Phosphorus acquisition and allocation patterns in species of *Hakea* follow phylogenetic relationships under high phosphorus supply, but not low phosphorus supply

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Abstract

Phosphorus (P) is essential for plant growth and development. The sources of P in the environment are finite and disappearing due to continued human dependence. Thus, the price of P fertilisers is likely to increase in future and may result in increases in food prices; therefore, it is important to find ways to reduce the P usage by crop plants to decrease their reliance on P fertiliser. Understanding the mechanisms that P-efficient plants use to acquire and remobilise P within their tissues may assist us in the long-term production of P-efficient crops, helping us to produce enough food when facing P limitation in the environment. The main form of plant available P is phosphate (Pi). The genus *Hakea* (Proteaceae) contains species that are able to live and survive on some of the most P-impoverished soils in the world , such as those in Western Australia. It has been reported that *Hakea prostrata* was highly efficient in P acquisition and utilisation and showed P toxicity symptoms at lower P supplies than other plants. However, there is not much information available on the variability of these traits within the *Hakea* genus. In this study, seven phylogenetically closely-related species of *Hakea* were compared with respect to Pi acquisition and P allocation within the tissues under low Pi supply. *H. pritzelii* was found to be somewhat more efficient than *H. prostrata* in Pi accumulation and allocation, and in the conversion of Pi to organic P. In addition, four of these *Hakea* spp. were tested for Pi allocation and remobilisation within their tissues, and P sensitivity under low and high Pi supplies. Results of this experiment illustrated that although some other species of *Hakea* were more sensitive to Pi, *H. prostrata* was the most efficient species in Pi accumulation and allocation to its leaves under high Pi supplies. In addition, while all the examined species were able to regulate Pi accumulation and allocation to the leaves to some extent, *H. drupacea* was the most efficient species in regulating Pi accumulation under high Pi supply. In conclusion, this study shed light on the mechanisms used by *Hakea* spp. for Pi acquisition and P allocation within the tissues. This information, in the long term, may assist in the production of crop species that are capable of surviving with less P fertilisation.

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I dedicate this thesis to my kind mother and my devoted father.

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Statement of candidate contribution

I declare that this thesis is my own research and writing of this thesis was done by myself in consultation with my supervisors.

Bahram Mirfakhraei

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1. Introduction and literature review

1.1 Phosphorus and Hakea prostrata

Phosphorus (P) is essential for plant growth and development (Smith *et al*., 2003; Chiou *et al*., 2006; Vance, 2010). Inorganic phosphate (Pi) is the main form of P that can be absorbed directly through roots (Schachtman *et al*., 1998). Pi is one of the main components in nucleic acids and also plays major roles in enzymatic regulation and metabolism (Raghothama, 1999). Pi is also the main form of P that is transported between plant cells (Schachtman *et al*., 1998). To take up Pi from the soil, roots need to use a strong positively charged proton gradient to overcome the negative electrochemical gradient that is created due to difference in Pi concentrations outside and inside the root cells (Smith *et al*., 2003). A number of PHOSPHATE TRANSPORTER 1 (PHT1) family members are responsible for Pi transport from the soil into the roots (Smith *et al*., 2003). The mechanism of Pi uptake has been studied in plants such as *Arabidopsis*, *Medicago truncatula* and rice (Smith *et al*., 2003). However, the Pi absorption mechanism is not well characterised in plants that are found on soils with extremely low P content, such as *Hakea prostrata* (Proteaceae).

P is non-renewable, therefore, as time goes on the price of P fertilisers will increase (Lambers *et al*., 2006), consequently leading to increases in the price of food. Thus, it is important to understand the mechanisms that are being used by plants that are highly efficient in Pi uptake. Such information may assist in the breeding of more P-efficient crops. Members of Proteaceae family are P-efficient plants (Handreck, 1997*b*). In addition, *H. prostrata* shows decreased ability to down-regulate P uptake and illustrates P toxicity symptoms at low available P concentrations that are not toxic for other species (Shane and Lambers, 2005).

Despite extremely low levels of nutrients, especially P, in the sandy soils of south-west Australia, various species of Proteaceae are able to survive, grow and thrive in these soils (Shane *et al*., 2004*a*). *H. prostrata* is one of these Proteaceae species (Lambers *et al*., 2008). Adaptations have evolved in such species, including photosynthesis with low P levels in the leaves, retranslocation of P from senescing tissues, efficient P absorption mechanisms (Lambers *et al*., 1998), cluster root formation (Purnell, 1960) and exudation of organic acids from the roots (Grierson, 1992). Cluster roots are short and unbranched lateral roots (Lamont, 1972) that appear along the lateral root in form of clustered rootlets (McCully, 1999). They increase the surface area and are capable of

exudation of organic anions (Shane *et al*., 2004*a*) that can result in mobilisation of P from insoluble P sources (Neumann and Martinoia, 2002) that results in an increase in available P for plants and P uptake by plants. Production of cluster roots is one of the mechanisms that some plant families such as Fabaceae, Casuarinaceae, Myricaceae and Proteaceae use to increase their nutrient uptake under nutrient-impoverished soils (Shane and Lambers, 2005). In addition cluster root production is not limited to these families and some of the crop species (such as *Cucurbita pepo*, *lupinus albus* and *Macadamia integrifolia*) are also able to develop such root systems (Shane and Lambers, 2005) (Fig. 1).

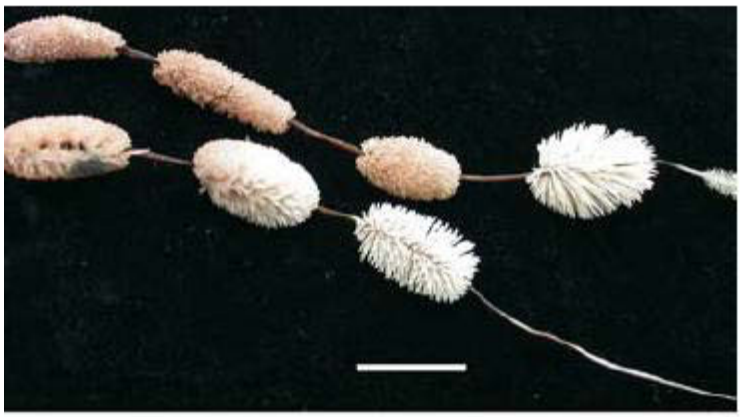

Fig. 1. Development of cluster roots in *H. prostrata* (Shane and Lambers *et al*., 2005).

1.2 Phosphate absorption and PHT *genes*

Members of the PHT1 protein family are present in plasma membranes of plants (Nussaume *et al*., 2011) and play major roles in absorption and distribution of Pi (Smith *et al*., 2003). These proteins are $H_2PO_4^-/H^+$ symporters (Smith *et al*., 2003). All PHT1 members have 12 membrane-spanning domains that are divided into two groups of six due to the presence of an extended hydrophilic region (Raghothama, 1999; Rausch and Bucher, 2002). The expression of *PHT1* genes in roots of plants generally increases following P starvation (Smith *et al*., 2003). These transporters include both high and low-affinity transporters (Rae *et al*., 2003). However, the high-affinity transporters are responsible for Pi absorption in low P conditions (Muchhal *et al*., 1996) . Nine members of this family have been found in *Arabidopsis thaliana* (Smith *et al*., 2003) and their homologs have been detected in all plant species examined (Nussaume *et al*., 2011).

The PHT2 family of Pi transporters are proposed to be structurally and mechanistically distinct from PHT1 family members (Daram *et al*., 1999). It is likely that PHT2;1 is mainly responsible for Pi remobilisation within the shoots of plants (Daram *et al*., 1999), the role that is performed in contribution with some members of PHT1 family (Smith *et al*., 2003). There is not much information available about the PHT3 family. It is suggested that members of this family are mitochondrial PHTs and three members of this family are present in *Arabisopsis* (Rausch and Bucher, 2002). PHT4 family are likely to be responsible for Pi transport inside the plant cells between heterotrophic plastids and Golgi apparatus and also between cytosol and chloroplast (Guo *et al*., 2008).

1.3 Response of plant organs to P supply

Increase in available P usually results in a increase in plant biomass and leaf P under low P conditions (Elser *et al*., 2007; Ostertag, 2010). It has been shown that in wheat increase in P supply results in an increase in total fresh mass of plants, while growth response of white lupin to P supply is not as major (Shane *et al*., 2004*a*). In *H. prostrata* total plant fresh mass in plants receiving P supply was higher than plants grown under P deficiency, however, high application of P could lower the plant fresh mass and develop P toxicity (Shane *et al*., 2004*a*). One hydroponic study showed that in *H. prostrata*, the dry mass of leaves and stems increased when the external P supply was increased from 1.2 µmol P d^{-1} to 3 µmol P d^{-1} (Shane *et al*., 2004*b*). Moreover, a further increase in dry mass of leaves and stems was reported when the P supply was increased to 6 µmol P d^{-1}(Shane *et al*., 2004*b*). In this study, as P supply increased, the rate of photosynthesis in young leaves also increased (Shane *et al*., 2004*b*). The growth of *Acacia xanthina*, *A. truncata* and *Banksia menziesii* also increased under higher levels of P supply up to a moderate P supply (de Campos *et al*., 2013). Therefore, P is an essential nutrient for plants, although, its impact may vary among different species and tissues. In *H. prostrata* the dry mass of cluster roots was similar under external P of 1.2 µmol P d^{-1} to 3 µmol P d^{-1} and 6 µmol P d^{-1} (Shane *et al*., 2004*b*). It has been reported that in *H. prostrata* initiation of cluster root formation is dependent on available P concentration in the root area and cluster root production was initiated under low and suppressed under high P concentrations (Shane *et al*., 2003). In addition, white lupin showed a similar pattern of cluster root production under P supply (Keerthisinghe *et al*., 1998; Shane *et al*., 2004*a*). The percentage contribution of cluster roots to total root

system in white lupin also decreased as the P supply increased (Keerthisinghe *et al*., 1998). Total root mass ratio in white lupin was similar over a range of low P supplies and decreased following exposure to a higher P supply, but stayed somewhat similar after further increase in available P and total root mass ratio of *H. prostrata* showed a similar pattern (Shane *et al*., 2004*a*). The root mass ratio in wheat responded negatively to increase in P supply (Shane *et al*., 2004*a*). Root production in *B. menziesii* was also reduced under high P supply (de Campos *et al*., 2013).

