The potential for phosphorus benefits through root placement in the rhizosphere of phosphorus-mobilising neighbours

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Abstract

Plants that produce specialised cluster roots, which mobilise large quantities of poorly-available nutrients such as phosphorus (P), can provide a benefit to neighbouring plants that produce roots in the cluster-rhizosphere, as demonstrated previously in pot studies. To be effective, such roots must be present within the short time of peak cluster activity. We tested if this requirement is met, and quantified potential P benefits, in a hyperdiverse Mediterranean woodland of southwest Australia where cluster-rooted species are prominent. Using minirhizotrons, we monitored root dynamics during the wet season in the natural habitat. We found non-cluster roots intermingling with all 57 of the observed cluster roots of the studied tree species, *Banksia attenuata*. Almost all (95%) of these cases were observed in a high-moisture treatment simulating the 45-year average, but not present when we intercepted some of the rainfall. We estimate that cluster-root activity can increase P availability to intermingling roots to a theoretical maximum of 80% of total P in the studied soil. Due to their high P-remobilisation efficiency (89%), which results from P rapidly being relocated from cluster roots within the plant, senesced *Banksia* cluster roots are a negligible P source for other roots. We conclude that, rather than serving as a P source, it is the cluster-root activity, particularly the exudation of carboxylates, that may improve the coexistence of interacting species that are capable of root intermingling, thus potentially promoting species diversity in nutrient-poor habitats, and that this mechanism will be less effective in a drying climate.

Keywords: *Banksia attenuata* and *B. menziesii*, climate change, minirhizotron, phosphorus-mobilisation facilitation, phosphorus remobilisation.

Introduction

When plant roots intermingle, are there interactions among interspecific neighbours, and if so, what are the potential consequences of such interactions? Classical plant root studies demonstrate that roots of competing species tend to avoid each other, and thus reduce belowground resource competition for soil nutrients and water (Schenk 2006). Yet, recent root research, often using advanced tools to study roots *in situ* (Metzner et al. 2015; Schmittgen et al. 2015; van Dusschoten et al. 2016), shows that root intermingling is perhaps more common than previously thought (Mommer et al. 2016; Oburger and Schmidt 2016). The main scenarios that promote root intermingling, rather than root avoidance, have been well-documented, and include: i) kin recognition (intraspecific roots), ii) nitrogen-uptake facilitation (interspecific roots), where N₂-fixing plants increase the availability of N to other plants, and iii) patchiness of soil resources (intra- and interspecific roots) where the presence of localised high resource levels (e.g., N) leads to root proliferation (Chen et al. 2018; Faget et al. 2013; Hodge 2012; Li et al. 2016). However, it is not known if root intermingling is common when phosphorus (P) is the key limiting nutrient for plant growth, and what the potential benefits of intermingling are.

In severely P-impooverished soils of the hyperdiverse sandplains of southwest Australia's biodiversity hotspot, many plants form cluster roots that are among the most efficient roots to acquire poorly-available P from such soils (Lambers et al. 2015). Cluster roots comprise a main axis and many determinate ephemeral rootlets (Shane and Lambers 2005). Large quantities of carboxylates, but also likely acid phosphatases, protons, and chelating substances, are released in an 'exudative burst' from cluster roots in several species (Delgado et al. 2013; Shane et al. 2004; Watt and Evans 1999), whereas in *Banksia* species, which have more complex cluster roots, exudation appears to occur at slower rates but for longer periods (Beeck 2017; Shi et al. 2020). The release of carboxylates effectively increases the

concentration of P and some micronutrients in the soil solution, thereby making them available for uptake (Shane and Lambers 2005). The development of cluster roots that exude large quantities of carboxylates into the cluster rhizosphere is referred to as ‘cluster-root activity’. As such, cluster-root activity involves a time-dependent process that increases P uptake by the cluster roots. Furthermore, there is evidence that, as a ‘side effect’, this process promotes P-uptake facilitation, which appears to occur when neighbouring plants grow along cluster-rooted plants (Gardner and Boundy 1983; Li et al. 2007; Muler et al. 2014). Yet, we lack empirical evidence showing *in situ* that other plant roots actually physically intermingle during these rapid biochemical rhizosphere processes (Lambers et al. 2018; Teste et al. 2018).

Root intermingling may be prevalent in P-limited environments to acquire sorbed P mobilised by carboxylates released by neighbours, but it is unclear what might direct the growth towards P-mobilising cluster roots, and what level of P-uptake benefits may result from such root interactions. Intermingling might be a random process, due to non-cluster roots being attracted by signals associated with cluster-root activity, or both non-cluster and cluster roots may be attracted by the same trigger (e.g., a high resource patch). A first step in elucidating mechanisms of intermingling with cluster roots is to demonstrate that intermingling is not random, to quantify its frequency, and to determine its timing relative to peak cluster-root activity. If non-cluster roots show a tendency to arrive in the rhizosphere of cluster roots when these are most active, this is likely an adaptive response to temporarily-enhanced resource availability.

Our ongoing work on foliar nutrient patterns within and in the neighbourhood of cluster-rooted plant species is pointing to a considerable potential for P-mobilisation based facilitation in nutrient-poor soils (Abrahão et al. 2019). We have found increases in foliar manganese (Mn) levels in plant species neighbouring cluster-rooted species compared with

controls without cluster-rooted neighbours (Muler et al. 2014). More recent studies show that plants that are potentially facilitated by carboxylate-releasing neighbours exhibit increased leaf [Mn] (Yu et al. in press; Yu et al. 2020). Here, for the first time, we present evidence that the fundamental conditions, relating to root positioning and rhizosphere-P dynamics, enable a considerable P benefit for other roots occurring within the rhizosphere of cluster roots. Global climate change is leading to substantial drying climates in most Mediterranean ecosystems (Diffenbaugh and Field 2013; Indian Ocean Climate Initiative 2012). Southwest Australia's Mediterranean ecosystems, which host a biodiversity hotspot and many cluster-rooted plant species (Lambers et al. 2014; Lamont 1982; Myers et al. 2000), could be affected by the drying climate. The production of cluster roots is stimulated by high surface soil moisture levels (Lamont 2003), and it appears that a minimum-threshold soil moisture level is required to trigger the plant into forming cluster roots (Lamont 1976; Lamont 2003). Our recent study in this ecosystem demonstrated a greater presence of cluster roots at high soil moisture levels compared with drier controls (Teste et al. 2018). However, effects of declining soil moisture levels on root interactions remain poorly explored in the field. Since plants that produce cluster roots are abundant and widespread in this biodiverse ecosystem, yet are particularly sensitive to changes in soil moisture, we proposed that plant community compositional shifts may continue to occur (Crosti et al. 2007; Fisher et al. 2009; Teste et al. 2018). In addition, we suggest that reductions in plant diversity may occur: i) if cluster-rooted plants become less efficient at mining soil P due to narrower 'windows' of favourable soil moisture conditions (Teste et al. 2018); ii) P is less mobile in dry soil than in moist soil thus exacerbating point i) (Lambers et al. 2008); and iii) P and micronutrient uptake of coexisting plants is indeed facilitated by the root activity of cluster-rooted plants (Lambers et al. 2018).

The overall aim of this study was to determine whether the spatial and temporal dynamics of cluster roots and roots of neighbouring plants provide clues for a potential P-mobilisation facilitative role of cluster roots. Our previous work (Muler et al. 2014; Teste et al. 2014) provided the theoretical underpinning and experimental support for facilitation by cluster-rooted species, but we did not yet provide visual evidence of root intermingling *in situ*, which is fundamental to a P-facilitation hypothesis (Lambers et al. 2018). Here, we test if root placement and rhizosphere P conditions are such that they would make root-to-root facilitation possible. Using a combination of minirhizotron field and glasshouse experiments, and P budgets during root intermingling, we estimate the potential benefits of P mobilisation by cluster roots to interacting non-cluster rooted plants. The study aimed to test: i) is there evidence of root intermingling between cluster-rooted species and other plant species? ii) Do roots from neighbouring plant species intermingle before, during, or after cluster-root activity; and does greater soil moisture availability intensify intermingling? iii) How much P is potentially made available in the cluster rhizosphere?

Kleptoparasitism is parasitism by theft of nutriment or a key resource. Kleptoparasitism is a well-documented animal-animal interaction (Gorman et al. 1998; Thompson 1986), where the optimal foraging of one species of animal is taken advantage of by another species by timely ‘robbery’ of a key resource (e.g., food). This type of biological interaction has also been found between carnivorous plants and predatory insects (Zomora 1995). Kleptoparasitism between plants has yet to be shown, yet it may exist in P-impoverished systems, particularly if timely intermingling by non-cluster rooted plant species occurs during the peak-P foraging activity of cluster-rooted plants.

Banksia attenuata and *B. menziesii* (Proteaceae) were used as the focal tree species, since they produce an abundance of large compound cluster roots (Denton et al. 2007b; Shane and

Lambers 2005). These *Banksia* species are long-lived trees with extensive lateral and deep roots where cluster roots are commonly found just below the leaf litter and top layers (0 to 20 cm) of the soil profile (Lambers 2014; Pate and Watt 2002; Teste et al. 2018). Root dynamics and interactions of all roots near the *B. attenuata* trees were monitored using installed minirhizotron tubes and cameras, and we quantified P concentration and remobilisation from cluster roots of *B. menziesii* in a glasshouse. These two *Banksia* species are physiologically similar and prominent components of these woodlands (Denton et al. 2007a; Lambers et al. 2012; Pate and Dell 1984). Finally, to evaluate the ways in which other roots might benefit from intermingling with cluster roots, the potential P availability in cluster-rhizospheres and after cluster root senescence was estimated.

Methods

Study site

Experimental irrigated and unirrigated plots were established in banksia-eucalypt woodland at The University of Western Australia's (UWA) Shenton Park Field Station (31.96°S, 115.79°E) (Supporting Information Fig. S1). The site is located in the kwongan vegetation type on the Swan Coastal Plain within the Southwest Australian Floristic Region (sensu Hopper and Gioia 2004). The site is part of the Spearwood coastal dune system (McArthur and Bettenay 1974; Wyrwoll et al. 2014). The sandy soils are severely nutrient-impoverished (Turner et al. 2018), and the climate is Mediterranean with a mean annual temperature of 19.2°C and average annual rainfall of 702 mm (Australia's Bureau of Meteorology; <http://www.bom.gov.au/climate/data/>; 1967 to 2011), where most of the rain occurs between May and September, the cooler "winter months" of the Southern Hemisphere. During these winter months, most of the root growth including cluster roots occurs (Lamont 2003; Pate and Beard 1982), thus the likelihood of root intermingling is high. A complete description of the study site and experimental design are included in Teste et al. (2018).

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180 In May 2011, 40 minirhizotron clear tubes (Bartz Technology Corp., Carpinteria, CA, USA,
181 internal diameter = 50.8 mm) were installed to a depth of 0.5 m at 1 m and 2.5 m from the
182 basal stem of ten *B. attenuata* trees with two irrigation treatments (i.e. on 10 plots x 2
183 distances x 2 irrigation treatments = 40 tubes). Tubes were prepared following similar
184 procedures outlined in Smit et al. (2000) and Johnson et al. (2001) to eliminate artefactual
185 effects of light and temperature. Prior to imaging roots with a BTC2 Minirhizotron Video
186 Microscope (Bartz Technology Corp., Carpinteria, CA, USA), the minirhizotron tubes were
187 left to stabilise for 11 months. From April 2012, sprinklers distributed water evenly to a
188 radial distance of 4 m around the minirhizotron tubes. This irrigation treatment started a
189 month before the winter rains and produced a 25% increase in monthly winter water inputs
190 (from April to October) based on 45 years of rainfall data (1967 to 2011) from a nearby
191 weather station (Subiaco treatment plant, Station # 009151, Lat: 31.96°S, Long: 115.79°E;
192 Bureau of Meteorology, <http://www.bom.gov.au/climate/averages/>). The plots were irrigated
193 every month, and by October, we had added a total of 148 mm of supplementary water to
194 each plot. The minirhizotron tubes around the mature *Banksia* focal trees were positioned to
195 be within the radial extent of the water spray provided by the sprinklers used in the irrigation
196 treatment (Teste et al. 2018). Furthermore, we inserted the tubes within the main root zone of
197 each focal tree. As for soil depth, the range of depths used to image roots was mainly
198 determined by the limitations of the minirhizotron equipment (tubes and camera) and the
199 feasibility of gathering images within the same day in the field. Furthermore, we knew that
200 imaging beyond 50 cm depth was unlikely to render observations of cluster roots, since they
201 are typically restricted to the top 15 cm of the soil profile (Denton et al. 2007b; Pate and Watt
202 2002).

