawthray (2003).

Statistics

The comparisons of plant growth traits, biomass allocation, P and Mn concentrations, and carboxylate exudation between *B. attenuata* and *B. sessilis* were analysed by *t* test. In experiment 2, the differences for the two species among soil types were analysed by two-way ANOVAs. To determine the differences in P mobilisation of extracting media (citrate, *iso*-citrate and DI water) on sand, laterite gravel or limestone gravel, one-way ANOVAs were applied with *post hoc* LSD test. The relationship between non-cluster root mass and plant mass, effective carboxylate amount and plant mass, effective carboxylate amount and acquired P content, and leaf Mn concentration and acquired P content were explored by linear regression analysis. All statistical analyses were performed using the SPSS 16.0 (SPSS Inc., Chicago, US), and graphed with OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA).

Results

Experiment 1: Deep-pot experiment

Plant growth and biomass-allocation pattern

Plants of *B. attenuata* were larger than those of *B. sessilis* ($P < 0.001$, $df = 11$), whereas the relative growth rate (RGR) of *B. attenuata* was significantly less than that of *B. sessilis* ($P < 0.001$, $df = 11$) (Fig. 1). *Banksia attenuata* and *B. sessilis* both allocated over 75% of their total fresh mass to their roots, while a small proportion was allocated to the leaves and stem (Fig. 2). Based on fresh mass, the leaf mass ratio (LMR) of *B. attenuata* was significantly greater than that of *B. sessilis* ($P < 0.05$, $df = 11$), the reverse being the case for the root mass ratio (RMR) ($P < 0.05$, $df = 11$). As for investment in different root parts, cluster roots and non-cluster roots, the two species exhibited distinctly different strategies. *Banksia attenuata* invested 1.34 times

more in non-cluster roots than *B. sessilis* did, and *B. sessilis* invested 4.45 times more in cluster roots than *B. attenuata* did (Fig. 2).

Carboxylates

The composition of carboxylates exuded by *B. attenuata* and *B. sessilis* was similar (Fig. S1). The effective carboxylates (citrate, malate and *trans*-aconitate) accounted for over 96% of total carboxylates, and others included shikimate, *cis*-aconitate and fumarate. The amount of effective carboxylates per unit cluster root fresh mass of *B. attenuata* was 2.20 times greater than that of *B. sessilis* ($P < 0.05$, $df = 11$) (Fig. S2). The correlation between plant fresh mass and effective carboxylate concentration in the collecting solution was tighter for *B. attenuata* ($r^2 = 0.76$, $P = 0.01$, $df = 6$) than for *B. sessilis* ($r^2 = 0.50$, $P = 0.12$, $df = 5$) (Fig. S3).

Experiment 2: Shallow-pot experiment

Plant growth and biomass-allocation pattern

The size of *B. attenuata*, with larger seeds than those of *B. sessilis*, was significantly greater than that of *B. sessilis* in all soil types ($P < 0.001$, $df = 18$ for all) (Fig. 3a). Plant biomass of both species was greater when grown in sand than that in SLAT, and least in SLIM. *Banksia attenuata* and *B. sessilis* grown in SLIM produced 36% and 47% less biomass than those grown in sand, respectively. *Banksia attenuata* and *B. sessilis* both allocated over 50% biomass to aboveground parts in different soil types, with the exception of *B. sessilis* in sand (Fig. 3b). However, *B. attenuata* allocated more biomass to the aboveground parts than *B. sessilis* did in all soil types. *Banksia sessilis* invested more biomass in non-cluster roots and cluster roots in all soil types than *B. attenuata* did (Fig. 3c, d). About one third of roots were cluster roots for the two species, and their cluster-root mass ratio ranged from 0.10 to 0.17, with the lowest value in SLAT. The RGR of *B. sessilis* was greater than that of *B. attenuata* in all soil types ($P < 0.001$, $df = 18$ for all) (Fig. 3e). The two species showed similar growth trends across different soil types, with the RGR slightly decreasing from sand to SLAT, and to SLIM. Specific leaf area (SLA) of *B. sessilis* was more than twice that of *B. attenuata* in all soil types ($P < 0.001$, $df = 18$ for all) (Fig. 3f).