1.4 Hakea *species and P sensitivity*

Triticum aestivum, *Medicago truncatula* and *Lupinus albus* previously showed the ability to down-regulate their P uptake whereas *H. prostrata* showed inability to down-regulate its P uptake (Shane *et al*., 2004*a*). Development of P toxicity symptoms has been reported in leaves of *H. prostrata* plants following exposure to 700 μmol P $week^{-1}$ (Shane *et al*., 2004*a*). These symptoms started as translucent patches that later changed to necrotic patches (Shane *et al*., 2004*a*). Whereas development of P toxicity symptoms on leaves of white lupin was reported after exposure to 14 mmol P $week^{-1}$ while the leaf P concentration of these two species were similar (Shane *et al*., 2004*a*). However, wheat plants did not show P toxicity symptoms even after exposure to 14 mmol P $week^{-1}$ (Shane *et al*., 2004*a*). This shows the inability of *H. prostrata* in down-regulating P uptake that has been suggested previously (Shane *et al*., 2003, Shane *et al*., 2004*a*).

H. prostrata has been found to be sensitive to P fertilisation and some of the leaves in plants treated with 150 and 300 μmol P d^{-1} showed P toxicity symptoms (Shane *et al*., 2004*b*). The leaf total P concentration in the leaves that showed toxicity symptoms were around 10 mg P g^{-1} dry mass, while this value was around 0.44 mg P g^{-1} dry mass in plants grown in their natural environment (Shane *et al*., 2004*b*). Another study recorded higher levels of photosynthesis as the P concentration in the leaves increased (Shane *et al*., 2003). This study also tested the impact of different P supplies on root halves of *H. prostrata* and reported an increase in P absorption and plant total P content as P supply in one of the root halves increased (Shane *et al*., 2003) showing that external P supply can affect the tissue P content. It has been suggested that P uptake in *H. prostrata* is not responsive to plant P status and that the decreased ability of Proteaceae to regulate P absorption is likely to be responsible for P toxicity in these species (Shane *et al*., 2003).

However, this hypothesis has not been tested on species that are phylogenetically closely related to *H. prostrata*.

Compatibility of *H. prostrata* with P-impoverished soils, in combination with the lack of information on the cause of P toxicity symptoms, the transcript profiles of *PHT1* genes in this species and mechanisms that allow this species to thrive on P limited soils make *H. prostrata* appropriate for studying the P absorption mechanism in a Pi-efficient plant. Other species of *Hakea*, such as *H. drupacea*, are less sensitive to Pi supply than *H. prostrata* (Handreck, 1997*a*). *H. drupacea* is the phylogenetically closest relative to *H. prostrata* that previously has been tested for P supply response (Clopton, 2012). Other species that are phylogenetically more closely related to *H. prostrata* than *H. drupacea* (Clopton, 2012) have not been tested for P sensitivity. To understand P uptake mechanisms and their regulation in *Hakea* species, it is important to study and compare species that are phylogenetically close to each other, but show contrasting P sensitivity responses.

Although some studies have focused on the impact of Pi fertilisation on some physiological parameters of *H. prostrata*, these physiological responses have not been compared to those in *Hakea* species that are more resistant to Pi fertilisation. Understanding the P sensitivity relationships among the *Hakea* species under various Pi supply regimes will help us to elucidate the basis of maintaining Pi homeostasis in this P-efficient group of plants. Results of this research may inform the production of plants that are able to absorb P more efficiently from the soil solution and can reproduce in soils with low levels of P with less reliance on P fertilisers.

1.5 Research direction

The main aim of this research was to elucidate and compare the Pi acquisition and allocation patterns in different *Hakea* species to better understand the mechanisms that are used by these plants. Therefore, the capacity for Pi acquisition, P allocation and the responsiveness of these processes to regulation by P under low Pi supply was compared in seven phylogenetically closely related species of *Hakea*. In addition, the capacity of four of these *Hakea* species for Pi accumulation, and the responsiveness of this process to Pi supply was compared under low and increasing Pi supplies.

2.Materials and methods

2.1 Plant material for the first experiment: Low Pi supply

Seeds of seven phylogenetically closely related species of *Hakea* (Fig. 2) (*H. amplexicaulis, H. denticulata, H. drupacea, H. megalosperma, H. pritzelii, H. prostrata, H. ruscifolia*) were obtained from Nindethana seed service, Albany, Western Australia. Seeds were sterilised by soaking in a solution containing 0.6% (w/v) NaClO and 0.5% (w/v) SDS for 5 min. Seeds were rinsed six times with water and imbibed in water for 24 h. Subsequently, seeds were kept on water-moistened filter papers in a Petridish until germination. Germinated seeds were transferred to pots (9 cm x 9 cm x 18 cm) containing sterilised river sand and watered weekly with nutrient solution (200 µM $Ca(NO_3)_2 \cdot 4H_2O$, 100 µM K_2SO_4, 54 µM $MgSO_4 \cdot 7H_2O$, 0.24 µM $MnSO_4 \cdot H_2O$, 0.1 µM $ZnSO_4 \cdot 7H_2O$, 0.018 µM $CuSO_4 \cdot 5H_2O$, 2.4 µM H_3BO_3, 0.03 µM $Na_2MoO_4 \cdot 2H_2O$, 10 µM Fe-EDTA, 20 µM KCl (Shane *et al*., 2003), various concentrations of KH_2PO_4, pH 5.8 to 6.1).

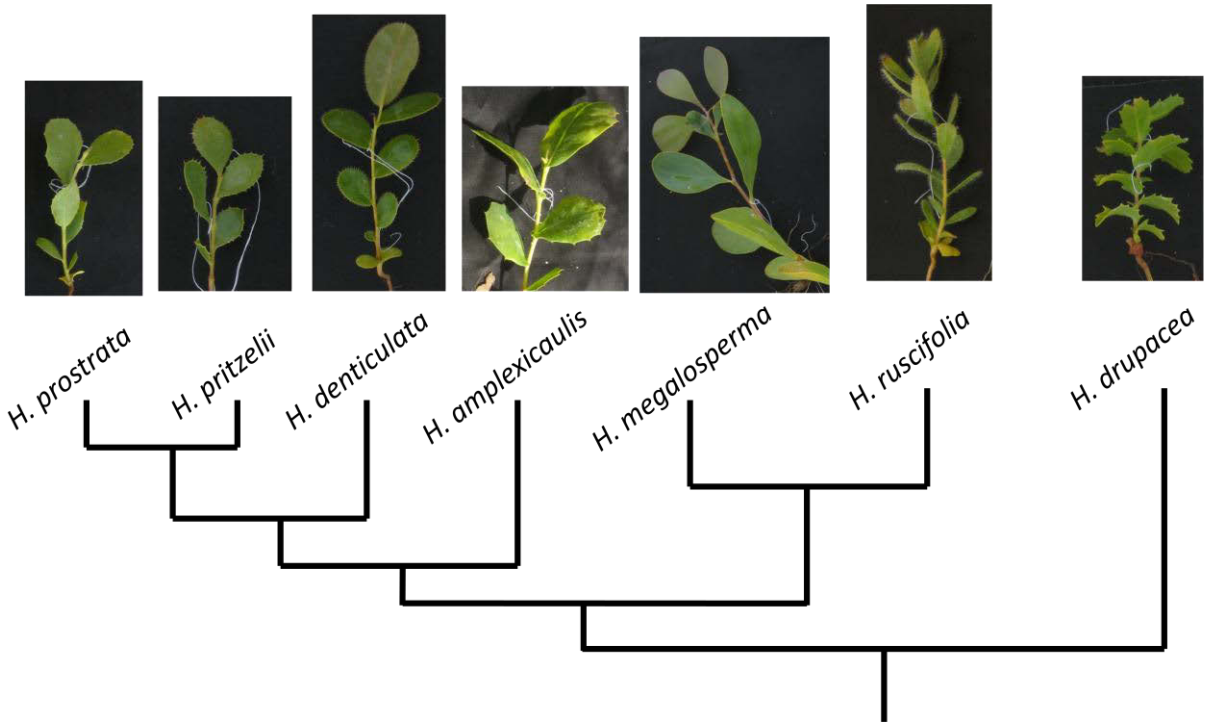

Fig. 2. Seven phylogenetically closely related species of *Hakea* (Clopton, 2012) used in this study. The lines represent the phylogenetic relationships among the species. Plants were supplied with increasing supplies of Pi over a period of 10 weeks.

The rate of Pi application was kept between 0 µmol Pi $week^{-1}$ to 0.25 µmol Pi $week^{-1}$ before start of the experiment and plants were grown in a shade-house with automated

irrigation. Following the establishment of the seedlings, they were transferred to a glasshouse and when they were about six months old. The positions of the plants within the experimental space were randomly allocated using a random number generator. The amount of Pi that was applied to the plants was increased gradually from 0.05 µmol Pi $week^{-1}$ (6 weeks) to 0.25 µmol Pi $week^{-1}$ (2 weeks), 0.75 µmol Pi $week^{-1}$ (1 week) and finally 7.5 µmol Pi $week^{-1}$ (1 week) and after application of 7.5 µmol Pi $week^{-1}$, plants were harvested (Fig. 3). The greater increase in Pi supply from 0.75 µmol Pi $week^{-1}$ to 7.5 µmol Pi $week^{-1}$ in the final week was an attempt to boost internal Pi concentrations and trigger P toxicity symptoms in the species before the final harvest.