203

204 *Image capture and analysis*

A complete description of the procedures involved in the root image capture and analysis is available in Teste et al. (2018). In summary, roots were imaged between 4 April 2012 and 5 October 2012, and root dynamics and soil moisture were monitored on a weekly basis when possible. The captured minirhizotron images were then analysed with Rootfly v. 2.0.2, an open-source software application (Zeng et al. 2010). Rootfly allowed us to annotate (with a computer mouse) the roots, determine the type of root (cluster (Fig. 1, S2), woody or herbaceous roots, based on structure and colour), and calculate the birth and death rates of roots through time. In this study the minirhizotron approach was used to track root intermingling over time in a non-destructive manner *in situ*. We annotated a ‘cluster-root’ type only when we saw rootlets at some point in time during root-imaging from April to October. *Banksia* roots that did not produce rootlets could not be differentiated from other woody roots; therefore, they were categorised as a ‘woody’ root type. A ‘cluster-root intermingling’ response was calculated based on minirhizotron images (image frame = 13.5 mm by 18 mm) that had co-occurring root types of which at least one was a cluster root.

Glasshouse experiment

In August 2012, a glasshouse experiment with *B. menziesii* seedlings grown in nutrient-impooverished Bassendean sand (see Laliberté et al. (2012) for specific soil characteristics) was established with the objective to quantify cluster-root nutrient concentrations and -remobilisation during senescence. *Banksia menziesii* seedlings were grown in 14 rhizoboxes (2.2 L) and in plastic bags inside 40 polyvinyl chloride (PVC) pots (2.1 L). Our aim was to simultaneously photograph and sample cluster roots at their various developmental stages. Four stages were morphologically identified: stage 1, immature cluster roots; stage 2, fully-formed cluster roots; stage 3, cluster roots with rusty brown colour and some loss of vigour (shrinking determined visually); stage 4, dark brown or grey cluster roots with noticeable loss of vigour (widespread shrinking and wrinkling determined visually). Between 10-15 January

2013, half of the seedlings were harvested, washed and cluster roots were then cleaned and oven-dried at 70 °C for 48 hours.

Due to the nature of cluster roots, small sand particles often tightly adhere to the root cluster. Therefore, once dried, the cluster roots were again cleaned to remove adhering sand to accurately quantify dry weight and chemical composition of the roots. Thus, cluster roots were placed between two fine mesh holders and subjected to pressurised air until most of the sand was removed. Any remaining sand was accounted for, by subtracting its weight from the sample dry weight, during root digests, prior to chemical analysis (see *Chemical analysis of cluster roots* below).

Chemical analyses of cluster roots

After sand removal, fragmented cluster roots were weighed and analysed for total P and silicon (Si) by atomic absorption spectrometry (Perkin Elmer 500 DV ICP-AES, Massachusetts, USA), with an adapted protocol to deal with sand particles, at the ChemCentre (<http://www.chemcentre.wa.gov.au/>), Perth, Australia. Since many cluster roots still contained traces of adhering sand particles, the digestion in closed capsules was carried out to avoid possible loss of acid digest solution (nitric-perchloric acid mixture) due to explosive boiling-over. All remaining root samples were then re-analysed for P and SiO₂ using an Elementar Vario Macro (Hanau, Germany) directly after combustion. Further analysis of the evolved gases and digests was done with an inductively coupled plasma mass spectrometer (Perkin Elmer, Elan 6000 ICP-MS, Norwalk, CT, USA) or axially-configured ICP-OES for low-weight samples. Total N was quantified with the Elementar Vario Macro (Hanau, Germany).

Cluster-root vitality

A modified Evans blue test was used to determine root vitality (Peterson et al. 2008). Two approaches to stain the four stages of cluster roots were used; first, whole cluster roots were stained by dipping them in Evans blue solution, and, second, the main root axes without the rootlets were hand-sectioned prior to dipping. Cluster roots boiled in water for 5 minutes were used as controls for this vitality assessment. Stained rootlets were observed under a dissecting light microscope to visually determine if the root was dead (i.e. stained) or alive (i.e. clear, not stained). The proportion of dead rootlets per 5 mm cluster root fragment was determined with the gridline intersect method (Giovannetti and Mosse 1980).

Structural dry matter

Structural dry matter was determined for cluster-root stages 2 to 4. First, 200 mg of dried root material was pulverised with a Retsch Tissue Lyser (TissueLyser Qiagen, Haan, Germany) at 25 Hz for 5 minutes. Then 700 μ L of NaH_2PO_4 (0.1 M, pH 7.3) and 300 μ l Tween-20 (0.1%, v/v) were added to the pulverised material, which was then vortexed, pulverised again, vortexed for 1 minute, and finally centrifuged at 8,164 g for 10 minutes. The supernatant was removed, the material allowed to dry at 60 °C for 72 hours, then re-weighed. The structural dry matter was then calculated as the difference between the initial dry weight minus the weight after releasing soluble metabolites. Structural dry matter was quantified to express the concentrations of N and P during the development of cluster roots on a structural dry weight basis; this avoids the bias of any changes in non-structural dry weight (e.g., non-structural carbohydrates) during cluster development.

Estimation of the amount of phosphorus made available by cluster roots

We consider two ways in which other roots might benefit from intermingling with cluster roots: 1) by taking up P made available through the action of cluster-root exudates (carboxylates and phosphatases), i.e. novel P pools that would not be available for non-cluster

roots; or 2) by taking up P left behind in senesced cluster roots. To estimate the P potentially made available through the release of cluster-root exudates, 10 soil samples were collected at the study site, regardless of the irrigation treatment given that this field sampling was done six years after the temporary irrigation treatment ceased. These field samples were taken below *Banksia* trees from 0-0.05 m depth, adjacent to where cluster roots were imaged (Teste et al. 2018). Total P was quantified by extraction in 1 M HCl (16 hours, 1:50 soil to solution ratio) of ashed samples (550 °C, 1 hour) modified after Saunders and Williams (1955). Free Pi (resin-P) was extracted with anion-exchange membranes (Turner and Romero 2009). Carboxylate-extractable Pi (citrate-extractable P) was assessed using a 0.05 M solution of citric acid (3 hours). Organic P (Soil organic P) was calculated as the difference between the 1 M HCl-extracted P after and before ashing. This method may cause a slight overestimate of the organic P, because of a potential increase in acid-extractability of P in soil ash (Williams et al. 1970). Results are expressed on an oven-dry (105°C) weight basis.

To estimate the potential maximum amount of P available from decomposing cluster roots, first, cluster root per soil weight ratio was determined, and then the P concentration of senesced cluster roots (see above) was used to estimate the amount of P left behind by cluster roots per unit soil dry weight. Ten patches of mature cluster roots were located by removing the litter layer below *Banksia* trees at the study site, and pieces of this cluster-root mat (depth ~0-50 mm, area ~5000 mm²) were collected. *Banksia* cluster roots are easily found given their large size and characteristic mat-like structures (Shane and Lambers 2005). In the lab, one mini-core from each of the samples (diameter 21.5 mm, depth 11 mm, volume 3990 mm³) was taken, and live cluster roots from the soil (sand, organic matter, any other roots) were separated by adding water and using end-over-end shaking, sonification, and manual separation. Cluster root samples and soil samples were dried at 70 °C, weighed, and then ashed at 550 °C. The cluster root dry weight was adjusted for contamination with sand,

assuming that the ash content of cluster roots was 5% of their dry weight, based on values from other slow-growing tree species (George et al. 2003; Helmisaari et al. 2009; Ostonen et al. 2005).

Data analysis

The frequency of roots intermingling with cluster roots was analysed as count data with Poisson and negative binomial regression models (Zuur et al. 2009). Based on the image analysis described above, one of three ‘interaction’ types was assigned to each observation based on whether the co-occurring ‘other roots’ were first observed before (Fig. 1), during (Fig. S3), or after (Fig. S4) the presence of live cluster roots. The average lifespan of cluster-roots, including the immature and mature stage, was 23 days (Teste et al. 2018). For cluster roots that did not fully develop by the end of the study, interaction type ‘before’ or ‘during’ could still be assigned, but not ‘after’. As such, we analysed the interaction type and irrigation treatment as fixed effects (Table S1). When the irrigation treatment or other root types affected the frequency of interaction, negative binomial models were fitted to account for over-dispersion (Cameron and Trivedi 2005).

Total P and total N in cluster roots of different stages were compared with analysis of variance and confidence intervals followed by the Tukey HSD test. We calculated a cluster-root P-remobilisation efficiency (%) based on the mean values of stage I and other stages (e.g. [stage I – stage III] / stage I). Cluster-root vitality was analysed as proportion data with generalised linear models. The assumptions for the statistical models were assessed graphically, with plots of residuals and boxplots for treatment variances. All graphing and statistical analyses were conducted in R (R Core Team 2020) and ‘ggplot2’ packages (Therneau 2015; Wickham 2016).

Results

Root intermingling with cluster roots

A total of 2091 root fragments were imaged between April and October 2012, where 57 of these developed into mature cluster roots such as shown in Figs S2, S3, S4, and S5. As a cluster root appeared and developed, other roots were seen in very close proximity (i.e. within the same 13.5 x 18 mm image frame), likely interacting and possibly acquiring P mobilised by the cluster root (Figs S3, S4, S5). Intermingling roots of non-cluster-root species were distinguished by colour, size, and lateral branching.

In all 57 image frames with cluster roots, at least one other root type was observed before, during or after the development of a live cluster root. This is in strong contrast with the overall probability of observing roots: only 11.3% of images had roots. We also found 257 cases of intermingling between non-cluster herbaceous and woody roots out of a total of 2034 non-cluster roots (i.e. 12.6% of non-cluster herbaceous roots were intermingling during their emergence with non-cluster woody roots). As such, the frequency of intermingling between non-cluster roots is considerably lower than that of intermingling between cluster roots and non-cluster roots ($X^2 = 331$, $df = 1$, $P < 0.001$). Furthermore, the maximum number of non-cluster roots intermingling with cluster roots within the same image frame was six, which occurred in an irrigated plot (data not shown). The majority of root intermingling with cluster roots (95%) was observed under irrigation (155 roots) compared with non-irrigated plots (9 roots, Fig. 2). Furthermore, in 90% of cases, intermingling roots appeared when live cluster roots were present, as opposed to before their appearance or after their senescence (Fig. 2; for photographs see Figs S2, S3, S4). The majority of intermingling cluster and non-cluster roots were found at the surface and upper-middle layers of the soil profile, rather than in deeper layers (Fig. S5).

Remobilisation of phosphorus and nitrogen from senescing cluster roots

To test if senesced cluster roots could serve as important sources of P or N, we determined total P and N concentrations in cluster roots at the first three stages of development (see Methods – Glasshouse experiment). Total P concentrations of cluster roots reached very low levels at stage III, the senesced stage (Fig. 3). We found a cluster-root P-remobilisation efficiency of 88.5% based on the mean values of stages I and III ($[\text{stage I} - \text{stage III}] / \text{stage I}$) from Fig. 3. Total N concentration in cluster roots peaked at developmental stage I (fully-formed cluster root) and was lowest at stage III (Fig. 3). Cluster-root N-remobilisation efficiency was 89.2%.

Root vitality of cluster rootlets

We found a substantial decrease in rootlet vitality, as expected, yet there were still some unstained, possibly live, rootlets at stage III (Fig. 4). However, the stain does not always bind to dead roots, in this case rootlets, especially those that only recently died. Therefore, these data may overestimate live rootlet percentage.