Phosphorus-distribution pattern

The P acquired by *B. attenuata* and *B. sessilis* from soil was greatest in sand, less in SLAT and least in SLIM (Fig. 4a). When acquired plant P content was expressed per unit cluster-root dry mass, the values for *B. attenuata* were slightly greater than those for *B. sessilis* for plants grown in sand and SLAT ($P > 0.05$, $df = 18$ for both), while it was the reverse in SLIM; the value for *B. attenuata* was significantly lower than that for *B. sessilis* ($P < 0.05$, $df = 18$) (Fig. 4b). There were no significant differences between *B. attenuata* and *B. sessilis* in leaf [P] for all soil types ($P > 0.05$, $df = 18$ for all) (Fig. 4c). However, stem [P] and non-cluster root [P] of *B. attenuata* were greater than those of *B. sessilis* in all soil types (Fig. 4d, e). Especially in sand, the average values of *B. attenuata* for stem [P] and non-cluster root [P] were 1.9-fold and 1.7-fold those of *B. sessilis*, respectively ($P < 0.001$, $df = 18$ for both). By contrast, [P] in cluster roots of *B. attenuata* were significantly lower than those of *B. sessilis* in all soil types ($P < 0.05$, $df = 18$ for SLAT, $P < 0.01$, $df = 18$ for sand and SLIM) (Fig. 4f).

Carboxylates and leaf manganese

The carboxylate composition in the rhizosphere soil for *B. attenuata* and *B. sessilis* was similar in the same soil. The effective carboxylates were citrate, malate and *trans*-aconitate for both species in all soil types, and *iso*-citrate was an additional effective carboxylate that we found when plants were grown in SLIM for both species (Fig. 5). These effective carboxylates accounted for over 86% of total carboxylates. Acetate, lactate, shikimate, *cis*-aconitate, fumarate and maleate were classified as ‘others’. *Banksia sessilis* released more effective carboxylates per unit cluster root dry mass than *B. attenuata* did in all soil types (Fig. 6a). Moreover, both species strikingly enhanced carboxylate exudation in SLIM compared with that in sand and SLAT. Leaf Mn concentrations ([Mn]) of *B. sessilis* were much greater than those of *B. attenuata* in all soil types ($P < 0.001$, $df = 18$ for all) (Fig. 6b). Comparing leaf [Mn] in different soils, there was a similar trend for both species, with leaf [Mn] decreasing from sand to SLAT and to SLIM. However, the leaf [Mn] ratio of *B. sessilis* to *B. attenuata* increased from 3.6 to 6.0, and to 7.1 for sand, SLAT and SLIM, respectively.

The correlations between acquired P content and effective carboxylate amount for *B. sessilis* was strong and positive ($r^2 = 0.36$, $P < 0.001$, $df = 29$), while the correlation

for *B. attenuata* was weak ($r^2 = 0.040$, $P = 0.298$, $df = 29$) (Fig. 7a). The acquired P content of *B. attenuata* increased with an increase in effective carboxylate amount for each soil type, but the correlation was not significant ($P > 0.05$, $df = 9$). Furthermore, both *B. attenuata* and *B. sessilis* showed a significant positive correlation between acquired P content and leaf [Mn] (*B. attenuata* $r^2 = 0.42$, $P < 0.001$, $df = 29$; *B. sessilis* $r^2 = 0.17$, $P = 0.02$, $df = 29$) (Fig. 7b), and a tighter relationship between acquired P content and leaf Mn content (*B. attenuata* $r^2 = 0.52$, $P < 0.001$, $df = 29$; *B. sessilis* $r^2 = 0.53$, $P < 0.001$, $df = 29$) (Fig. 7c). However, leaf [Mn] was not significantly associated with carboxylate amount for both species (*B. attenuata* $r^2 = 0.034$, $P = 0.33$, $df = 29$; *B. sessilis* $r^2 = 0.004$, $P = 0.75$, $df = 29$) (Fig. 7d).

Experiment 3: Hydroponic experiment

The effective carboxylates in the hydroponic experiment were *trans*-aconitate, malate, citrate and *cis*-aconitate, and the minor carboxylates fumarate, maleate and shikimate were classified as ‘others’, which accounted for about 1% of total carboxylate amounts (Fig. 8). The carboxylate composition was similar for *B. attenuata* and *B. sessilis* in nutrient solution, but it was slightly different from that in soils, because *cis*-aconitate was absent from the major carboxylates found in experiment 1 (Fig. S1) and experiment 2 (Fig. 5). Carboxylate-exudation rates of *B. attenuata* and *B. sessilis* were very similar, for both effective carboxylates and total carboxylates (Fig. 8).

Experiment 4: Phosphorus-extraction experiment

A P-extraction experiment showed that citrate extracted more P from sand and laterite gravel than *iso*-citrate and deionised water did (Fig. 9). The least P was extracted from limestone, while the most P was extracted from sand. *Iso*-citrate, a carboxylate that only appeared in exudates from plants grown in limestone soil (Fig. 5), extracted more P from limestone gravel than citrate and deionised water did.