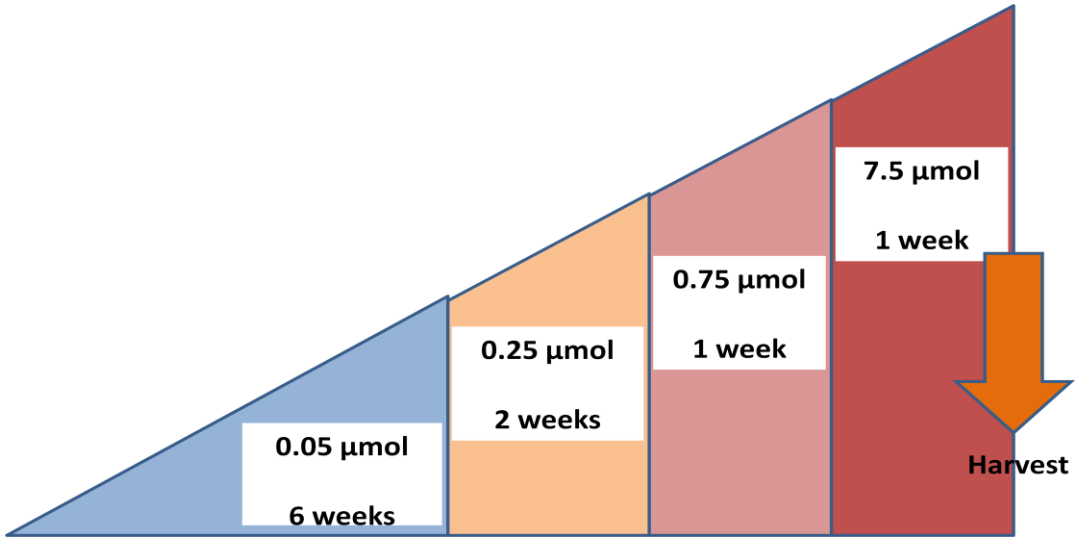

Fig. 3. Pi supply pattern in the first experiment. The increasing Pi supply used in this experiment for a period of 10 weeks (0.05 µmol $week^{-1}$ Pi for 6 weeks, 0.25 µmol Pi $week^{-1}$ for 2 weeks, 0.75 µmol Pi $week^{-1}$ for 1 week and 7.5 µmol Pi $week^{-1}$ for 1 week). The increase in Pi supply from 0.75 µmol Pi $week^{-1}$ to 7.5 µmol Pi $week^{-1}$ in the final week was an attempt to trigger P toxicity in the more tolerant species.

2.2 Plant material for the second experiment: Low versus high Pi supply

Seeds of four phylogenetically closely related species of *Hakea* (Fig. 2) (*H. denticulata, H. drupacea, H. pritzelii and H. prostrata*) were ontained from Nindethana seed service, Albany, Western Australia. Seeds were sterilised by soaking in a solution containing 0.6% (w/v) NaClO and 0.5% (w/v) SDS for 5 min. Seeds were rinsed six times with water and imbibed in water for 24 h. Subsequently, seeds were kept on moist sand until germination. Germinated seeds were transferred to pots (9 cm x 9cm x 18 cm) containing river sand and watered weekly with nutrient solution (200 µM $Ca(NO_3)_2.4H_2O$, 100 µM K_2SO_4, 54 µM $MgSO_4.7H_2O$, 0.24 µM $MnSO_4.H_2O$, 0.1 µM $ZnSO_4.7H_2O$, 0.018 µM $CuSO_4.5H_2O$, 2.4 µM H_3BO_3, 0.03 µM $Na_2MoO_4.2H_2O$, 10 µM Fe-EDTA, 20 µM KCl (Shane *et al*., 2003), various concentrations of KH_2PO_4, pH 5.8 to 6.1). The Pi supply was kept between 0.05 µmol Pi $week^{-1}$ to 20 µmol Pi $week^{-1}$. Before start of the experiment plants were grown in a shade-house with automated irrigation. Following the establishment of the seedlings they were transferred to a glasshouse. The positions of plants within the experimental space were randomly allocated using a random number generator. When seedlings were almost one year old, two Pi supplies were applied to the plants of each species.

2.3 Time-course dependent Pi accumulation in leaves of Hakea *species under low versus high Pi supply over a period of 12 weeks*

A batch of these species (*H. denticulata, H. drupacea, H. pritzelii and H. prostrata*) were exposed to either a constant low amount (10 µmol Pi $week^{-1}$) or increasing amounts of Pi for a period of 12 weeks. The highest Pi supply applied was 1.7 mmol Pi $week^{-1}$ (Fig. 4). Leaf samples from these plants were harvested every three weeks and these samples were used for a time-course measurement of leaf Pi concentration.

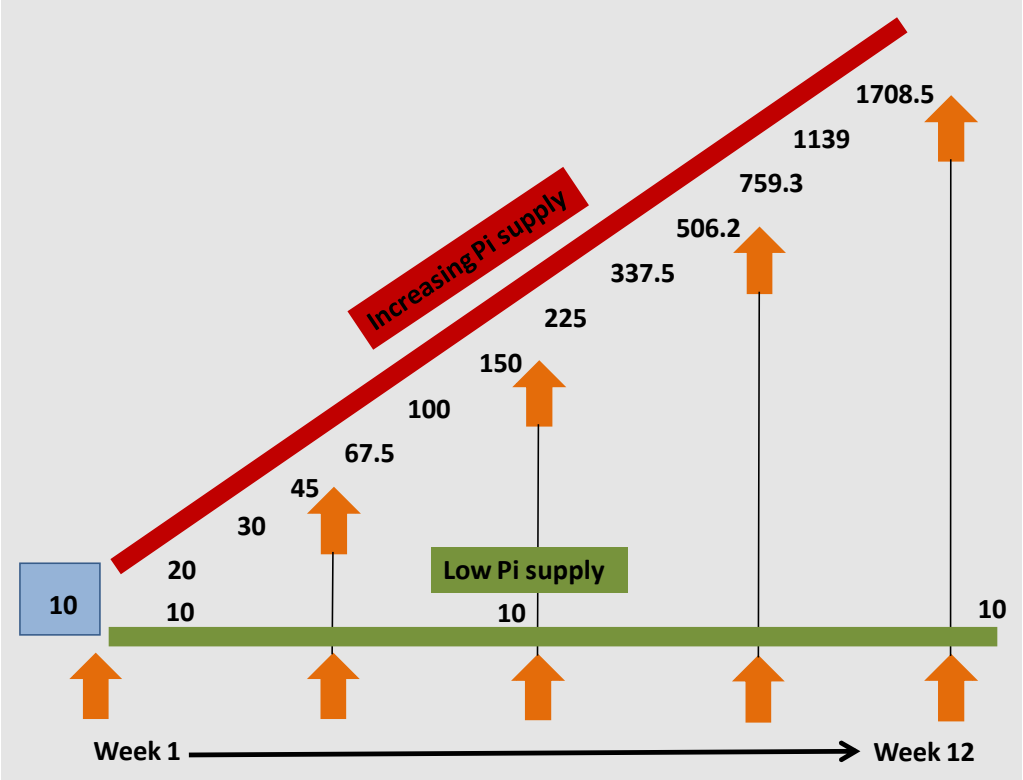

Fig. 4. Time-course study of leaf Pi accumulation in four *Hakea* species under low and high Pi supplies. All the Pi supply units are (µmol Pi week^{-1}). Leaf samples were collected at the start of the experiment and every three weeks (arrows) for Pi determination from both low and high Pi treated plants for a period of 12 weeks.

2.4 End point analysis of organ P accumulation patterns in Hakea *species under low versus high Pi supply*

Another batch of these species (*H. denticulata, H. drupacea, H. pritzelii and H. prostrata*) were exposed to constant low (10 µmol Pi week^{-1}) and increasing supply of Pi from 10 µmol Pi week^{-1} to 2.5 mmol Pi week^{-1} over a period of 13 weeks (Fig. 5). Plants from each species were harvested individually at the indicated time points following the development of necrosis lesions (percentage of leaf area showing necrosis) in plants treated with high Pi supply, a symptom believed to be associated with P toxicity. Only *H. drupacea* did not show leaf necrosis at the time of harvest.