Phosphorus available to intermingling roots in developing and mature cluster rhizospheres

In order to calculate the amount of P possibly available to roots capable of intermingling with cluster roots, during their development, we gathered several values from field samples and the literature. First, at the study site, mean total soil P concentration is estimated to be 21.1 mg kg⁻¹, very similar to 17.8 mg kg⁻¹ which was measured on nearby Spearwood dunes (Turner et al. 2018). Part of this P, estimated as resin-extracted inorganic P (Pi; 1.35 mg kg⁻¹ and 1.27 mg kg⁻¹ in Turner et al. (2018)) is available to any root. Additional to this freely available Pi, cluster roots extract additional amounts of P by bringing inorganic and organic P in solution (mobilised by carboxylates) and hydrolysing organic P (i.e. phosphatase-extractable P)

(Delgado et al. 2013; Shane et al. 2004; Watt and Evans 1999). Carboxylate-extractable Pi was estimated as 2.16 mg kg⁻¹ dry soil from older studies (McPharlin et al. 1994; Ozanne and Shaw 1968; Pathan et al. 2003; Watson et al. 1982). We measured Carboxylate-extractable Pi (citrate-extractable P) as 1.59 mg P kg⁻¹ dry soil and organic P that could potentially be accessed (i.e. Organic-P available) by our *Banksia* cluster roots. This Organic-P available fraction was estimated as 16.7 mg P kg⁻¹ dry soil, similar to the value of 12.5 mg P kg⁻¹ dry soil found in recent work from our group (Zhou et al. unpublished) and similar to the NaOH-extractable organic P of 10.6 mg P kg⁻¹ dry soil from Turner et al. (2018).

Phosphorus available to intermingling roots in senesced cluster rhizospheres

In order to calculate the amount of P possibly available to roots capable of intermingling with senesced cluster roots, we gathered several values from field samples and the literature. We made these calculations, given that cluster roots produce an abundance of fine root biomass in a small volume of soil, and despite efficient remobilisation of P from senescing cluster roots, we hypothesised that some remaining P might be available to intermingling roots. As such, we first estimated this amount of P, expressed as a soil P concentration, as the product of the P concentration in senesced clusters and the dry mass density of cluster roots in soil.

Senesced clusters contain 118 mg P kg⁻¹ dry weight; stage III in Fig. 3). Estimates for the density of cluster roots in soil were 0.0284 g cluster roots per g dry rhizosphere soil, similar to what was measured in another *Banksia* study (Pate and Watt 2002). Therefore, the amount of P remaining in senesced cluster roots is estimated at 118 mg P kg⁻¹ cluster roots x 0.0284 g cluster roots g⁻¹ soil = 3.36 mg P g⁻¹ dry rhizosphere soil (Fig. 5). As such, we estimate the volume used by these cluster roots is approximately 37.8 mg of cluster roots per cm³ of soil in these sandy soils.

Phosphorus available to intermingling roots during cluster-root development

Combining the information presented in the preceding sections, we expect non-cluster roots to have access to 1.35 mg P kg⁻¹ soil (Free Pi, resin-P values from Turner et al. (2018) and mentioned above) outside cluster rhizospheres, increasing to a potential 19.6 mg P kg⁻¹ soil (i.e. Free Pi (1.35) + Carboxylate-extractable Pi (1.59) + Organic-P available (16.68) Fig. 5) in cluster rhizospheres (but in competition with cluster roots), and then decreasing to a potential 3.36 mg P kg⁻¹ soil (senesced cluster roots) after clusters have senesced (assuming that P in senesced clusters is completely available to other roots or their mycorrhizas) (Fig. 5). Based on our previous work, we estimated a mean lifespan of 23 days for *Banksia* cluster roots (Teste et al. 2018); as such we suggest that P from senescing cluster roots could become available to other roots from ~23 days after the start of CR development.

Discussion

We documented the *in situ* seasonal dynamics of *Banksia* cluster-root formation with minirhizotrons, and determined, for the first time, the degree of intermingling events by roots of non-cluster-rooted species. The very high frequency of roots intermingling with cluster roots, relative to the cluster roots detected with our minirhizotrons, during their development and period of peak activity represents the first evidence of physical interaction between such roots, and shows that P-mobilisation facilitation may potentially operate in these nutrient-poor soils. Although intermingling certainly occurred between non-cluster roots, we found that the presence of cluster roots significantly promoted intermingling events, since we always observed the appearance of at least one other non-cluster root. Our estimates of soil P availability before, during and after peak cluster-root activity, support the hypothesis that potential benefits exist for non-cluster roots of invading cluster rhizospheres, and that these benefits are greatest during periods of cluster-root exudation.

Conditions for roots intermingling with cluster-roots

Root intermingling was prominent, and P-uptake facilitation likely. We found that roots of other non-cluster rooted species freely intermingled with all *B. attenuata* cluster roots that we observed, especially when cluster roots were mature or developing. These intermingling events were particularly pronounced when soil moisture conditions were at a historical, rather than current precipitation level for this Mediterranean woodland (Teste et al. 2018). We recently demonstrated that *Banksia* spp. may be sensitive to a drying climate, with a lower incidence of cluster roots under drier soil conditions (Teste et al. 2018). Further research is needed to elucidate the mechanism of intermingling, and specifically the signal(s) triggering growth of roots towards cluster roots. It is unlikely that the signal is P itself, because P migration in soil is very slow (Lambers and Oliveira 2019). Biogenic volatile organic signals may be involved (Peñuelas et al. 2014a): cluster roots may release VOCs into surrounding soil and prime more root proliferation from intraspecific roots. This would be a novel mechanism for VOCs, but would resemble the root-to-root signalling that has been documented between unstressed plants to drought-stressed plants (Falik et al. 2011; Peñuelas et al. 2014b). It is also possible that both cluster roots and intermingling roots respond to a common signal indicating a locally-high resource level such as resource-rich patches (Hodge 2009). The signal could be a mobile nutrient, such as nitrate, or a volatile signal generated by roots or soil microbes. The possibility of nitrate as a signal for root proliferation, which may provide a large potential benefit for nutrients such as P in our study system, suggests an alternative explanation for the observation in the study of van Vuuren et al. (1996), where many roots grew after most of the N in an enriched patch had been depleted. Those late-growing roots probably still had significant benefits from nutrients other than N.

Phosphorus benefits to roots intermingling with cluster roots

The P-acquisition benefits that other roots could gain from intermingling with cluster roots in terms of P availability in soil during cluster-root development and senescence (i.e. during and after cluster activity; Fig. 2) is considerable (Fig. 5). Such a benefit would come from the P-mobilising activity of cluster roots through exuded carboxylates and phosphatases. However, the P availability in both living and senesced cluster rhizospheres are maximum estimates, given that cluster roots are likely strong competitors for P uptake, and P in senesced clusters may largely be in organic forms, or immobilised by microbes, and thus not available to other roots. We expect that the peak of the P-acquisition benefits would be short-lived and occur between ~20 to 30 days after the initiation of the development of the cluster root, based on our glasshouse and field observations of the development stages of *Banksia* cluster roots (Teste et al. 2018) and well-documented observations of cluster-root physiology in hydroponics (Shane & Lambers 2005). We estimated that 16.9 mg P kg⁻¹ soil represents the theoretical maximum amount of P available to intermingling roots during the presence of an active cluster root, assuming all P made available by the cluster roots could be taken up by intermingling roots. While clusters are active (matching the period of maximum exudation of carboxylates and phosphatases), plant-available soil P increases from 1.35 mg kg⁻¹ (i.e. resin-P) to a maximum of 16.9 mg kg⁻¹ (Fig. 4). Due to the very high density of cluster rootlets relative to intermingling roots in the rhizosphere, cluster roots would have a competitive advantage for uptake of the P mobilised by themselves. Nevertheless, intermingling roots that invade the cluster rhizosphere, or are at the outer boundary of the clusters, are positioned within a zone of influence of carboxylates and phosphatases where they may benefit from mobilised P. Our estimates indicate that there is more P in living cluster roots than extractable soil P in that same soil volume (Fig. 5), demonstrating that both efficient soil P extraction and efficient P remobilisation from senescent roots are essential for cluster roots to be effective and a functional investment benefitting the plant's P economy. Intermingling roots need to compete with cluster roots to access mobilised P pools; and we expected that effects from the large

amounts of carboxylates exuded by cluster roots and their high-affinity P transporters would maximise the share of that mobilised P for the cluster roots. However, the theoretical maximum amounts of P that could be gained are worth further empirical testing.

Remobilisation of root phosphorus during cluster root senescence

The amount of P remaining after senescence of the cluster roots might be accessible to non-cluster roots entering or staying in that “senesced-cluster region”, and although the amounts are small, such roots might derive a P benefit from decomposing cluster roots in these impoverished soils. We found a very high root P-remobilisation efficiency of 89% based on determining the root P concentrations during the development and fully senesced stages of our *Banksia* cluster roots. Physiological studies of *Hakea* cluster roots with controlled hydroponic systems found similarly high root P-remobilisation efficiency of 95% (Shane et al. 2004). These results show only a very small amount of P remains after a cluster has senesced for uptake by intermingling non-cluster roots (Fig. 5), compared with the amount that may be gained by intermingling with active cluster roots. Our observation that intermingling roots arrive when cluster roots are alive, rather than senesced (Fig. 2), supports the concept that intermingling roots respond to a signal indicating the imminent opportunity of enhanced resource availability (P and micronutrients), and that most resources become available through cluster-root induced mobilisation, rather than cluster-root decomposition.

Evidence for the potential of facilitated uptake of phosphorus

The facilitated uptake of P that may occur between cluster-rooted species and other interacting plant species such as mycorrhizal plant species falls under two of the three broad facilitative mechanisms proposed by Wright et al. (2017). First, indirect biotic facilitation may result if other roots can scavenge the P that is mobilised by the active cluster root. Second, abiotic facilitation due to local P enrichment by senesced cluster roots remains a

possibility in the most severely-impoverished soils where very small amounts of P can still increase plant growth of other plant species capable of taking up the P ‘mined’ by the cluster roots. Our findings suggest that the first mechanism, indirect biotic facilitation, is probable with some level of P enrichment of the cluster rhizosphere during mobilisation of soil P as previously proposed (Gardner and Boundy 1983; Lambers et al. 2013; Li et al. 2007). Nutrient-mobilisation-based facilitation as proposed in Lambers et al. (2018) and demonstrated in crop plants (e.g., lupin) may also be ecologically relevant for iron (Fe), zinc (Zn) and manganese (Mn), since these micronutrients are also mobilised during cluster-root activity (Li et al. 2014). Nutrient (P, Fe, Zn and Mn)-mobilisation-based facilitation is mainly driven by: i) the release of large amounts of organic acids and chelating substances from cluster roots that increase the concentrations of these nutrients in the soil solution (Lambers et al. 2018); and ii) the ability of other roots to first sense the activity of cluster roots and then grow in the rhizosphere of cluster roots directly or via their extra-radical mycorrhizal hyphae (i.e. in the case where mycorrhizal roots are intermingling). Microcosm studies using *Banksia* as the cluster-rooted plants lend some support to this principle; improved plant growth of neighbouring and interacting plants was shown (Muler et al. 2014; Teste et al. 2014). We suggest that nutrient-mobilisation-based facilitation is more common than previously thought; however, more formal testing is required (see section on *Future directions* below).

Potential impacts of a drying climate on interactions with cluster roots

Plant species compositional shifts may occur due to a drying climate, if P and micronutrient uptake of coexisting plants is facilitated by the root activity of cluster-rooted plants (Lambers et al. 2018; McIntire and Fajardo 2014). Cluster-root development and its capacity to mobilise P from poorly-available forms in soil is affected by soil moisture levels (Lamont 1976; Lamont 2003). We found that lower soil moisture levels reduce cluster-root occurrence

in this system (Teste et al. 2018); thus their P-mobilising activities would also be reduced in a given plant community. We also found that the majority of root intermingling between non-cluster roots and cluster roots occurred in the surface soil layers, where soil drying is typically faster than that in deeper soil layers. Our findings suggest that a considerable amount of P benefits may be obtained when coexisting plants intermingle their roots in the rhizospheres of cluster-rooted plant species. Thus, plant species that depend on this type of interaction to meet their P demand may be the first to succumb in plant communities dominated by cluster-rooted plants during further drying of this Mediterranean environment.