Discussion

Investment strategy and species distribution

Our first hypothesis that resprouter *B. attenuata* invests more in deep roots for water uptake than seeder *B. sessilis* does to persist in deep sand during the dry season, was

fully supported. A high biomass investment in roots supports plant drought-resistance (Markesteijn and Poorter 2009). To develop a strong root system for water uptake at depth is vital for plant to survive the first summer in a Mediterranean climate region (Canham and Froend 2015; Witkowski and Lamont 1996). In our experiments, *B. sessilis* invested more in superficial P-mobilising cluster roots, whereas *B. attenuata* invested more in deep non-cluster roots for water-scavenging at depth during the dry season. Sufficient water supply is essential for cluster-root functioning (Lambers et al. 2006); in accordance, *B. attenuata* released more carboxylates per unit cluster root mass than *B. sessilis* did (Fig. S2), and showed more growth in the deep-pot experiment (Fig. S3). We conclude that greater investment in non-cluster roots is responsible for the survival of *B. attenuata* in the dry season. This agrees with findings of Poot and Lambers (2003), who showed that edaphically narrow-endemic species allocate more biomass to deep roots than widespread species do, and enable their roots to access water from cracks and fissures in the underlying ironstone rock. Conversely, the low investment in deep roots limited *B. sessilis* to acquire sufficient water from deep soil and thus restricted cluster-root functioning and plant growth (Fig. S2 and S3), even though *B. sessilis* had a higher cluster-root mass ratio. One plant of *B. sessilis* died during the course of the experiment, which is consistent with field observations of high mortality rates for *B. sessilis* (Causley et al. 2016) and for other species (Frazer and Davis 1988; Lamont and Markey 1995; Poot and Lambers 2008; Richards et al. 1997) during summer drought. This investment pattern is likely responsible for the absence of *B. sessilis* in deep sand (Hayes et al. 2019; Pate and Bell 1999).

Cluster-root functioning and species distribution

Our second hypothesis that both *B. attenuata* and *B. sessilis* change the rhizosphere carboxylate composition with soil types, and *B. sessilis* releases specific carboxylates which *B. attenuata* does not exude in limestone soil, was partly supported. The two species slightly changed their effective carboxylate composition, with *cis*-aconitate only appearing in SLIM and *iso*-citrate only appearing in hydroponics. However, *B. sessilis* exhibited the same carboxylate composition as *B. attenuata* did on all

substrates, including SLIM (Figs 5, 8, S1). Many studies showed that carboxylate composition changes with substrate (Denton et al. 2007; Lambers et al. 2002; Suriyagoda et al. 2012; Veneklaas et al. 2003), but the exact mechanism accounting for this remains unknown. Moreover, previous studies indicated that the P-mobilisation efficiency of carboxylates depends on specific carboxylate species and soil substrates (Denton et al. 2007; Lambers et al. 2002; Ström et al. 1994; Suriyagoda et al. 2012; Veneklaas et al. 2003). Our results also show that citrate and *iso*-citrate have a different P-mobilisation efficiency for sand, laterite gravel and limestone gravel. However, as the two species exuded the same carboxylates in all soil treatments, carboxylate composition cannot account for their different distribution.

Our third hypothesis that *B. sessilis* allocates more biomass to cluster roots and enhances cluster-root P-mobilising efficiency by releasing more carboxylates and/or exuding at a faster rate than *B. attenuata* does to allow more P acquisition from limestone was partly supported. Based on the results of P extraction and P acquired by plants, SLIM was the hardest substrate to mobilise P from. Both species greatly enhanced carboxylate exudation in SLIM compared with that in sand and SLAT, but the amount of effective carboxylates per unit cluster root dry mass of *B. sessilis* was greater than that of *B. attenuata* in SLIM (Fig. 6a). Most importantly, *B. sessilis* allocated more biomass to cluster roots than *B. attenuata* did in SLIM, and thus *B. sessilis* released more carboxylate than *B. attenuata* did, and eventually acquired more P from limestone soil (Fig. 4b). Likewise, Ström et al. (1994) found that calcicole species exude more effective carboxylates (e.g., oxalic acid, citric acid) to mobilise P from soil than calcifuge species do. Our findings supports the claim in previous studies that species with a high capacity of acquiring P, can grow in more severely P-impooverished habitats (Lambers et al. 2006; Pearse et al. 2007; Ström et al. 1994). Kidd et al. (2018) showed that the exudation rate of carboxylates differs among 12 species of pasture and crop legumes. However, the carboxylate-release rates of *B. sessilis* and *B. attenuata* were identical, which is contrary to our hypothesis. Therefore, greater release of carboxylates, rather than carboxylate-exudation rate is likely a physiological basis for the occurrence of *B. sessilis* on limestone soil.