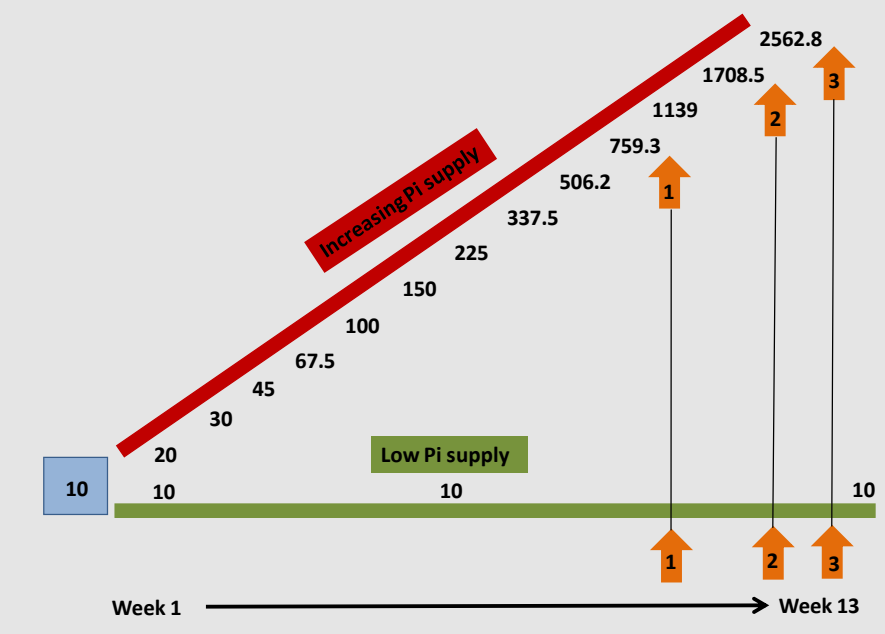

Fig. 5. Experimental set-up to study differences in P sensitivity between the four selected *Hakea* species over a period of 13 weeks. All the Pi supply units are (µmol Pi week^{-1}). All the plants were exposed to 10 µmol Pi week^{-1} before the start of the experiment. Subsequently, five plants from each species were supplied with either low or high Pi treatments. High Pi treated plants of each species were harvested after development of necrotic lesions in plants exposed to high Pi supply, except *H. drupacea* that did not show necrosis symptoms. As controls, plants grown under low Pi supply were harvested at the same time. Arrows show time of harvest. *H. denticulata* was harvested after exposure of high-Pi supplied plants to 759 µmol Pi week^{-1} (arrow 1), *H. pritzelii* was harvested after exposure of high-Pi supplied plants to 1708 µmol Pi week^{-1} (arrow 2), *H. prostrata* and *H. drupacea* both were harvested after exposure of high-Pi supplied plants to 2562 µmol Pi week^{-1} (arrow 3).

2.5 P sensitivity ranking

Plants exposed to low and high Pi supply over a period of 13 weeks were ranked for the necrotic lesions that were believed to be caused by P toxicity over the last five weeks of the experiment. The percentage of the total leaf area that was showing necrotic lesions was estimated and recorded for each plant.

2.6 Pi and total P determination

2.6.1 Phosphate extraction

Tissues from each organ (whole leaves, roots and cluster roots) were ground to a fine powder under liquid nitrogen (same ground samples were used for Pi and total P determination). Ten to fifty mg of tissue were combined with two zirconium oxide beads (Bertin technologies) and 500 µl of 1% (v/v) acetic acid before homogenising (Precellys 24 Tissue Disruptor, Bertin Technologies, Saint Quentin en Yvelines Cedex, France) using three cycles of 5000 rpm for 45 s each. Samples were cooled on ice for 15 min. Another 500 µl 1% acetic acid was added to samples treated with more than 10 µmol Pi, to dilute the samples, and homogenised again as above before incubating on ice in the dark for 15 min. Samples were clarified by centrifugation at 20817 xg for 15 minutes at 4 ^{o}C. Supernatants were transferred to fresh tubes and the centrifugation step was repeated.

2.6.2 Plant tissue digestion

Plant tissue (100 mg to 200 mg) was dried in the oven for 2 to 3 nights under approximately 70 ^{o}C. Subsequently plant tissue was digested at 100 ^{o}C in 3 ml nitric acid and cooled to room temperature (about 25 ^{o}C) before continued digestion at 140 ^{o}C to 150 ^{o}C in 1 ml perchloric acid. Subsequently, samples were cooled to room temperature (about 25 ^{o}C) before heating at 160 ^{o}C to 180 ^{o}C for 10 minutes and cooling down to room temperature. Samples were mixed with approximately 5 ml water and heated to 80 ^{o}C to 90 ^{o}C to dissolve the salts and silicates before diluting to a final volume of 10 ml.

2.6.3 Phosphate, total phosphorus and organic P measurement using ammonium molybdate assay

Assay solution (210 µl six parts 42% ammonium molybdate in 1N H_2SO_4 to one part 10% ascorbic acid) was added to 90 µl of either undiluted or diluted sample in 96-well microplates (Greiner, Bio-one) before incubating at 37 ^{o}C for 60 min. Absorbance was measured at 620 nm (Multiskan Spektrum Platereader, Thermo Scientific, Waltham, Massachusetts, USA) and the Pi concentration calculated from a standard curve constructed using KH_2PO_4. The contribution of organic P (Po) to the total P (Pt) concentration from the acid digests was calculated by subtracting the Pi concentration

from the tissue Pt concentration. In addition the Pi and Po content of tissues in different species were calculated based on fresh weight.

2.7 Chlorophyll and carotenoid measurement

Three leaf disks with the total area of 0.21 cm^2, were taken from young, fully expanded leaves, mixed with 2 ml of methanol and incubated overnight (approximately 16 h) at 4 ^oC in the dark. Subsequently, clear supernatant was transferred to 96- well microplates (Greiner, Bio-one) to record the optical density of samples at 470 nm, 653 nm and 666 nm (Multiskan Spektrum, Thermo Scientific, Waltham, Massachusetts, USA). The optical density of methanol alone was subtracted from the optical density of the samples and the concentration (µg cm^{-2}) of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (C_{x+c}) pigments in tested tissues calculated: $Chl_a = 15.65\ OD_{666} - 7.34\ OD_{653}$, $Chl_b = 27.05\ OD_{653} - 11.21\ OD_{666}$, $C_{x+c} = (1000\ OD_{470} - 2.86\ Chl_a - 129.2\ Chl_b)/221$ (Wellburn, 1994).

2.8 Statistical analysis

Data were analysed using Genstat (Genstat fifteenth edition, version-15.2.0.8821 (64-bit edition), supplied by VSN International Ltd.) by analysis of variance (ANOVA) and Tukey's multiple comparison. Data were tested for normality. One-way ANOVA and two-way ANOVA were used where appropriate. The tables associated with statistical analysis are included in the appendices. Due to diverse germination rate of the species it was not possible to acquire same replication numbers in the first experiment and when plants were only exposed low Pi supplies (Fig. 3). However, this issue was resolved in next experiments (Fig. 4 and Fig. 5) by using more seeds in the germination process and therefore, equal replication numbers were acquired.

3. Results

3.1. Impact of low Pi supply on Hakea *species*

An experiment was done to elucidate and compare the Pi acquisition and P allocation patterns in seven *Hakea* species that are phylogenetically closely related to one another and included *H. prostrata* a species that is believed to be unable to down-regulate P uptake (Shane *et al*., 2004*a*).Well-established seedlings growing in sand were given increasing doses of Pi over 10 weeks (0.05 µmol $week^{-1}$ Pi for 6 weeks, 0.25 µmol Pi $week^{-1}$ for 2 weeks, 0.75 µmol Pi for 1 week and 7.5 µmol Pi for 1 week). Since previously it was reported that plants treated with 2.1 mmol P $week^{-1}$ (Shane *et al*., 2004*b*) and 700 µmol P $week^{-1}$ (Shane *et al*., 2004*a*) showd symptoms of P toxicity, Pi levels used in this experiment are indicated as low Pi supply.

3.2. Growth and cluster root production under low Pi supply

H. megalosperma tended to have the higher leaf, stem, root and cluster root fresh weights among the species at the final harvest (Fig. 6). However, there was no significant difference between *H. megalosperma* and *H. prostrata* in terms of root and cluster root fresh weight. *H. drupacea* and *H. pritzelii* tended to have lower amount of cluster roots and there was no significant difference between the cluster root fresh weight in these two species (Fig. 6). Root fresh weight of *H. pritzelii* tended to be lower than that of the other species. *H. megalosperma* and *H. pritzelii* tended to have the highest and lowest leaf fresh weights, respectively.

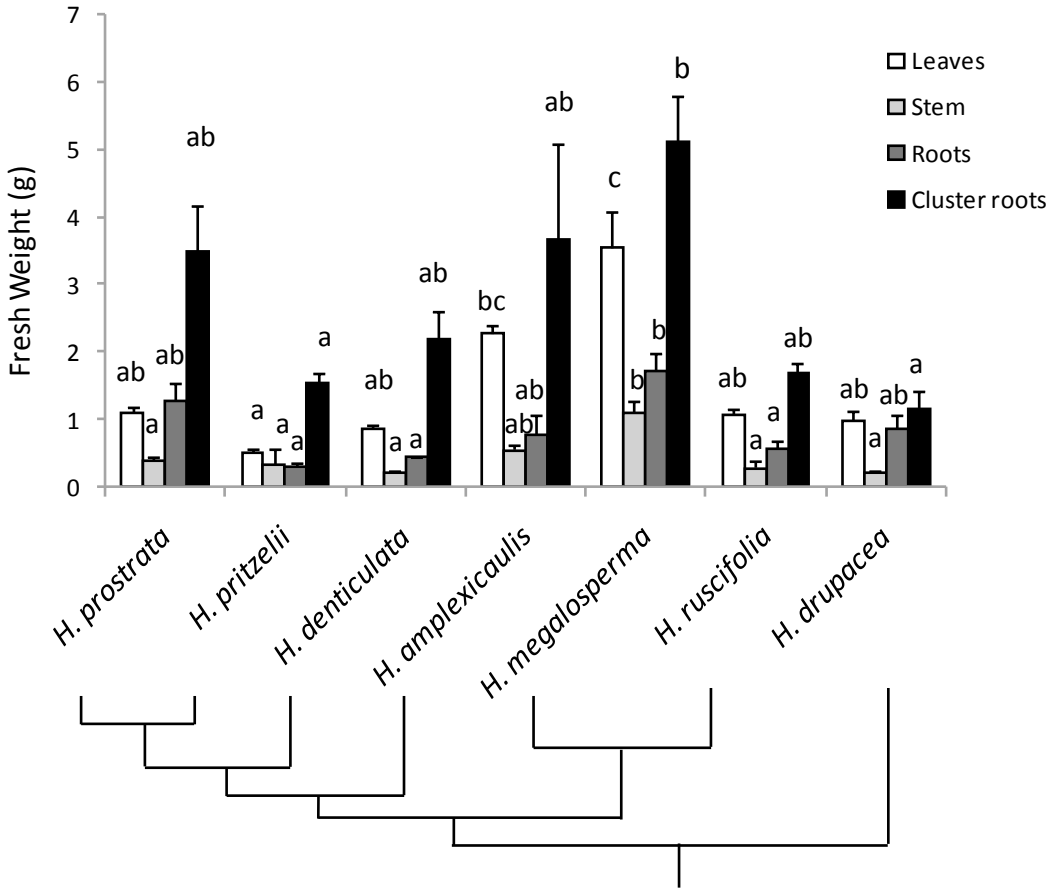

Fig. 6. Leaf, stem, root and cluster root fresh weight of seven *Hakea* species. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see the Methods. The values are means and bars represent standard errors (n = 2 to 5). Results were tested using one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. The values for each organ were tested separately and letters show the multiple comparison ($P < 0.05$).