Future directions

Radio-isotope P work (i.e. ^{33}P and ^{32}P) would help quantify the magnitude of P-mobilisation facilitation, but documenting radioactive P movement in megadiverse habitats would be very challenging, if not impossible. Analysis of the spatial pattern between cluster-rooted *Banksia* plants and plants potentially interacting could convey evidence for facilitation, but the lateral extent of *Banksia* cluster roots is far reaching (~2x tree height in *B. prionotes* (Jeschke and Pate 1995)), so the distance between plants may not be a good predictor of the intensity of facilitative relationships. As such, current methodology such as minirhizotrons, nutrient tracers, and microcosm experiments will still be needed to progress our understanding of the roles of belowground facilitation in plant species coexistence. Finally, to robustly test for facilitation, future studies could entail: i) identifying the plant species whose roots intermingle with cluster-rooted plants; ii) quantify the differences between foliar P concentrations with and without neighbouring *Banksia*; and iii) quantify a metric that could serve as a proxy for improved fitness (e.g., seed production).

Conclusions

We provide evidence for *in situ* root interactions between cluster-rooted *Banksia* species and roots of other species with implications for species coexistence when soil moisture conditions are favourable. We calculated the potential P benefits to roots growing in or near the rhizosphere of clusters during and after their peak activity. The most frequent root interaction type described here was when soils were relatively moist, and can be viewed as a plant version of ‘kleptoparasitism’ (Gorman et al. 1998; Thompson 1986; Zomora 1995). These are the first accounts of a kleptoparasitism-type root interaction, and we suggest that such interactions occur between other cluster-rooted species and neighbouring plants with considerable potential for nutrient-mobilisation-based facilitation. These interactions and facilitative mechanisms are likely also relevant for other specialised root strategies (e.g., carboxylate-releasing simple cluster roots, dauciform roots, sand-binding roots, vellozioid roots) and in other P-impoverished ecosystems such as campos rupestres (Abrahão et al. 2019; Teodoro et al. 2019). Such interactions may promote species co-existence and act as a means of increasing species diversity in severely nutrient-impoverished soils.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data accessibility

Data will be made available as part of a separate digital file in the Electronic Supplementary Material section (Data S1).

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Figure legends

Fig. 1* Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster root (shown with white arrow) and other roots (shown with black arrow) of other species (*i.e.* herbaceous and woody plants) in a banksia-eucalypt woodland (Tube 20, Window 4 from 9 September to 5 October, 2012). In top image, a cluster root starts to form with other roots seen below. Based on this series of images, a frequency of intermingling response was generated; here, frequency = 2, since two non-cluster roots are seen in the same image frame. Since ‘other roots’ were present before the appearance of the cluster root, we named this root interaction ‘before cluster root’ (BeforeCR)). Also see Fig. S3 and Fig. S4 for photographic examples of the other interaction types, and see Fig. 2 for differences in the frequency of interactions with irrigation and amongst the two general root types. See Teste et al. (2018) for an estimate of *in situ* survival rates of cluster roots and their rootlets. Also see Shane and Lambers (2005) for photographs of *Banksia* cluster roots that clearly show their ‘compound’ shape. [*Figure to be possibly reworked by the journal’s illustrator].

Fig. 2 Frequency of intermingling of cluster roots and herbaceous or woody roots during April – October 2012 in a banksia-eucalypt woodland. This intermingling response was based on the joint occurrence of live cluster roots with other live roots in small minirhizotron image frames (height = 1.35 cm, width = 1.8 cm) that hosted at least one live cluster root. The other roots appeared before the cluster root developed (BeforeCR; Fig. 1), while the cluster root was alive (DuringCR; Fig. S3), or during senescence (AfterCR; Fig. S4). We found the negative binomial model to be the best fit (AIC = 199, Theta = 1.986) for these data; these showed a statistically significant interaction between the irrigation treatment and type of interaction (P -value = 0.011), while no significant effect of root type was found (see Table S1 for summary statistics).

Fig. 3 Concentrations of total nitrogen (N) and phosphorus (P) across the developmental stages of cluster roots. Values are means with 95% confidence intervals, and different letters to indicate statistically-significant differences between means (Tukey HSD tests with $P \leq 0.05$). Shown here are four developmental stages of cluster roots: stage pre-cluster (immature cluster roots; $n=21$); stage I (fully-formed cluster roots; $n=16$); stage II (cluster roots with rusty brown colour and some loss of vigour; $n=34$); and stage III (dark brown or grey cluster roots with noticeable loss of vigour; $n=29$).

Fig. 4 Percent of live (*i.e.* unstained) rootlets across the developmental stages of cluster roots. Values are mean percent of live rootlets with 95 % confidence intervals, and different letters indicate statistically-significant differences between means (Tukey HSD tests with $P \leq 0.05$). Shown here are three developmental stages of cluster roots: stage I (fully-formed cluster roots; $n=16$); stage II (cluster roots with rusty brown colour and some loss of vigour; $n=16$); and stage III (dark brown or grey cluster roots with noticeable loss of vigour; $n=31$).

864 **Fig. 5** Phosphorus (P) concentrations typically found in Spearwood sandy soils, as well
865 as P contained in *Banksia* cluster roots (CR), all expressed per unit soil dry mass.
866 Carboxylate-extractable inorganic P (Pi) and organic-P are potentially available to
867 intermingling roots (indicated with root illustrations), in the short period that cluster
868 roots exude P-mobilising compounds (carboxylates and phosphatases). Phosphorus
869 remaining in senesced cluster roots is potentially available to intermingling roots after
870 CR death (indicated with root illustrations).

871

09 Sept.

872

19 Sept.

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28 Sept.

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05 Oct.

875 **Fig. 1***

876

877 **Fig. 2**

878

879 **Fig. 3**

880

881 **Fig. 4**

882

883 **Fig. 5**

884 **ELECTRONIC SUPPLEMENTARY MATERIAL**

885 **Table S1.** Analysis of deviance tables of the Poisson and negative binomial regression models (full and minimal adequate model determined via
886 the stepAIC function) for the Frequency response data (Fig. 2) of roots intermingling with live cluster roots (CR).

887

888 **Analysis of Deviance (Dev) summary tables**

889

890 **Poisson full model**

Source of Deviance	df	Deviance	Residual		Pr(>Chi)
			df	Dev	
NULL	-	-	40	177.39	-
Irrigation	1	20.109	39	157.28	<0.001
InteracWhen	2	13.298	37	143.98	0.001
RTInterac	1	0.215	36	143.76	0.643
Irrigation:InteracWhen	2	15.329	34	128.44	<0.001
Irrigation:RTInterac	1	0.700	33	127.73	0.403
InteracWhen:RTInterac	2	2.561	31	125.17	0.278
Irrigation:InteracWhen:RTInterac	2	0.719	29	124.45	0.698

891 InteracWhen: BeforeCR, DuringCR, or AfterCR. RTInterac: the other types of roots (Herbaceous or Woody) interacting with CR.

892

893 **Poisson minimal adequate model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	177.39	-
Irrigation	1	20.109	39	157.28	<0.001
InteracWhen	2	13.298	37	143.98	0.001
Irrigation:InteracWhen	2	15.196	35	128.78	0.001

894 df: degrees of freedom

895

896 **Negative binomial full model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	58.4737	-
Irrigation	1	8.2489	39	50.2248	0.0041
InteracWhen	2	4.8166	37	45.4082	0.09
RTInterac	1	1e-04	36	45.4081	0.9913
Irrigation:InteracWhen	2	9.2123	34	36.1958	0.01
Irrigation:RTInterac	1	0.2084	33	35.9874	0.648
InteracWhen:RTInterac	2	1.5634	31	34.424	0.4576
Irrigation:InteracWhen:RTInterac	2	0.5945	29	33.8295	0.7429

897

898 **Negative binomial minimal adequate model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	57.0737	-
Irrigation	1	8.059	39	49.0146	0.0045
InteracWhen	2	4.7076	37	44.307	0.095
Irrigation:InteracWhen	2	9.0556	35	35.2514	0.0108

899 df: degrees of freedom.

900 **Figure legends for ESM**

901 **Fig. S1** Location of the research plots (white triangle) where this study using
902 minirhizotron tubes was carried out. A photo of the irrigation treatment in action on Plot
903 6 from Teste et al. (2018) with a *Banksia attenuata* tree in the centre with white-capped
904 minirhizotron tubes all around. Also shown is the Bartz BTC2 Minirhizotron video
905 camera imaging on Plot 2. Plots were situated in remnant banksia-eucalypt (*Eucalyptus*
906 *gomphocephala*) banksia woodland of the UWA Shenton Park Field Station (31°56' S,
907 115°47' E) that is situated on the Swan Coastal Plain, Western Australia.

908 **Fig. S2*** Photographs of *Banksia attenuata* cluster-root appearance (shown with arrow)
909 and development *in situ* in a banksia-eucalypt woodland (Tube 2, Window 4 from 29
910 August to 5 October 2012). See Teste et al. (2018) for an estimate of *in situ* survival
911 rates of cluster roots and their rootlets. Also please see Shane and Lambers (2005) for
912 photographs of *Banksia* cluster roots that clearly show their 'compound' shape.
913 [*Figure to be possibly reworked by the journal's illustrator].

914 **Fig. S3*** Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster
915 root and other roots of other species (i.e. herbaceous and woody plants) in a banksia-
916 eucalypt woodland (Tube 20, Window 5 from 29 August to 5 October, 2012). Since
917 'other roots' appeared during the development of a live cluster root we named this root
918 interaction 'during cluster root (DuringCR)'. See Fig. 2 for differences in the frequency
919 of interactions with irrigation and amongst the two general root types. [*Figure to be
920 possibly reworked by the journal's illustrator].

921 **Fig. S4*** Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster
922 root and other roots of other species (i.e. herbaceous and woody plants) in a banksia-
923 eucalypt woodland (Tube 2, Window 19 from 22 August to 19 September, 2012). Since
924 'other roots' appeared after the development of the cluster root we named this root
925 interaction 'after cluster root (AfterCR)'. See Fig. 2 for differences in the frequency of
926 interactions with irrigation and amongst the two general root types. [*Figure to be
927 possibly reworked by the journal's illustrator].

928 **Fig. S5** Frequency of other roots (herbaceous and woody) intermingling with cluster
929 roots over distinct soil depth ranges (0 to 10 cm, 11 to 20 cm, and 21 to 40 cm) during
930 months of peak root growth (April – October) of 2012 in a banksia-eucalypt woodland.
931 This intermingling response was based on the joint occurrence of live cluster roots with
932 other live roots in small minirhizotron image frames (height = 1.35 cm, width = 1.8 cm)
933 that hosted at least one live cluster root. The other roots appeared before the cluster root
934 developed (BeforeCR; Fig. 1), while the cluster root was alive (DuringCR; Fig. S3), or
935 during senescence (AfterCR; Fig. S4).

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942

943 **Fig. S1.**

944

29 Aug.

945

09 Sept.

946

19 Sept.

947

28 Sept.

948

05 Oct.

949

Fig. S2*

950

No roots observed

951

952

root

Appearance of an axis of a cluster root and a woody

953

954

955

956

Fig. S3*

957

Senescent cluster root

958

Growing woody root in proximity to senescent

959 cluster root

960

961

962

Fig. S4*

963

964

965 **Data S1**

966 Data table S2 (separate Microsoft Excel file) has all data associated with this study.

[Click here to view linked References](#)

**The potential for phosphorus benefits through root placement in the rhizosphere of
phosphorus-mobilising neighbours**

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of the manuscript and all authors collected data and contributed substantially to revisions.