Cluster roots are ephemeral structures with a rapid turnover rate (Shane et al. 2004), and the collected cluster roots or carboxylates provided only an instantaneous estimate. As P and Mn are mobilised by carboxylates simultaneously (Gardener and Boundy 1983; Shane and Lambers 2005), the Mn content gives the cumulative effect of carboxylates, and thus can be used as a proxy for P-acquisition potential. In agreement with this contention, our results show that the correlation between leaf Mn content and acquired P content was significantly tighter than the correlation between effective carboxylates and acquired P content for both *B. attenuata* and *B. sessilis* (Fig. 7). Leaf [Mn] was proposed as a proxy of carboxylate release (Lambers et al. 2015; Pang et al. 2018), and this is supported by our findings. Although leaf [Mn] was not significantly associated with carboxylate amount, it was tightly correlated with acquired P content for both species. This indicates that leaf Mn content, as well as leaf [Mn], was a stronger predictor for acquired P content by plant than effective carboxylate amount measured at a single moment.

Ecophysiological traits are associated with life history

Our fourth hypothesis that the traits supporting the distribution pattern of *B. attenuata* and *B. sessilis* match their life histories was fully supported (Fig. 10). Resprouter *B. attenuata* produces few and large seeds (101 mg dry mass per seed) compared with seeder *B. sessilis* (only 7 mg dry mass per seed) (Hayes et al. 2019). Furthermore, *B. attenuata* can resprout from epicormic buds or lignotubers (Groom and Lamont 2011; Pate et al. 1991), therefore, does not need to grow fast and complete its life cycle quickly (Bowen and Pate 2017; Knox and Clarke 2005; Pate et al. 1990). Thus, *B. attenuata* stored more resources such as P (Fig. 4d, e) and starch (Bowen and Pate 2017; Knox and Clarke 2005; Pate et al. 1990, 1991) in stems or/and non-cluster roots for regrowth after fire. In addition, *B. attenuata* allocated more biomass to non-cluster roots for water uptake from deep soil (Fig. 2), favouring *B. attenuata* persisting in a water-limited habitat. Generally, the seed size, resource allocation and root-investment strategy are the bases to ensure its occupation in deep sand, and they are also the traits that support the K selection life history of *B. attenuata*.

In contrast with *B. attenuata*, the seeds of *B. sessilis* are small, less than one

fourteenth of those of *B. attenuata* (Hayes et al. 2019). The lighter seeds allow further dispersal. Moving even tens of metres away increases the possibility of germination in a different soil type in the Southwest Australian Floristic Region (Hopper 2009), but *B. sessilis* has a high P-mobilisation ability to colonise across different P-impooverished soils (sand over laterite or limestone), which improves its chances of survival on different substrates. Moreover, as a short-lived obligate seeder, *B. sessilis* needs to grow fast (Clarke et al. 2013), and to produce seeds before the next catastrophe, i.e. fire or drought (Bowen and Pate 2017; Knox and Clarke 2005; Pate et al. 1990). In the present study, *B. sessilis* invested more biomass in cluster roots and, in the shallow-pot experiment, released more carboxylates per unit cluster mass to mobilise more P for growth. It also had a higher SLA. All these traits provide a physiological basis to support its faster growth, but the lower investment in non-cluster root for water uptake restricts its distribution on deep sand. In other word, the small seeds, combined with the investment and functioning of its cluster roots determine the distribution of *B. sessilis* and allow it to function as a coloniser. Finally, as *B. sessilis* occurs in contrasting soil habitats (sand over laterite and limestone), its physiological ecotypic differentiation is worthy of further investigation.

Conclusions

Banksia attenuata can persist in deep sand due to its greater investment in deep non-cluster roots for water uptake, while low investment in deep non-cluster root of *B. sessilis* is a likely cause of its absence from deep sand. On the other hand, the sand over limestone habitat, where *B. attenuata* does not occur, was likely inhabited by *B. sessilis* because of a larger investment in cluster roots and larger amount of released carboxylate (except in deep pots), rather than fast carboxylate-exudation rates or carboxylate composition. The trade-offs in investment strategy and cluster-root functioning account for the different distribution of *B. attenuata* and *B. sessilis*, and match their contrasting life history.