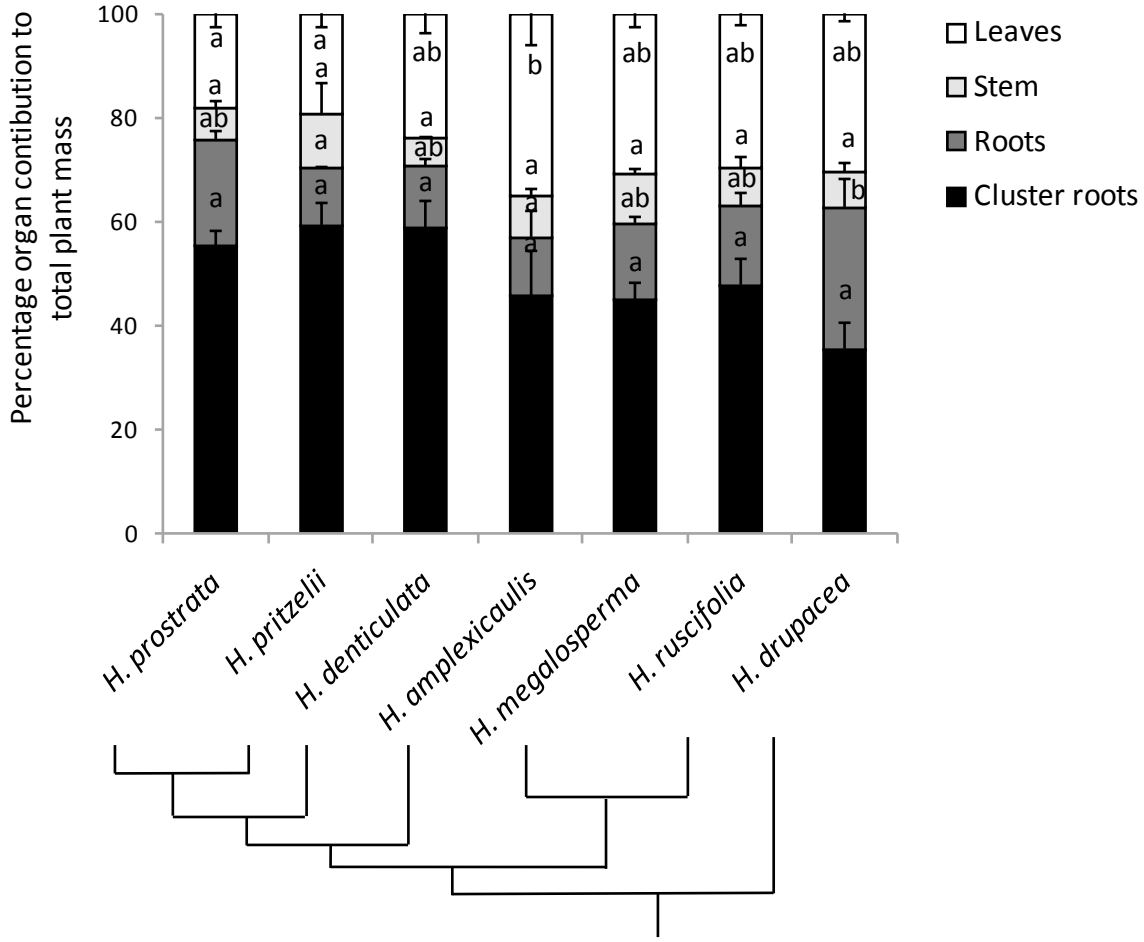

Fig. 7. Average contribution made by leaves, stem, roots and cluster roots to the whole plant fresh weights of seven *Hakea* species. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Bars represent standard errors (n = 2 to 5). Results were tested by one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. The values for each organ were tested separately and letters show the multiple comparison ($P < 0.05$).

H. denticulata, *H. amplexicaulis*, *H. megalosperma* and *H. ruscifolia* allocated more resources to leaves than to non-cluster roots and stems (Fig. 7). Conversely, *H. prostrata* and *H. drupacea* allocated more resources to non-cluster roots and stems and less resources to leaves. *H. pritzelii* allocated somewhat similar resources to leaves, stems and non-cluster roots.

The number of cluster roots at the final harvest tended to be greater for *H. megalosperma* followed by *H. ruscifolia* (Fig. 8).

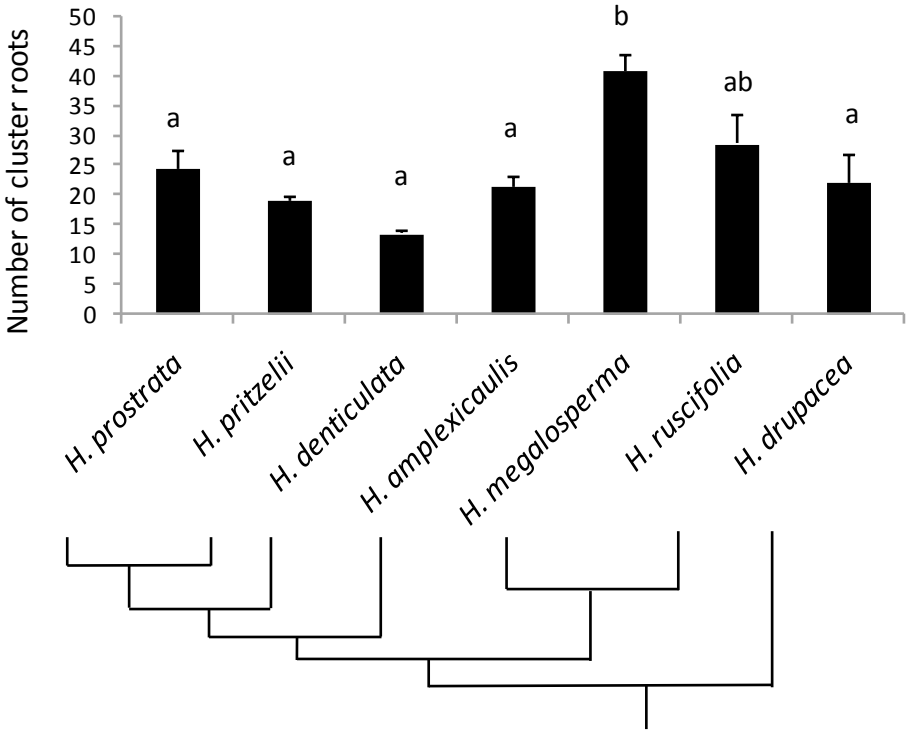

Fig. 8. Cluster root production among seven *Hakea* species. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Values represent means and bars are standard errors (n = 2 to 5). Results were tested by one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Different letters show the multiple comparison ($P < 0.05$).

The other species were not significantly different from each other in terms of cluster root production (Fig. 8).

3.3 Leaf photosynthetic pigment concentration under low Pi supply

As leaf pigments, especially Chl a and Chl b and carotenoids, may represent the photosynthetic potential in the leaves (Chappelle *et al*., 1992), the concentration of these pigments was measured. At the end of the experiment, all species had a higher concentration of Chl a than of Chl b or carotenoids (Fig. 9), and there was no significant difference among the species in terms of Chl a concentration.