Abstract

Plants that produce specialised cluster roots, which mobilise large quantities of poorly-available nutrients such as phosphorus (P), can provide a benefit to neighbouring plants that produce roots in the cluster-rhizosphere, as demonstrated previously in pot studies. To be effective, such roots must be present within the short time of peak cluster activity. We tested if this requirement is met, and quantified potential P benefits, in a hyperdiverse Mediterranean woodland of southwest Australia where cluster-rooted species are prominent. Using minirhizotrons, we monitored root dynamics during the wet season in the natural habitat. We found non-cluster roots intermingling with all 57 of the observed cluster roots of the studied tree species, *Banksia attenuata*. Almost all (95%) of these cases were observed in a high-moisture treatment simulating the 45-year average, but not present when we intercepted some of the rainfall. We estimate that cluster-root activity can increase P availability to intermingling roots to a theoretical maximum of 80% of total P in the studied soil. Due to their high P-remobilisation efficiency (89%), which results from P rapidly being relocated from cluster roots within the plant, senesced *Banksia* cluster roots are a negligible P source for other roots. We conclude that, rather than serving as a P source, it is the cluster-root activity, particularly the exudation of carboxylates, that may improve the coexistence of interacting species that are capable of root intermingling, thus potentially promoting species diversity in nutrient-poor habitats, and that this mechanism will be less effective in a drying climate.

Keywords: *Banksia attenuata* and *B. menziesii*, climate change, minirhizotron, phosphorus-mobilisation facilitation, phosphorus remobilisation.

Introduction

When plant roots intermingle, are there interactions among interspecific neighbours, and if so, what are the potential consequences of such interactions? Classical plant root studies demonstrate that roots of competing species tend to avoid each other, and thus reduce belowground resource competition for soil nutrients and water (Schenk 2006). Yet, recent root research, often using advanced tools to study roots *in situ* (Metzner et al. 2015; Schmittgen et al. 2015; van Dusschoten et al. 2016), shows that root intermingling is perhaps more common than previously thought (Mommer et al. 2016; Oburger and Schmidt 2016). The main scenarios that promote root intermingling, rather than root avoidance, have been well-documented, and include: i) kin recognition (intraspecific roots), ii) nitrogen-uptake facilitation (interspecific roots), where N₂-fixing plants increase the availability of N to other plants, and iii) patchiness of soil resources (intra- and interspecific roots) where the presence of localised high resource levels (e.g., N) leads to root proliferation (Chen et al. 2018; Faget et al. 2013; Hodge 2012; Li et al. 2016). However, it is not known if root intermingling is common when phosphorus (P) is the key limiting nutrient for plant growth, and what the potential benefits of intermingling are.

In severely P-impooverished soils of the hyperdiverse sandplains of southwest Australia's biodiversity hotspot, many plants form cluster roots that are among the most efficient roots to acquire poorly-available P from such soils (Lambers et al. 2015). Cluster roots comprise a main axis and many determinate ephemeral rootlets (Shane and Lambers 2005). Large quantities of carboxylates, but also likely acid phosphatases, protons, and chelating substances, are released in an 'exudative burst' from cluster roots in several species (Delgado et al. 2013; Shane et al. 2004; Watt and Evans 1999), whereas in *Banksia* species, which have more complex cluster roots, exudation appears to occur at slower rates but for longer periods (Beeck 2017; Shi et al. 2020). The release of carboxylates effectively increases the

concentration of P and some micronutrients in the soil solution, thereby making them available for uptake (Shane and Lambers 2005). The development of cluster roots that exude large quantities of carboxylates into the cluster rhizosphere is referred to as ‘cluster-root activity’. As such, cluster-root activity involves a time-dependent process that increases P uptake by the cluster roots. Furthermore, there is evidence that, as a ‘side effect’, this process promotes P-uptake facilitation, which appears to occur when neighbouring plants grow along cluster-rooted plants (Gardner and Boundy 1983; Li et al. 2007; Muler et al. 2014). Yet, we lack empirical evidence showing *in situ* that other plant roots actually physically intermingle during these rapid biochemical rhizosphere processes (Lambers et al. 2018; Teste et al. 2018).

Root intermingling may be prevalent in P-limited environments to acquire sorbed P mobilised by carboxylates released by neighbours, but it is unclear what might direct the growth towards P-mobilising cluster roots, and what level of P-uptake benefits may result from such root interactions. Intermingling might be a random process, due to non-cluster roots being attracted by signals associated with cluster-root activity, or both non-cluster and cluster roots may be attracted by the same trigger (e.g., a high resource patch). A first step in elucidating mechanisms of intermingling with cluster roots is to demonstrate that intermingling is not random, to quantify its frequency, and to determine its timing relative to peak cluster-root activity. If non-cluster roots show a tendency to arrive in the rhizosphere of cluster roots when these are most active, this is likely an adaptive response to temporarily-enhanced resource availability.

Our ongoing work on foliar nutrient patterns within and in the neighbourhood of cluster-rooted plant species is pointing to a considerable potential for P-mobilisation based facilitation in nutrient-poor soils (Abrahão et al. 2019). We have found increases in foliar manganese (Mn) levels in plant species neighbouring cluster-rooted species compared with

controls without cluster-rooted neighbours (Muler et al. 2014). More recent studies show that plants that are potentially facilitated by carboxylate-releasing neighbours exhibit increased leaf [Mn] (Yu et al. in press; Yu et al. 2020). Here, for the first time, we present evidence that the fundamental conditions, relating to root positioning and rhizosphere-P dynamics, enable a considerable P benefit for other roots occurring within the rhizosphere of cluster roots. Global climate change is leading to substantial drying climates in most Mediterranean ecosystems (Diffenbaugh and Field 2013; Indian Ocean Climate Initiative 2012). Southwest Australia's Mediterranean ecosystems, which host a biodiversity hotspot and many cluster-rooted plant species (Lambers et al. 2014; Lamont 1982; Myers et al. 2000), could be affected by the drying climate. The production of cluster roots is stimulated by high surface soil moisture levels (Lamont 2003), and it appears that a minimum-threshold soil moisture level is required to trigger the plant into forming cluster roots (Lamont 1976; Lamont 2003). Our recent study in this ecosystem demonstrated a greater presence of cluster roots at high soil moisture levels compared with drier controls (Teste et al. 2018). However, effects of declining soil moisture levels on root interactions remain poorly explored in the field. Since plants that produce cluster roots are abundant and widespread in this biodiverse ecosystem, yet are particularly sensitive to changes in soil moisture, we proposed that plant community compositional shifts may continue to occur (Crosti et al. 2007; Fisher et al. 2009; Teste et al. 2018). In addition, we suggest that reductions in plant diversity may occur: i) if cluster-rooted plants become less efficient at mining soil P due to narrower 'windows' of favourable soil moisture conditions (Teste et al. 2018); ii) P is less mobile in dry soil than in moist soil thus exacerbating point i) (Lambers et al. 2008); and iii) P and micronutrient uptake of coexisting plants is indeed facilitated by the root activity of cluster-rooted plants (Lambers et al. 2018).

127 The overall aim of this study was to determine whether the spatial and temporal dynamics of
128 cluster roots and roots of neighbouring plants provide clues for a potential P-mobilisation
129 facilitative role of cluster roots. Our previous work (Muler et al. 2014; Teste et al. 2014)
130 provided the theoretical underpinning and experimental support for facilitation by cluster-
131 rooted species, but we did not yet provide visual evidence of root intermingling *in situ*, which
132 is fundamental to a P-facilitation hypothesis (Lambers et al. 2018). Here, we test if root
133 placement and rhizosphere P conditions are such that they would make root-to-root
134 facilitation possible. Using a combination of minirhizotron field and glasshouse experiments,
135 and P budgets during root intermingling, we estimate the potential benefits of P mobilisation
136 by cluster roots to interacting non-cluster rooted plants. The study aimed to test: i) is there
137 evidence of root intermingling between cluster-rooted species and other plant species? ii) Do
138 roots from neighbouring plant species intermingle before, during, or after cluster-root activity;
139 and does greater soil moisture availability intensify intermingling? iii) How much P is
140 potentially made available in the cluster rhizosphere?

141

142 Kleptoparasitism is parasitism by theft of nutriment or a key resource. Kleptoparasitism is a
143 well-documented animal-animal interaction (Gorman et al. 1998; Thompson 1986), where the
144 optimal foraging of one species of animal is taken advantage of by another species by timely
145 ‘robbery’ of a key resource (e.g., food). This type of biological interaction has also been
146 found between carnivorous plants and predatory insects (Zomora 1995). Kleptoparasitism
147 between plants has yet to be shown, yet it may exist in P-impooverished systems, particularly if
148 timely intermingling by non-cluster rooted plant species occurs during the peak-P foraging
149 activity of cluster-rooted plants.

150

151 *Banksia attenuata* and *B. menziesii* (Proteaceae) were used as the focal tree species, since they
152 produce an abundance of large compound cluster roots (Denton et al. 2007b; Shane and

Lambers 2005). These *Banksia* species are long-lived trees with extensive lateral and deep roots where cluster roots are commonly found just below the leaf litter and top layers (0 to 20 cm) of the soil profile (Lambers 2014; Pate and Watt 2002; Teste et al. 2018). Root dynamics and interactions of all roots near the *B. attenuata* trees were monitored using installed minirhizotron tubes and cameras, and we quantified P concentration and remobilisation from cluster roots of *B. menziesii* in a glasshouse. These two *Banksia* species are physiologically similar and prominent components of these woodlands (Denton et al. 2007a; Lambers et al. 2012; Pate and Dell 1984). Finally, to evaluate the ways in which other roots might benefit from intermingling with cluster roots, the potential P availability in cluster-rhizospheres and after cluster root senescence was estimated.

Methods

Study site

Experimental irrigated and unirrigated plots were established in banksia-eucalypt woodland at The University of Western Australia's (UWA) Shenton Park Field Station (31.96°S, 115.79°E) (Supporting Information Fig. S1). The site is located in the kwongan vegetation type on the Swan Coastal Plain within the Southwest Australian Floristic Region (sensu Hopper and Gioia 2004). The site is part of the Spearwood coastal dune system (McArthur and Bettenay 1974; Wyrwoll et al. 2014). The sandy soils are severely nutrient-impooverished (Turner et al. 2018), and the climate is Mediterranean with a mean annual temperature of 19.2°C and average annual rainfall of 702 mm (Australia's Bureau of Meteorology; <http://www.bom.gov.au/climate/data/>; 1967 to 2011), where most of the rain occurs between May and September, the cooler "winter months" of the Southern Hemisphere. During these winter months, most of the root growth including cluster roots occurs (Lamont 2003; Pate and Beard 1982), thus the likelihood of root intermingling is high. A complete description of the study site and experimental design are included in Teste et al. (2018).

179

180 In May 2011, 40 minirhizotron clear tubes (Bartz Technology Corp., Carpinteria, CA, USA,
181 internal diameter = 50.8 mm) were installed to a depth of 0.5 m at 1 m and 2.5 m from the
182 basal stem of ten *B. attenuata* trees with two irrigation treatments (i.e. on 10 plots x 2
183 distances x 2 irrigation treatments = 40 tubes). Tubes were prepared following similar
184 procedures outlined in Smit et al. (2000) and Johnson et al. (2001) to eliminate artefactual
185 effects of light and temperature. Prior to imaging roots with a BTC2 Minirhizotron Video
186 Microscope (Bartz Technology Corp., Carpinteria, CA, USA), the minirhizotron tubes were
187 left to stabilise for 11 months. From April 2012, sprinklers distributed water evenly to a
188 radial distance of 4 m around the minirhizotron tubes. This irrigation treatment started a
189 month before the winter rains and produced a 25% increase in monthly winter water inputs
190 (from April to October) based on 45 years of rainfall data (1967 to 2011) from a nearby
191 weather station (Subiaco treatment plant, Station # 009151, Lat: 31.96°S, Long: 115.79°E;
192 Bureau of Meteorology, <http://www.bom.gov.au/climate/averages/>). The plots were irrigated
193 every month, and by October, we had added a total of 148 mm of supplementary water to
194 each plot. The minirhizotron tubes around the mature *Banksia* focal trees were positioned to
195 be within the radial extent of the water spray provided by the sprinklers used in the irrigation
196 treatment (Teste et al. 2018). Furthermore, we inserted the tubes within the main root zone of
197 each focal tree. As for soil depth, the range of depths used to image roots was mainly
198 determined by the limitations of the minirhizotron equipment (tubes and camera) and the
199 feasibility of gathering images within the same day in the field. Furthermore, we knew that
200 imaging beyond 50 cm depth was unlikely to render observations of cluster roots, since they
201 are typically restricted to the top 15 cm of the soil profile (Denton et al. 2007b; Pate and Watt
202 2002).