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Figures

Fig. 1 Comparison of plant fresh mass (FM) and fresh mass-based relative growth rate (RGR) between resprouter *Banksia attenuata* and seeder *B. sessilis*. Values are means $\pm$ SE; *B. attenuata* ($n = 7$), *B. sessilis* ($n = 6$, one plant that died was excluded). Asterisks show significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2 Comparison of organ fresh mass ratio between resprouter *Banksia attenuata* and seeder *B. sessilis*. Values are means $\pm$ SE; *B. attenuata* ($n = 7$), *B. sessilis* ($n = 6$, one plant that died was excluded). Asterisks show significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3 The plant biomass (a), shoot : root ratio (b), non-cluster root mass ratio (c), cluster root mass ratio (d), relative growth rate (RGR) (e), and specific leaf area (SLA) (f) of resprouter *Banksia attenuata* and seeder *B. sessilis* grown in three soil types. Values are means ($n = 10$) $\pm$ SE; Asterisks show significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Different capital letters indicate significant differences for the two species among soil types ($P < 0.05$). SLAT: sand plus laterite, SLIM: sand plus limestone.

Fig. 4 The acquired phosphorus (P) content (a), acquired P per unit cluster root dry mass (b), leaf P concentration (c), stem P concentration (d), non-cluster root P concentration (e) and cluster root P concentration (f) of resprouter *Banksia attenuata* and seeder *B. sessilis* grown in three soil types. Values are means ($n = 10$) $\pm$ SE; Asterisks show significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Different capital letters indicate significant differences for the two species among soil types ($P < 0.05$). SLAT: sand plus laterite, SLIM: sand plus limestone.

Fig. 5 The composition and amounts of carboxylates exuded by resprouter *Banksia attenuata* and seeder *B. sessilis* grown in three soil types. Values are means ($n = 10$) $\pm$ SE; BA: *B. attenuata*, BS: *B. sessilis*. SLAT: sand plus laterite, SLIM: sand plus limestone.

Fig. 6 The effective carboxylates per unit cluster root dry mass **(a)** and leaf manganese (Mn) concentration **(b)** of resprouter *Banksia attenuata* and seeder *B. sessilis* grown in three soil types. Values are means ($n = 10$) $\pm$ SE; Asterisks show significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Different capital letters indicate significant differences for the two species among soil types ($P < 0.05$). SLAT: sand plus laterite, SLIM: sand plus limestone.

Fig. 7 The regression of effective carboxylate amounts **(a)**, leaf manganese (Mn) concentration **(b)** and leaf Mn content **(c)** on acquired phosphorus (P) content, and the regression of leaf Mn concentration on effective carboxylate amounts **(d)**. The broken line indicates a non-significant regression; solid lines indicate significant regressions. BA: *Banksia attenuata* ($n = 30$), resprouter; BS: *B. sessilis* ($n = 30$), seeder. SLAT: sand plus laterite, SLIM: sand plus limestone.

Fig. 8 The composition and rate of exudation of carboxylates in resprouter *Banksia attenuata* and seeder *B. sessilis* in a hydroponic experiment. Values are means ($n = 7$) $\pm$ SE, expressed per unit dry mass (DM) of cluster roots (CR); the error bar pointing downwards shows SE for each carboxylate component.

Fig. 9 The phosphorus (P) concentration in soil solution extracted by citrate and *iso*-citrate for different soil substrates. Values are means ($n = 5$) $\pm$ SE; different small letters indicate significant differences among different extraction solutions for the same substrate (LSD, $P = 0.05$).

Fig. 10 Diagrams summarising pivotal ecophysiological traits investigated in the present study, and distribution pattern and life history based on literature data for resprouter *Banksia attenuata* (**a**) and seeder *B. sessilis* (**b**). RGR: relative growth rate, SLA: specific leaf area, SRR: shoot: root ratio, P: phosphorus, non-CRMR: non-cluster root mass ratio, CRMR: cluster root mass ratio.

Fig. S1 Carboxylates composition exuded by *Banksia attenuata* ($n = 7$), resprouter; *B. sessilis*, seeder ($n = 6$, one plant that died was excluded)

Fig. S2 Effective carboxylates per unit cluster root fresh mass of resprouter *Banksia attenuata* and seeder *B. sessilis*. Values are means $\pm$ SE; *B. attenuata* ($n = 7$), *B. sessilis* ($n = 6$, one plant that died was excluded). Asterisk shows significant differences between the two species; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CR: cluster root, FM: fresh mass.

Fig. S3 Relationship between effective carboxylates and plant fresh mass. BA: *Banksia attenuata* ($n = 7$), resprouter; BS: *B. sessilis*, seeder ($n = 6$; one plant that died was excluded in the analysis).

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Fig. S1

Fig. S2

Fig. S3