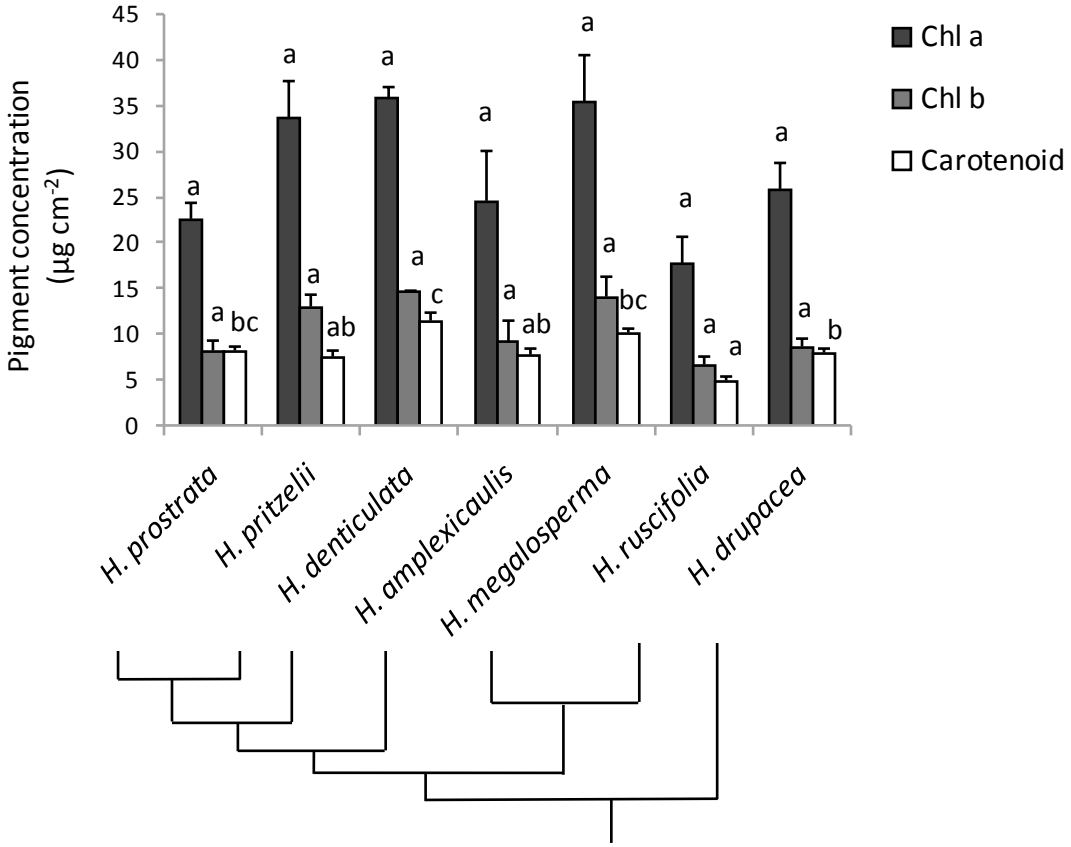

Fig. 9. Chl a, Chl b and carotenoid concentration per leaf area in seven *Hakea* species. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Values represent means and bars are standard errors (n = 2 to 5). Results were tested by one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. The values of each pigment were tested separately and letter show the multiple comparison ($P < 0.05$).

H. denticulata had the highest Chl b concentration and *H. ruscifolia* had the lowest Chl b concentration. Although the statistical analysis showed that overall there was a significant difference in Chl b concentration among the species, the multiple comparison did not show any significant difference. This contrast illustrated that it is likely that there is a significant difference between the Chl b concentration of *H. ruscifolia* and *H. denticulata* at lowest and highest extremes. *H. denticulata, H.megalosperma* and *H. prostrata* had similar carotenoid concentrations that tended to be somewhat higher than in the other species. *H. ruscifolia* was ranked last in terms of carotenoid concentration (Fig. 9). The ratios of Chl a to Chl b are shown in Table 1. *H. denticulata* and *H. drupacea* had the lowest and highest Chl a to Chl b ratio respectively.

Table 1. Chlorophyll a to chlorophyll b ratios under low Pi supply.

Species	Chl a / Chl b
H. prostrata	2.91
H. pritzelii	2.61
H. denticulata	2.44
H. amplexicaulis	2.76
H. megalosperma	2.55
H. ruscifolia	2.70
H. drupacea	2.99

3.4 Pi acquisition and P allocation patterns

Although the Pi concentration in the leaves of *H. pritzelii* tended to be higher than in leaves of the other species examined (Fig. 10), there was no significant difference among this species and *H. megalosperma*, *H. prostrata*, *H. drupacea*, *H. denticulata* and *H. ruscifolia* for this trait. Leaf Pi concentrations in *H. amplexicaulis* were trending lower than in the other species. *H. pritzelii* tended to show a higher leaf Po concentration compared to *H. prostrata* and *H. megalosperma*, *H. ruscifolia*, *H. drupacea* and *H. denticulata*. Only the leaf Po concentrations between *H. pritzelii* and *H. amplexicaulis* were significantly different.

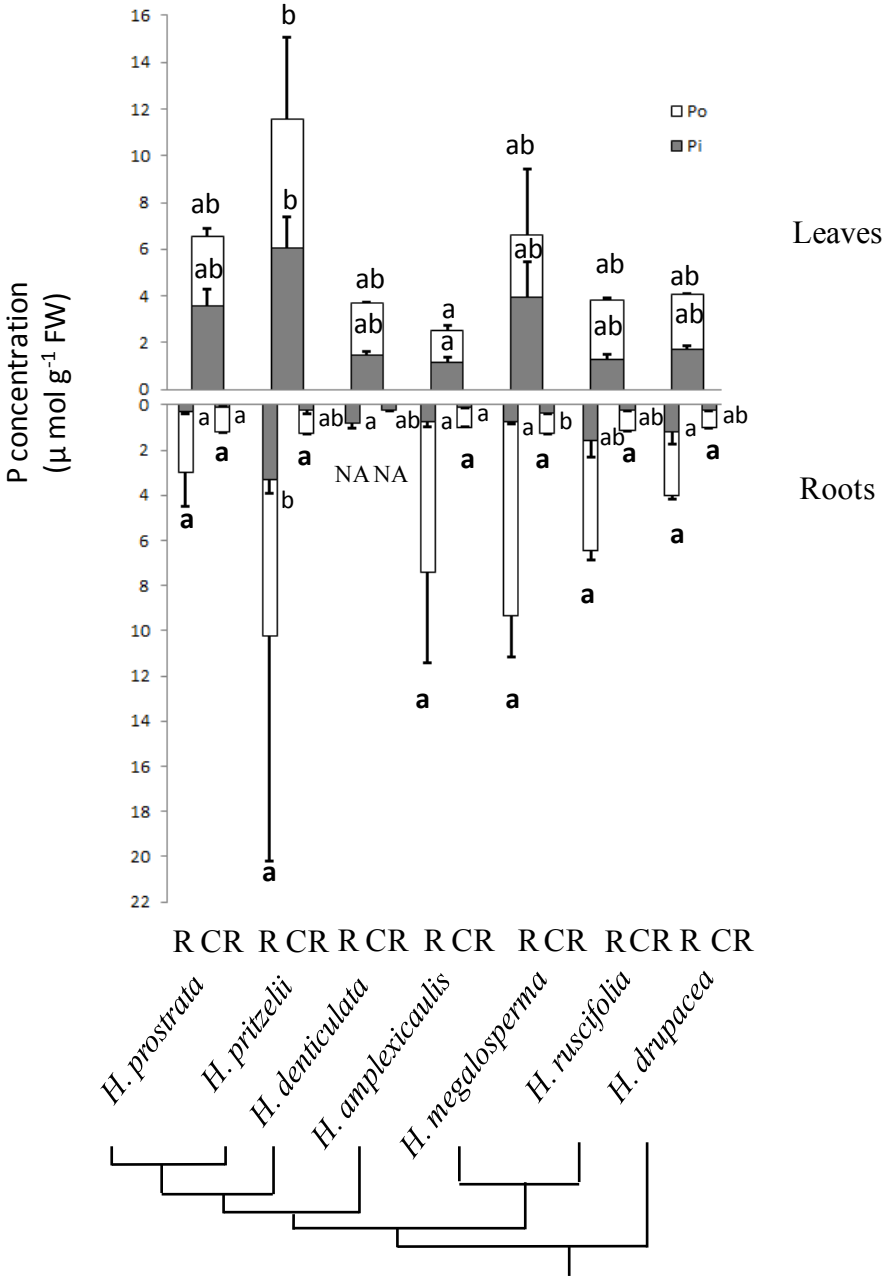

Fig. 10. Pi and Po concentrations in the leaves, roots (R) and cluster roots (CR) of seven *Hakea* species. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Values are means and bars represent standard errors (n = 2 to 5). Results were tested by one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Pi and Po of each organ were tested separately and letters show the multiple comparison ($P < 0.05$). The multiple comparisons for non-cluster roots are shown to the right of the bars and the multiple comparisons for cluster roots are shown in bold. NA indicates that values for Po are not available due to insufficient tissue for total P analysis.

Root Pi concentrations were significantly different between *H. pritzelii* and *H. prostrata*, the species that tended to have the highest and lowest root Pi concentrations, respectively. Overall, the concentration of Pi tended to be greater in the leaves than in the non-cluster roots of all the species except in *H. ruscifolia*, which had slightly greater levels of Pi in the roots than in the leaves. The trends for Pi concentrations in the cluster roots were lower than in both leaves and roots for all the species, and there was a significant difference between *H. megalosperma*, with highest levels, and *H. prostrata* and *H. amplexicaulis* with lower levels of cluster root Pi concentration. There were no significant differences among the root or cluster root Po concentrations of the species.

All the species had similar Pi and Po content in leaves, roots and cluster roots except *H. megalosperma*, which had higher amounts of both Pi and Po in its leaves and it also had higher amounts of Po in its roots and cluster roots than the other species (Fig. 11). A difference between P content and concentration was observed in the leaves of *H. prostrata* and *H. pritzelii*. While *H. pritzelii* had higher Pi and Po concentrations in its leaves than *H. prostrata*, the Pi and Po content in leaves for these species was similar (Fig. 10 and Fig. 11). In addition, *H. prostrata* and *H. megalosperma* had similar Pi and Po concentrations in their leaves but vastly different leaf P content.