203

204 *Image capture and analysis*

205 A complete description of the procedures involved in the root image capture and analysis is
206 available in Teste et al. (2018). In summary, roots were imaged between 4 April 2012 and 5
207 October 2012, and root dynamics and soil moisture were monitored on a weekly basis when
208 possible. The captured minirhizotron images were then analysed with Rootfly v. 2.0.2, an
209 open-source software application (Zeng et al. 2010). Rootfly allowed us to annotate (with a
210 computer mouse) the roots, determine the type of root (cluster (Fig. 1, [S2](#)), woody or
211 herbaceous roots, based on structure and colour), and calculate the birth and death rates of
212 roots through time. In this study the minirhizotron approach was used to track root
213 intermingling over time in a non-destructive manner *in situ*. We annotated a ‘cluster-root’
214 type only when we saw rootlets at some point in time during root-imaging from April to
215 October. *Banksia* roots that did not produce rootlets could not be differentiated from other
216 woody roots; therefore, they were categorised as a ‘woody’ root type. A ‘cluster-root
217 intermingling’ response was calculated based on minirhizotron images (image frame = 13.5
218 mm by 18 mm) that had co-occurring root types of which at least one was a cluster root.

219

220 *Glasshouse experiment*

221 In August 2012, a glasshouse experiment with *B. menziesii* seedlings grown in nutrient-
222 impoverished Bassendean sand (see Laliberté et al. (2012) for specific soil characteristics)
223 was established with the objective to quantify cluster-root nutrient concentrations and -
224 remobilisation during senescence. *Banksia menziesii* seedlings were grown in 14 rhizoboxes
225 (2.2 L) and in plastic bags inside 40 polyvinyl chloride (PVC) pots (2.1 L). Our aim was to
226 simultaneously photograph and sample cluster roots at their various developmental stages.
227 Four stages were morphologically identified: stage 1, immature cluster roots; stage 2, fully-
228 formed cluster roots; stage 3, cluster roots with rusty brown colour and some loss of vigour
229 (shrinking determined visually); stage 4, dark brown or grey cluster roots with noticeable loss
230 of vigour (widespread shrinking and wrinkling determined visually). Between 10-15 January

231 2013, half of the seedlings were harvested, washed and cluster roots were then cleaned and
232 oven-dried at 70 °C for 48 hours.
233
234 Due to the nature of cluster roots, small sand particles often tightly adhere to the root cluster.
235 Therefore, once dried, the cluster roots were again cleaned to remove adhering sand to
236 accurately quantify dry weight and chemical composition of the roots. Thus, cluster roots
237 were placed between two fine mesh holders and subjected to pressurised air until most of the
238 sand was removed. Any remaining sand was accounted for, by subtracting its weight from
239 the sample dry weight, during root digests, prior to chemical analysis (see *Chemical analysis*
240 *of cluster roots* below).
241
242 *Chemical analyses of cluster roots*
243 After sand removal, fragmented cluster roots were weighed and analysed for total P and
244 silicon (Si) by atomic absorption spectrometry (Perkin Elmer 500 DV ICP-AES,
245 Massachusetts, USA), with an adapted protocol to deal with sand particles, at the ChemCentre
246 (<http://www.chemcentre.wa.gov.au/>), Perth, Australia. Since many cluster roots still
247 contained traces of adhering sand particles, the digestion in closed capsules was carried out to
248 avoid possible loss of acid digest solution (nitric-perchloric acid mixture) due to explosive
249 boiling-over. All remaining root samples were then re-analysed for P and SiO₂ using an
250 Elementar Vario Macro (Hanau, Germany) directly after combustion. Further analysis of the
251 evolved gases and digests was done with an inductively coupled plasma mass spectrometer
252 (Perkin Elmer, Elan 6000 ICP-MS, Norwalk, CT, USA) or axially-configured ICP-OES for
253 low-weight samples. Total N was quantified with the Elementar Vario Macro (Hanau,
254 Germany).
255
256 *Cluster-root vitality*

257 A modified Evans blue test was used to determine root vitality (Peterson et al. 2008). Two
258 approaches to stain the four stages of cluster roots were used; first, whole cluster roots were
259 stained by dipping them in Evans blue solution, and, second, the main root axes without the
260 rootlets were hand-sectioned prior to dipping. Cluster roots boiled in water for 5 minutes
261 were used as controls for this vitality assessment. Stained rootlets were observed under a
262 dissecting light microscope to visually determine if the root was dead (i.e. stained) or alive
263 (i.e. clear, not stained). The proportion of dead rootlets per 5 mm cluster root fragment was
264 determined with the gridline intersect method (Giovannetti and Mosse 1980).

265

266 *Structural dry matter*

267 Structural dry matter was determined for cluster-root stages 2 to 4. First, 200 mg of dried root
268 material was pulverised with a Retsch Tissue Lyser (TissueLyser Qiagen, Haan, Germany) at
269 25 Hz for 5 minutes. Then 700 µL of NaH₂PO₄ (0.1 M, pH 7.3) and 300 µl Tween-20 (0.1%,
270 v/v) were added to the pulverised material, which was then vortexed, pulverised again,
271 vortexed for 1 minute, and finally centrifuged at 8,164 g for 10 minutes. The supernatant was
272 removed, the material allowed to dry at 60 °C for 72 hours, then re-weighed. The structural
273 dry matter was then calculated as the difference between the initial dry weight minus the
274 weight after releasing soluble metabolites. Structural dry matter was quantified to express the
275 concentrations of N and P during the development of cluster roots on a structural dry weight
276 basis; this avoids the bias of any changes in non-structural dry weight (*e.g.*, non-structural
277 carbohydrates) during cluster development.

278

279 *Estimation of the amount of phosphorus made available by cluster roots*

280 We consider two ways in which other roots might benefit from intermingling with cluster
281 roots: 1) by taking up P made available through the action of cluster-root exudates
282 (carboxylates and phosphatases), i.e. novel P pools that would not be available for non-cluster

283 roots; or 2) by taking up P left behind in senesced cluster roots. To estimate the P potentially
 284 made available through the release of cluster-root exudates, 10 soil samples were collected at
 285 the study site, regardless of the irrigation treatment given that this field sampling was done six
 286 years after the temporary irrigation treatment ceased. These field samples were taken below
 287 *Banksia* trees from 0-0.05 m depth, adjacent to where cluster roots were imaged (Teste et al.
 288 2018). Total P was quantified by extraction in 1 M HCl (16 hours, 1:50 soil to solution ratio)
 289 of ashed samples (550 °C, 1 hour) modified after Saunders and Williams (1955). Free Pi
 290 (resin-P) was extracted with anion-exchange membranes (Turner and Romero 2009).
 291 Carboxylate-extractable Pi (citrate-extractable P) was assessed using a 0.05 M solution of
 292 citric acid (3 hours). Organic P (Soil organic P) was calculated as the difference between the
 293 1 M HCl-extracted P after and before ashing. This method may cause a slight overestimate of
 294 the organic P, because of a potential increase in acid-extractability of P in soil ash (Williams
 295 et al. 1970). Results are expressed on an oven-dry (105°C) weight basis.
 296
 297 To estimate the potential maximum amount of P available from decomposing cluster roots,
 298 first, cluster root per soil weight ratio was determined, and then the P concentration of
 299 senesced cluster roots (see above) was used to estimate the amount of P left behind by cluster
 300 roots per unit soil dry weight. Ten patches of mature cluster roots were located by removing
 301 the litter layer below *Banksia* trees at the study site, and pieces of this cluster-root mat (depth
 302 ~0-50 mm, area ~5000 mm²) were collected. *Banksia* cluster roots are easily found given
 303 their large size and characteristic mat-like structures (Shane and Lambers 2005). In the lab,
 304 one mini-core from each of the samples (diameter 21.5 mm, depth 11 mm, volume 3990 mm³)
 305 was taken, and live cluster roots from the soil (sand, organic matter, any other roots) were
 306 separated by adding water and using end-over-end shaking, sonification, and manual
 307 separation. Cluster root samples and soil samples were dried at 70 °C, weighed, and then
 308 ashed at 550 °C. The cluster root dry weight was adjusted for contamination with sand,

assuming that the ash content of cluster roots was 5% of their dry weight, based on values from other slow-growing tree species (George et al. 2003; Helmisaari et al. 2009; Ostonen et al. 2005).

Data analysis

The frequency of roots intermingling with cluster roots was analysed as count data with Poisson and negative binomial regression models (Zuur et al. 2009). Based on the image analysis described above, one of three ‘interaction’ types was assigned to each observation based on whether the co-occurring ‘other roots’ were first observed before (Fig. S21), during (Fig. S3), or after (Fig. S4) the presence of live cluster roots. The average lifespan of cluster-roots, including the immature and mature stage, was 23 days (Teste et al. 2018). For cluster roots that did not fully develop by the end of the study, interaction type ‘before’ or ‘during’ could still be assigned, but not ‘after’. As such, we analysed the interaction type and irrigation treatment as fixed effects (Table S1). When the irrigation treatment or other root types affected the frequency of interaction, negative binomial models were fitted to account for over-dispersion (Cameron and Trivedi 2005).

Total P and total N in cluster roots of different stages were compared with analysis of variance and confidence intervals followed by the Tukey HSD test. We calculated a cluster-root P-remobilisation efficiency (%) based on the mean values of stage I and other stages (e.g. [stage I – stage III] / stage I). Cluster-root vitality was analysed as proportion data with generalised linear models. The assumptions for the statistical models were assessed graphically, with plots of residuals and boxplots for treatment variances. All graphing and statistical analyses were conducted in R (R Core Team 2020) and ‘ggplot2’ packages (Therneau 2015; Wickham 2016).

Results

Root intermingling with cluster roots

A total of 2091 root fragments were imaged between April and October 2012, where 57 of these developed into mature cluster roots such as shown in Figs S2, S3, S4, and S5. As a cluster root appeared and developed, other roots were seen in very close proximity (i.e. within the same 13.5 x 18 mm image frame), likely interacting and possibly acquiring P mobilised by the cluster root (Figs S3, S4, S5). Intermingling roots of non-cluster-root species were distinguished by colour, size, and lateral branching.

In all 57 image frames with cluster roots, at least one other root type was observed before, during or after the development of a live cluster root. This is in strong contrast with the overall probability of observing roots: only 11.3% of images had roots. We also found 257 cases of intermingling between non-cluster herbaceous and woody roots out of a total of 2034 non-cluster roots (i.e. 12.6% of non-cluster herbaceous roots were intermingling during their emergence with non-cluster woody roots). As such, the frequency of intermingling between non-cluster roots is considerably lower than that of intermingling between cluster roots and non-cluster roots ($X^2 = 331$, $df = 1$, $P < 0.001$). Furthermore, the maximum number of non-cluster roots intermingling with cluster roots within the same image frame was six, which occurred in an irrigated plot (data not shown). The majority of root intermingling with cluster roots (95%) was observed under irrigation (155 roots) compared with non-irrigated plots (9 roots, Fig. 2). Furthermore, in 90% of cases, intermingling roots appeared when live cluster roots were present, as opposed to before their appearance or after their senescence (Fig. 2; for photographs see Figs S2, S3, S4). The majority of intermingling cluster and non-cluster roots were found at the surface and upper-middle layers of the soil profile, rather than in deeper layers (Fig. S5).

Remobilisation of phosphorus and nitrogen from senescing cluster roots

To test if senesced cluster roots could serve as important sources of P or N, we determined total P and N concentrations in cluster roots at the first three stages of development (see Methods – Glasshouse experiment). Total P concentrations of cluster roots reached very low levels at stage III, the senesced stage (Fig. 3). We found a cluster-root P-remobilisation efficiency of 88.5% based on the mean values of stages I and III ([stage I – stage III] / stage I) from Fig. 3. Total N concentration in cluster roots peaked at developmental stage I (fully-formed cluster root) and was lowest at stage III (Fig. 3). Cluster-root N-remobilisation efficiency was 89.2%.

Root vitality of cluster rootlets

We found a substantial decrease in rootlet vitality, as expected, yet there were still some unstained, possibly live, rootlets at stage III (Fig. 4). However, the stain does not always bind to dead roots, in this case rootlets, especially those that only recently died. Therefore, these data may overestimate live rootlet percentage.