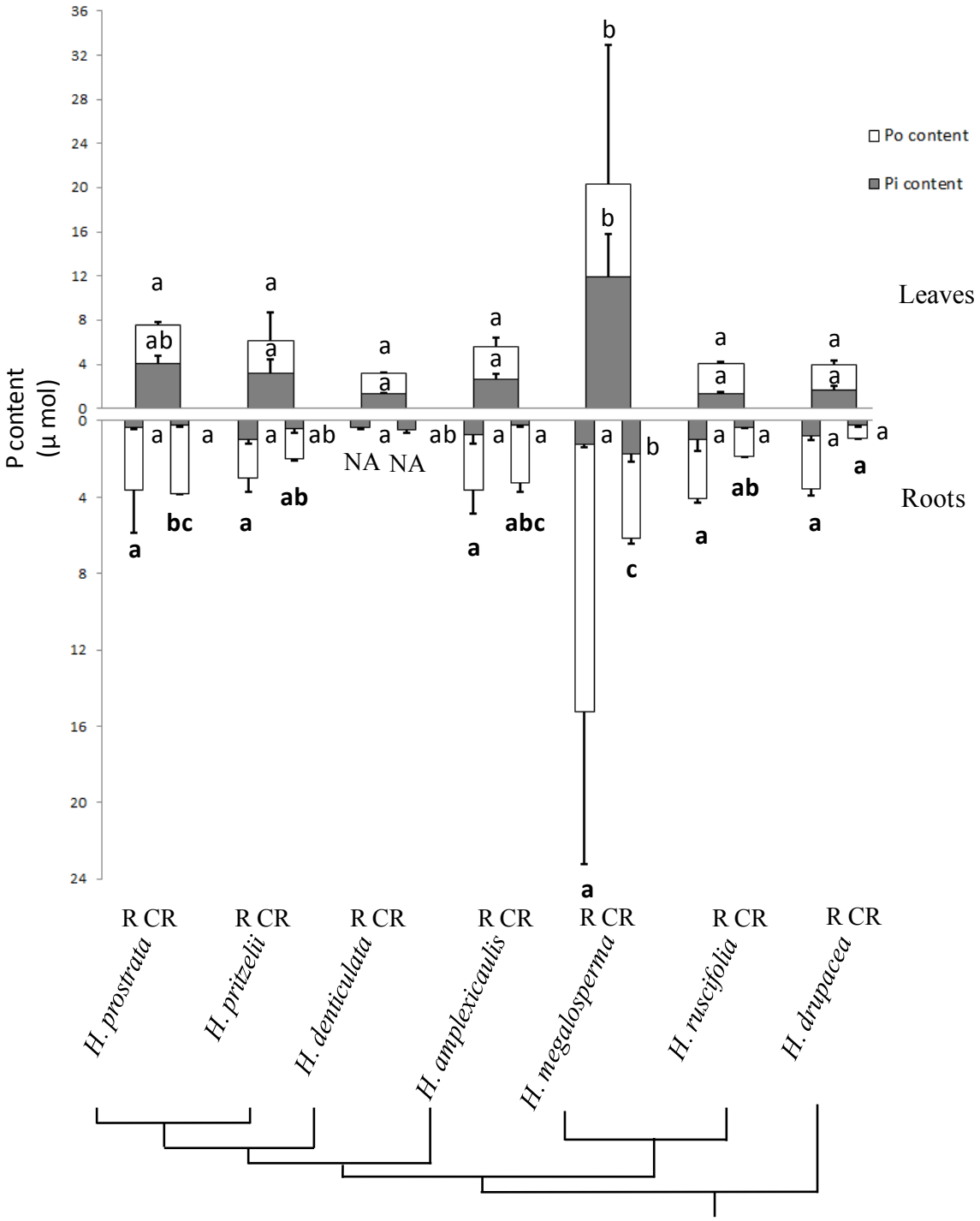

Fig. 11. Pi and Po content based on fresh weight of leaves, roots (R) and cluster roots (CR) of seven *Hakea* species. Species are ordered based on phylogenetic relationships (see Methods). For growth conditions see Methods.Values are means and bars represent standard errors (n = 2 to 5). Results were tested by one-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Pi and Po of each organ was tested separately and letters show the multiple comparison ($P < 0.05$). NA indicates that values for Po are not available due to insufficient tissue for analysis.

3.5 Impact of low versus high Pi supply on Hakea *species*

In the previous experiment and when plants were exposed to only low Pi supply (7.5 µmol Pi week^{-1}), *H. pritzelii* tended to have greater concentrations of Pi and Po in its leaves and therefore seemed somewhat more efficient in Pi accumulation and P utilisation than the other species. Moreover, this species had a similar leaf Pi concentration to its closest relative *H. prostrata*. *H. prostrata*, *H. pritzelii* and their closest and most distantly related relatives in the study, *H. denticulata* and *H. drupacea*, respectively, were selected to further examine the relationship between Pi accumulation, P utilisation and phylogenetic distance of *Hakea* species. The response of four *Hakea* species (*H. prostrata*, *H. pritzelii*, *H. denticulata* and *H. drupacea*) to low and high Pi supply was determined to assess the ability of these species to regulate Pi accumulation (see Methods).

3.6 Time-course dependent Pi accumulation in leaves of Hakea *species under low versus high Pi supply over a period of 12 weeks*

A set of plants were used to determine the ability of *H. prostrata*, *H. pritzelii*, *H. denticulata* and *H. drupacea* to regulate Pi accumulation and allocation to the leaves over the time-course of 12 weeks. In this experiment a set of plants were randomly distributed among the plants that were used for low versus high Pi supply experiment (see Methods) and treated with low (10 µmol Pi week^{-1}) or increasing Pi from 10 µmol Pi week^{-1} to 1.7 mmol Pi week^{-1} over a period of 12 weeks and leaf samples were collected from them every 3 weeks to examine and compare the Pi concentration in the included species.

Species treated with low Pi had quite constant Pi concentrations in their leaves during thecourse of the experiment (Fig. 12 A). By contrast, plants supplied with increasing Pi (Fig. 12 B) showed a similarly low concentration of Pi in their leaves until week 6 when the supply was 150 µmol Pi week^{-1}. The Pi in leaves subsequently increased when the supply was increased to 506 µmol Pi week^{-1} and 1.7 mmol Pi week^{-1}.

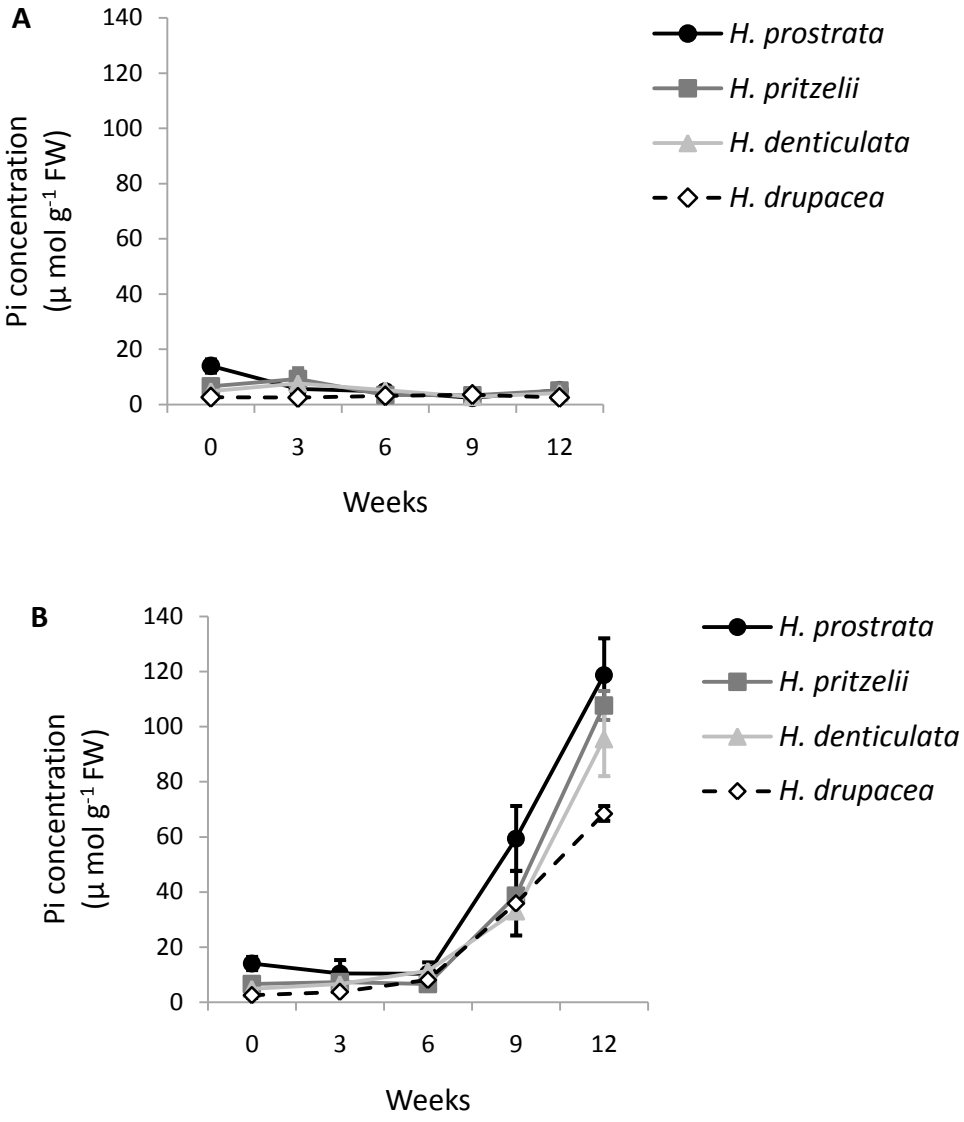

Fig 12. Pi concentration in the leaves of four *Hakea* species treated with (A) low Pi supply (10 µmol Pi week^{-1}) and (B) increasing Pi supply (week 0: 10 µmol Pi, week 3: 45 µmol Pi, week 6: 150 µmol Pi, week 9: 506 µmol Pi, week 12: 1.7 mmol Pi) over a time-course of 12 weeks. Values are means and bars are standard errors (n = 3). Leaf samples were taken every three weeks. For phylogenetic relationship between the species and growth conditions see Methods.