Phosphorus available to intermingling roots in developing and mature cluster rhizospheres

In order to calculate the amount of P possibly available to roots capable of intermingling with cluster roots, during their development, we gathered several values from field samples and the literature. First, ~~At~~ the study site, mean total soil P concentration is estimated to be 21.1 mg kg⁻¹, very similar to 17.8 mg kg⁻¹ which was measured on nearby Spearwood dunes (Turner et al. 2018). Part of this P, estimated as resin-extracted inorganic P (Pi; 1.35 mg kg⁻¹ and 1.27 mg kg⁻¹ in Turner et al. (2018)) is available to any root. Additional to this freely available Pi, cluster roots extract additional amounts of P by bringing inorganic and organic P in solution (mobilised by carboxylates) and hydrolysing organic P (i.e. phosphatase-extractable P)

(Delgado et al. 2013; Shane et al. 2004; Watt and Evans 1999). Carboxylate-extractable Pi was estimated as 2.16 mg kg⁻¹ dry soil from older studies (McPharlin et al. 1994; Ozanne and Shaw 1968; Pathan et al. 2003; Watson et al. 1982). We measured Carboxylate-extractable Pi (citrate-extractable P) as 1.59 mg P kg⁻¹ dry soil and organic P that could potentially be accessed (i.e. Organic-P available) by our *Banksia* cluster roots. This Organic-P available fraction was estimated as 16.7 mg P kg⁻¹ dry soil, similar to the value of 12.5 mg P kg⁻¹ dry soil found in recent work from our group (Zhou et al. unpublished) and similar to the NaOH-extractable organic P of 10.6 mg P kg⁻¹ dry soil from Turner et al. (2018).

Phosphorus available to intermingling roots in senesced cluster rhizospheres

In order to calculate the amount of P possibly available to roots capable of intermingling with senesced cluster roots, we gathered several values from field samples and the literature. We made these calculations, given that cluster roots produce an abundance of fine root biomass in a small volume of soil, and despite efficient remobilisation of P from senescing cluster roots, we hypothesised that some remaining P might be available to intermingling roots. As such, we first estimated this amount of P, expressed as a soil P concentration, as the product of the P concentration in senesced clusters and the dry mass density of cluster roots in soil. Senesced clusters contain 118 mg P kg⁻¹ dry weight; stage III in Fig. 3). Estimates for the density of cluster roots in soil were 0.0284 g cluster roots per g dry rhizosphere soil, similar to what was measured in another *Banksia* study (Pate and Watt 2002). Therefore, the amount of P remaining in senesced cluster roots is estimated at 118 mg P kg⁻¹ cluster roots x 0.0284 g cluster roots g⁻¹ soil = 3.36 mg P g⁻¹ dry rhizosphere soil (Fig. 5). As such, we estimate the volume used by these cluster roots is approximately 37.8 mg of cluster roots per cm³ of soil in these sandy soils.

Phosphorus available to intermingling roots during cluster-root development

Combining the information presented in the preceding sections, we expect non-cluster roots to have access to 1.35 mg P kg⁻¹ soil (Free Pi, resin-P values from Turner et al. (2018) and mentioned above) outside cluster rhizospheres, increasing to a potential 19.6 mg P kg⁻¹ soil (i.e. Free Pi (1.35) + Carboxylate-extractable Pi (1.59) + Organic-P available (16.68) Fig. 5) in cluster rhizospheres (but in competition with cluster roots), and then decreasing to a potential 3.36 mg P kg⁻¹ soil (senesced cluster roots) after clusters have senesced (assuming that P in senesced clusters is completely available to other roots or their mycorrhizas) (Fig. 5). Based on our previous work, we estimated a mean lifespan of 23 days for *Banksia* cluster roots (Teste et al. 2018); as such we suggest that P from senescing cluster roots could become available to other roots from ~23 days after the start of CR development.

Discussion

We documented the *in situ* seasonal dynamics of *Banksia* cluster-root formation with minirhizotrons, and determined, for the first time, the degree of intermingling events by roots of non-cluster-rooted species. The very high frequency of roots intermingling with cluster roots, relative to the cluster roots detected with our minirhizotrons, during their development and period of peak activity represents the first evidence of physical interaction between such roots, and shows that P-mobilisation facilitation may potentially operate in these nutrient-poor soils. Although intermingling certainly occurred between non-cluster roots, we found that the presence of cluster roots significantly promoted intermingling events, since we always observed the appearance of at least one other non-cluster root. Our estimates of soil P availability before, during and after peak cluster-root activity, support the hypothesis that potential benefits exist for non-cluster roots of invading cluster rhizospheres, and that these benefits are greatest during periods of cluster-root exudation.

Conditions for roots intermingling with cluster-roots

439 Root intermingling was prominent, and P-uptake facilitation likely. We found that roots of
 440 other non-cluster rooted species freely intermingled with all *B. attenuata* cluster roots that we
 441 observed, especially when cluster roots were mature or developing. These intermingling
 442 events were particularly pronounced when soil moisture conditions were at a historical, rather
 443 than current precipitation level for this Mediterranean woodland (Teste et al. 2018). We
 444 recently demonstrated that *Banksia* spp. may be sensitive to a drying climate, with a lower
 445 incidence of cluster roots under drier soil conditions (Teste et al. 2018).
 446 Further research is needed to elucidate the mechanism of intermingling, and specifically the
 447 signal(s) triggering growth of roots towards cluster roots. It is unlikely that the signal is P
 448 itself, because P migration in soil is very slow (Lambers and Oliveira 2019). Biogenic
 449 volatile organic signals may be involved (Peñuelas et al. 2014a): cluster roots may release
 450 VOCs into surrounding soil and prime more root proliferation from intraspecific roots. This
 451 would be a novel mechanism for VOCs, but would resemble the root-to-root signalling that
 452 has been documented between unstressed plants to drought-stressed plants (Falik et al. 2011;
 453 Peñuelas et al. 2014b). It is also possible that both cluster roots and intermingling roots
 454 respond to a common signal indicating a locally-high resource level such as resource-rich
 455 patches (Hodge 2009). The signal could be a mobile nutrient, such as nitrate, or a volatile
 456 signal generated by roots or soil microbes. The possibility of nitrate as a signal for root
 457 proliferation, which may provide a large potential benefit for nutrients such as P in our study
 458 system, suggests an alternative explanation for the observation in the study of van Vuuren et
 459 al. (1996), where many roots grew after most of the N in an enriched patch had been
 460 depleted. Those late-growing roots probably still had significant benefits ~~ferom~~ nutrients other
 461 than N.

462
 463 *Phosphorus benefits to roots intermingling with cluster roots*

464 The P-acquisition benefits that other roots could gain from intermingling with cluster roots in
 465 terms of P availability in soil during cluster-root development and senescence (i.e. during and
 466 after cluster activity; Fig. 2) is considerable (Fig. 5). Such a benefit would come from the P-
 467 mobilising activity of cluster roots through exuded carboxylates and phosphatases. However,
 468 the P availability in both living and senesced cluster rhizospheres are maximum estimates,
 469 given that cluster roots are likely strong competitors for P uptake, and P in senesced clusters
 470 may largely be in organic forms, or immobilised by microbes, and thus not available to other
 471 roots. We expect that the peak of the P-acquisition benefits would be short-lived and occur between
 472 ~20 to 30 days after the initiation of the development of the cluster root, based on our glasshouse and
 473 field observations of the development stages of *Banksia* cluster roots (Teste et al. 2018)- and well-
 474 documented observations of cluster-root physiology in hydroponics (Shane & Lambers 2005).
 475 We estimated that 16.9 mg P kg⁻¹ soil represents the theoretical maximum amount of P
 476 available to intermingling roots during the presence of an active cluster root, assuming all P
 477 made available by the cluster roots could be taken up by intermingling roots. While clusters
 478 are active (matching the period of maximum exudation of carboxylates and phosphatases),
 479 plant-available soil P increases from 1.35 mg kg⁻¹ (i.e. resin-P) to a maximum of 16.9 mg kg⁻¹
 480 (Fig. 4). Due to the very high density of cluster rootlets relative to intermingling roots in the
 481 rhizosphere, cluster roots would have a competitive advantage for uptake of the P mobilised
 482 by themselves. Nevertheless, intermingling roots that invade the cluster rhizosphere, or are at
 483 the outer boundary of the clusters, are positioned within a zone of influence of carboxylates
 484 and phosphatases where they may benefit from mobilised P.
 485 Our estimates indicate that there is more P in living cluster roots than extractable soil P in that
 486 same soil volume (Fig. 5), demonstrating that both efficient soil P extraction and efficient P
 487 remobilisation from senescent roots are essential for cluster roots to be effective and a
 488 functional investment benefitting the plant's P economy. Intermingling roots need to compete
 489 with cluster roots to access mobilised P pools; and we expected that effects from the large

amounts of carboxylates exuded by cluster roots and their high-affinity P transporters would maximise the share of that mobilised P for the cluster roots. However, the theoretical

maximum amounts of P that could be gained are worth further empirical testing.

Remobilisation of root phosphorus during cluster root senescence

The amount of P remaining after senescence of the cluster roots might be accessible to non-cluster roots entering or staying in that “senesced-cluster region”, and although the amounts are small, such roots might derive a P benefit from decomposing cluster roots in these impoverished soils. We found a very high root P-remobilisation efficiency of 89% based on determining the root P concentrations during the development and fully senesced stages of our *Banksia* cluster roots. Physiological studies of *Hakea* cluster roots with controlled hydroponic systems found similarly high root P-remobilisation efficiency of 95% (Shane et al. 2004). These results show only a very small amount of P remains after a cluster has senesced for uptake by intermingling non-cluster roots (Fig. 5), compared with the amount that may be gained by intermingling with active cluster roots. Our observation that intermingling roots arrive when cluster roots are alive, rather than senesced (Fig. 2), supports the concept that intermingling roots respond to a signal indicating the imminent opportunity of enhanced resource availability (P and micronutrients), and that most resources become available through cluster-root induced mobilisation, rather than cluster-root decomposition.

Evidence for the potential of facilitated uptake of phosphorus

The facilitated uptake of P that may occur between cluster-rooted species and other interacting plant species such as mycorrhizal plant species falls under two of the three broad facilitative mechanisms proposed by Wright et al. (2017). First, indirect biotic facilitation may result if other roots can scavenge the P that is mobilised by the active cluster root. Second, abiotic facilitation due to local P enrichment by senesced cluster roots remains a

possibility in the most severely-impoverished soils where very small amounts of P can still increase plant growth of other plant species capable of taking up the P ‘mined’ by the cluster roots. Our findings suggest that the first mechanism, indirect biotic facilitation, is probable with some level of P enrichment of the cluster rhizosphere during mobilisation of soil P as previously proposed (Gardner and Boundy 1983; Lambers et al. 2013; Li et al. 2007). Nutrient-mobilisation-based facilitation as proposed in Lambers et al. (2018) and demonstrated in crop plants (e.g., lupin) may also be ecologically relevant for iron (Fe), zinc (Zn) and manganese (Mn), since these micronutrients are also mobilised during cluster-root activity (Li et al. 2014). Nutrient (P, Fe, Zn and Mn)-mobilisation-based facilitation is mainly driven by: i) the release of large amounts of organic acids and chelating substances from cluster roots that increase the concentrations of these nutrients in the soil solution (Lambers et al. 2018); and ii) the ability of other roots to first sense the activity of cluster roots and then grow in the rhizosphere of cluster roots directly or via their extra-radical mycorrhizal hyphae (i.e. in the case where mycorrhizal roots are intermingling). Microcosm studies using *Banksia* as the cluster-rooted plants lend some support to this principle; improved plant growth of neighbouring and interacting plants was shown (Muler et al. 2014; Teste et al. 2014). We suggest that nutrient-mobilisation-based facilitation is more common than previously thought; however, more formal testing is required (see section on *Future directions* below).

Potential impacts of a drying climate on interactions with cluster roots

Plant species compositional shifts may occur due to a drying climate, if P and micronutrient uptake of coexisting plants is facilitated by the root activity of cluster-rooted plants (Lambers et al. 2018; McIntire and Fajardo 2014). Cluster-root development and its capacity to mobilise P from poorly-available forms in soil is affected by soil moisture levels (Lamont 1976; Lamont 2003). We found that lower soil moisture levels reduce cluster-root occurrence

in this system (Teste et al. 2018); thus their P-mobilising activities would also be reduced in a given plant community. We also found that the majority of root intermingling between non-cluster roots and cluster roots occurred in the surface soil layers, where soil drying is typically faster than that in deeper soil layers. Our findings suggest that a considerable amount of P benefits may be obtained when coexisting plants intermingle their roots in the rhizospheres of cluster-rooted plant species. Thus, plant species that depend on this type of interaction to meet their P demand may be the first to succumb in plant communities dominated by cluster-rooted plants during further drying of this Mediterranean environment.

Future directions

Radio-isotope P work (i.e. ^{33}P and ^{32}P) would help quantify the magnitude of P-mobilisation facilitation, but documenting radioactive P movement in megadiverse habitats would be very challenging, if not impossible. Analysis of the spatial pattern between cluster-rooted *Banksia* plants and plants potentially interacting could convey evidence for facilitation, but the lateral extent of *Banksia* cluster roots is far reaching (~2x tree height in *B. prionotes* (Jeschke and Pate 1995)), so the distance between plants may not be a good predictor of the intensity of facilitative relationships. As such, current methodology such as minirhizotrons, nutrient tracers, and microcosm experiments will still be needed to progress our understanding of the roles of belowground facilitation in plant species coexistence. Finally, to robustly test for facilitation, future studies could entail: i) identifying the plant species whose roots intermingle with cluster-rooted plants; ii) quantify the differences between foliar P concentrations with and without neighbouring *Banksia*; and iii) quantify a metric that could serve as a proxy for improved fitness (e.g., seed production).

Conclusions

567 We provide evidence for *in situ* root interactions between cluster-rooted *Banksia* species and
568 roots of other species with implications for species coexistence when soil moisture conditions
569 are favourable. We calculated the potential P benefits to roots growing in or near the
570 rhizosphere of clusters during and after their peak activity. The most frequent root interaction
571 type described here was when soils were relatively moist, and can be viewed as a plant
572 version of ‘kleptoparasitism’ (Gorman et al. 1998; Thompson 1986; Zomora 1995). These
573 are the first accounts of a kleptoparasitism-type root interaction, and we suggest that such
574 interactions occur between other cluster-rooted species and neighbouring plants with
575 considerable potential for nutrient-mobilisation-based facilitation. These interactions and
576 facilitative mechanisms are likely also relevant for other specialised root strategies (e.g.,
577 carboxylate-releasing simple cluster roots, dauciform roots, sand-binding roots, vellozioid
578 roots) and in other P-impooverished ecosystems such as campos rupestres (Abrahão et al.
579 2019; Teodoro et al. 2019). Such interactions may promote species co-existence and act as a
580 means of increasing species diversity in severely nutrient-impooverished soils.

581

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595

596 **Compliance with ethical standards**

597 **Conflict of interest**

598 The authors declare that they have no conflict of interest.

599

600 **Ethical approval**

601 This article does not contain any studies with human participants or animals performed by any
602 of the authors.

603

604 **Data accessibility**

605 Data will be made available as part of a separate digital file in the Electronic Supplementary

606 Material section (Data S1).

607

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Figure legends

Fig. 1* Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster root (shown with white arrow) and other roots (shown with black arrow) of other species (i.e. herbaceous and woody plants) in a banksia-eucalypt woodland (Tube 20, Window 4 from 9 September to 5 October, 2012). In top image, a cluster root starts to form with other roots seen below. Based on this series of images, a frequency of intermingling response was generated; here, frequency = 2, since two non-cluster roots are seen in the same image frame. Since ‘other roots’ were present before the appearance of the cluster root, we named this root interaction ‘before cluster root’ (BeforeCR)). Also see [Fig. S3 and Fig. S4 for photographic examples of the other interaction types](#), and see Fig. 2 for differences in the frequency of interactions with irrigation and amongst the two general root types. See Teste et al. (2018) for an estimate of *in situ* survival rates of cluster roots and their rootlets. Also see Shane and Lambers (2005) for photographs of *Banksia* cluster roots that clearly show their ‘compound’ shape. [*Figure to be possibly reworked by the journal’s illustrator].

Fig. 2 Frequency of intermingling of cluster roots and herbaceous or woody roots during April – October 2012 in a banksia-eucalypt woodland. This intermingling response was based on the joint occurrence of live cluster roots with other live roots in small minirhizotron image frames (height = 1.35 cm, width = 1.8 cm) that hosted at least one live cluster root. The other roots appeared before the cluster root developed (BeforeCR; Fig. [1S2](#)), while the cluster root was alive (DuringCR; Fig. S3), or during senescence (AfterCR; Fig. S4). We found the negative binomial model to be the best fit (AIC = 199, Theta = 1.986) for these data; these showed a statistically significant interaction between the irrigation treatment and type of interaction (P -value = 0.011), while no significant effect of root type was found (see Table S1 for summary statistics).

Fig. 3 Concentrations of total nitrogen (N) and phosphorus (P) across the developmental stages of cluster roots. Values are means with 95% confidence intervals, and different letters to indicate statistically-significant differences between means (Tukey HSD tests with $P \leq 0.05$). Shown here are four developmental stages of cluster roots: stage pre-cluster (immature cluster roots; $n=21$); stage I (fully-formed cluster roots; $n=16$); stage II (cluster roots with rusty brown colour and some loss of vigour; $n=34$); and stage III (dark brown or grey cluster roots with noticeable loss of vigour; $n=29$).

Fig. 4 Percent of live (i.e. unstained) rootlets across the developmental stages of cluster roots. Values are mean percent of live rootlets with 95 % confidence intervals, and different letters indicate statistically-significant differences between means (Tukey HSD tests with $P \leq 0.05$). Shown here are three developmental stages of cluster roots: stage I (fully-formed cluster roots; $n=16$); stage II (cluster roots with rusty brown colour and some loss of vigour; $n=16$); and stage III (dark brown or grey cluster roots with noticeable loss of vigour; $n=31$).

864 **Fig. 5** Phosphorus (P) concentrations typically found in Spearwood sandy soils, as well
865 as P contained in *Banksia* cluster roots (CR), all expressed per unit soil dry mass.
866 Carboxylate-extractable inorganic P (Pi) and organic-P are potentially available to
867 intermingling roots (indicated with root illustrations), in the short period that cluster
868 roots exude P-mobilising compounds (carboxylates and phosphatases). Phosphorus
869 remaining in senesced cluster roots is potentially available to intermingling roots after
870 CR death (indicated with root illustrations).

875 **Fig. 1***

876

877 **Fig. 2**

878

879 **Fig. 3**

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881 **Fig. 4**

882

883 **Fig. 5**

884 **ELECTRONIC SUPPLEMENTARY MATERIAL**

885 **Table S1.** Analysis of deviance tables of the Poisson and negative binomial regression models (full and minimal adequate model
886 determined via the stepAIC function) for the Frequency response data (Fig. 2) of roots intermingling with live cluster roots (CR).

887

888 **Analysis of Deviance (Dev) summary tables**

889

890 **Poisson full model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	177.39	-
Irrigation	1	20.109	39	157.28	<0.001
InteracWhen	2	13.298	37	143.98	0.001
RTInterac	1	0.215	36	143.76	0.643
Irrigation:InteracWhen	2	15.329	34	128.44	<0.001
Irrigation:RTInterac	1	0.700	33	127.73	0.403
InteracWhen:RTInterac	2	2.561	31	125.17	0.278
Irrigation:InteracWhen:RTInterac	2	0.719	29	124.45	0.698

891 InteracWhen: BeforeCR, DuringCR, or AfterCR. RTInterac: the other types of roots (Herbaceous or Woody) interacting with CR.

892

893 **Poisson minimal adequate model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	177.39	-
Irrigation	1	20.109	39	157.28	<0.001
InteracWhen	2	13.298	37	143.98	0.001
Irrigation:InteracWhen	2	15.196	35	128.78	0.001

894 df: degrees of freedom

895

896 **Negative binomial full model**

Source of Deviance	df	Deviance	Residual	Residual	Pr(>Chi)
			df	Dev	
NULL	-	-	40	58.4737	-
Irrigation	1	8.2489	39	50.2248	0.0041
InteracWhen	2	4.8166	37	45.4082	0.09
RTInterac	1	1e-04	36	45.4081	0.9913
Irrigation:InteracWhen	2	9.2123	34	36.1958	0.01
Irrigation:RTInterac	1	0.2084	33	35.9874	0.648
InteracWhen:RTInterac	2	1.5634	31	34.424	0.4576
Irrigation:InteracWhen:RTInterac	2	0.5945	29	33.8295	0.7429

897

898 **Negative binomial minimal adequate model**

Source of Deviance	df	Deviance	Residual df	Residual Dev	Pr(>Chi)
NULL	-	-	40	57.0737	-
Irrigation	1	8.059	39	49.0146	0.0045
InteracWhen	2	4.7076	37	44.307	0.095
Irrigation:InteracWhen	2	9.0556	35	35.2514	0.0108

899 df: degrees of freedom.

Figure legends for ESM

Fig. S1 Location of the research plots (white triangle) where this study using minirhizotron tubes was carried out. A photo of the irrigation treatment in action on Plot 6 from Teste et al. (2018) with a *Banksia attenuata* tree in the centre with white-capped minirhizotron tubes all around. Also shown is the Bartz BTC2 Minirhizotron video camera imaging on Plot 2. Plots were situated in remnant banksia-eucalypt (*Eucalyptus gomphocephala*) banksia woodland of the UWA Shenton Park Field Station (31°56' S, 115°47' E) that is situated on the Swan Coastal Plain, Western Australia.

Fig. S2* Photographs of *Banksia attenuata* cluster-root appearance (shown with arrow) and development *in situ* in a banksia-eucalypt woodland (Tube 2, Window 4 from 29 August to 5 October 2012). See Teste et al. (2018) for an estimate of *in situ* survival rates of cluster roots and their rootlets. Also please see Shane and Lambers (2005) for photographs of *Banksia* cluster roots that clearly show their ‘compound’ shape. [*Figure to be possibly reworked by the journal’s illustrator].

Fig. S3* Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster root and other roots of other species (i.e. herbaceous and woody plants) in a banksia-eucalypt woodland (Tube 20, Window 5 from 29 August to 5 October, 2012). Since ‘other roots’ appeared during the development of a live cluster root we named this root interaction ‘during cluster root (DuringCR)’. See Fig. 2 for differences in the frequency of interactions with irrigation and amongst the two general root types. [*Figure to be possibly reworked by the journal’s illustrator].

Fig. S4* Photographs of *in situ* root intermingling between a *Banksia attenuata* cluster root and other roots of other species (i.e. herbaceous and woody plants) in a banksia-eucalypt woodland (Tube 2, Window 19 from 22 August to 19 September, 2012). Since ‘other roots’ appeared after the development of the cluster root we named this root interaction ‘after cluster root (AfterCR)’. See Fig. 2 for differences in the frequency of interactions with irrigation and amongst the two general root types. [*Figure to be possibly reworked by the journal’s illustrator].

Fig. S5 Frequency of other roots (herbaceous and woody) intermingling with cluster roots over distinct soil depth ranges (0 to 10 cm, 11 to 20 cm, and 21 to 40 cm) during months of peak root growth (April – October) of 2012 in a banksia-eucalypt woodland. This intermingling response was based on the joint occurrence of live cluster roots with other live roots in small minirhizotron image frames (height = 1.35 cm, width = 1.8 cm) that hosted at least one live cluster root. The other roots appeared before the cluster root developed (BeforeCR; Fig. [S2](#)), while the cluster root was alive (DuringCR; Fig. S3), or during senescence (AfterCR; Fig. S4).

Fig. S1.

949 **Fig. S2***

957

Senescent cluster root

958

Growing woody root in proximity to senescent

959

cluster root

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962

Fig. S4*

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965 **Data S1**

966 Data table S2 (separate Microsoft Excel file) has all data associated with this study.