3.7 End point analysis of Pi allocation and organ P accumulation patterns in Hakea *species under low versus high Pi supplies*

Another set of well-established seedlings grown in sand was treated with constant Pi supply of 10 µmol Pi week^{-1} or with an incremental Pi supply from 10 µmol Pi week^{-1} to 2.5 mmol Pi week^{-1} over a period of 13 weeks. Plants were ranked weekly for their P

toxicity symptoms and harvested following the development of necrotic lesions to greater than 10% in the leaves of plants treated with high Pi supply.

3.8 Development of P toxicity symptoms under high Pi supply and leaf Pi concentration under low versus high Pi supply

Plants exposed to high levels of Pi were ranked for local leaf necrosis during the last 5 weeks of experiment to elucidate the P sensitivity of included species. Plants exposed to low Pi supply did not show any necrosis symptoms and therefore, those rankings are not shown.

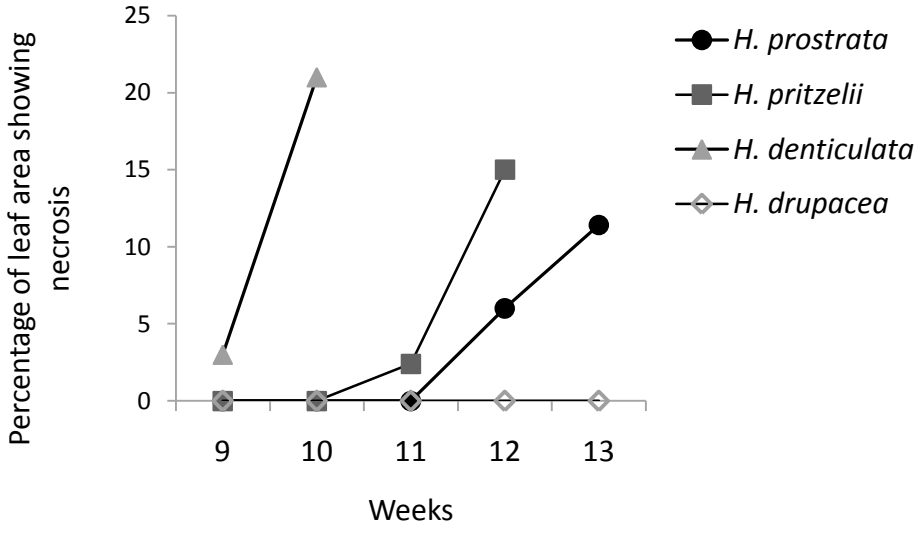

Fig. 13. Patterns of necrotic lesion development in four *Hakea* species under high Pi supply in the last 5 weeks of the experiment (week 9: 506 µmol Pi week^{-1}, week 10: 759 µmol Pi week^{-1}, week 11: 1.13 mmol Pi week^{-1}, week 12: 1.7 mmol Pi week^{-1} and week 13: 2.5 mmol Pi week^{-1}). For phylogenetic relationships between the species and growth conditions see Methods. Values are means (n = 5).

H. denticulata was the first species to develop necrosis in its leaves, following exposure to 759 µmol Pi week^{-1}. *H. pritzelii* was the next most sensitive, after exposure to 1.7 mmol Pi week^{-1}. *H. prostrata* displayed necrosis after exposure to 2.5 mmol Pi week^{-1}. *H. drupacea* plants did not show any signs of necrosis.

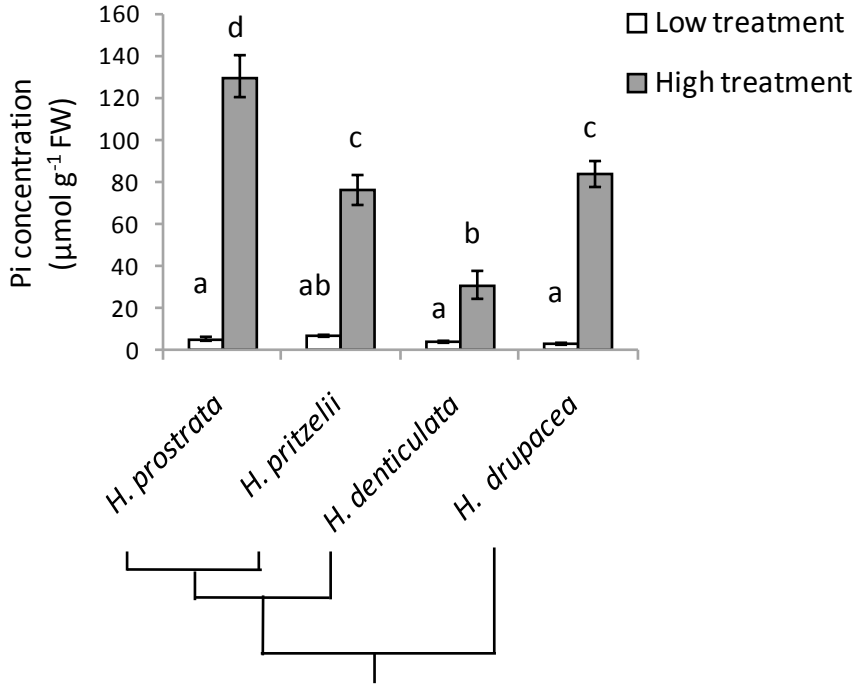

Fig. 14. Leaf Pi concentration at harvest of four *Hakea* species under low versus high Pi supply. Species are ordered based on phylogenetic relationships (see Methods). For growth conditions see Methods. Plants were harvested following development of necrosis symptoms in plants treated with high Pi supply. Values are means and bars are standard errors (n = 5). Results were tested by two-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Letters show the multiple comparison ($P < 0.05$).

There was a significant interaction between species and Pi treatments showing that species respond differently to Pi supplies. All the species had similar levels of Pi in their leaves when treated with low Pi supply, while when supplied with high Pi, *H. denticulata* had the lowest leaf Pi concentration and *H. prostrata* had the highest leaf Pi concentration when necrosis developed in their leaves (Fig. 14).

3.9 Growth and cluster root production at low versus high Pi supply

Overall, treatments did not have a significant impact on leaf fresh weight showing that Pi supply did not alter biomass allocation in the leaves; however, there was a significant difference among the species for leaf fresh weights. *H. drupacea* plants had higher leaf fresh weights compared to all other species examined (Fig. 15). *H. prostrata* and *H. drupacea* tended to have greater leaf fresh weight when treated with higher levels of Pi. Conversely, *H. pritzelii* and *H. denticulata* had lower leaf fresh weight trends following exposure to higher Pi levels (Fig.15).

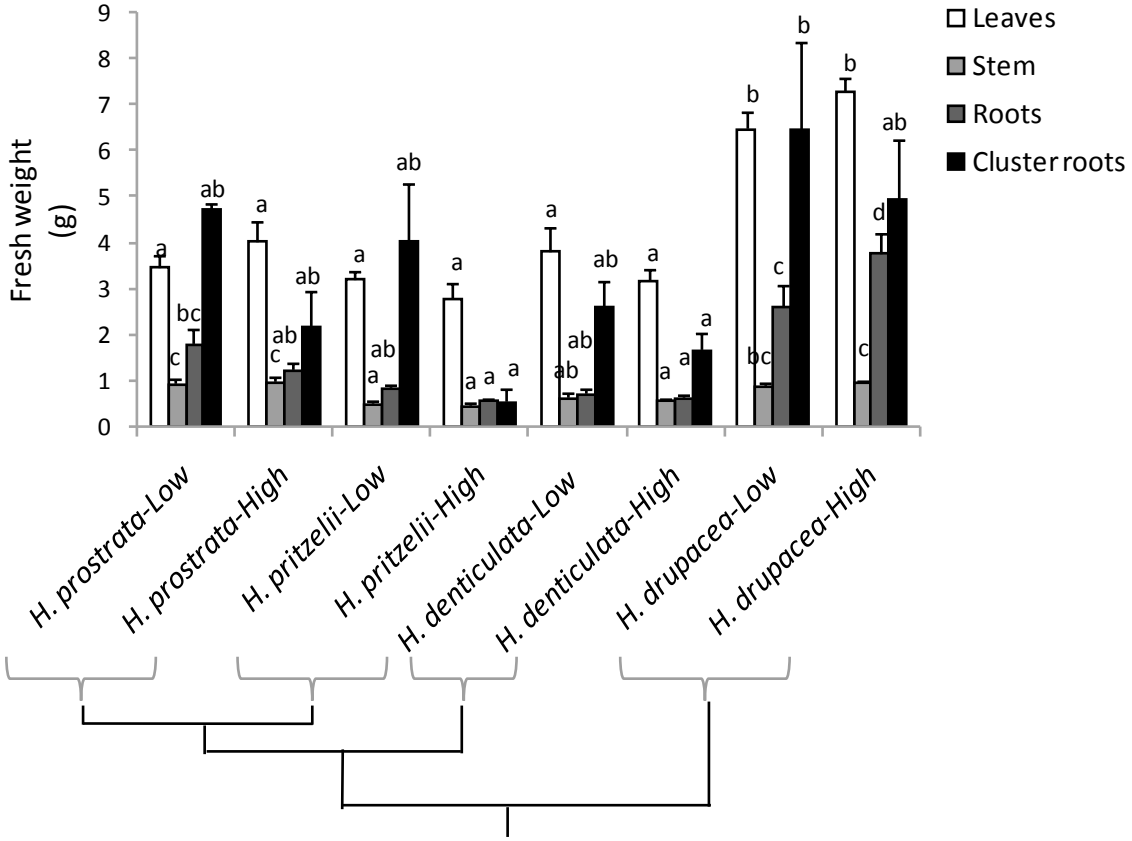

Fig. 15. Leaf, stem, root and cluster root fresh weight of four *Hake* species under low or high Pi supply. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Plants were harvested following development of necrotic lesions in plants treated with high Pi supply. Values are means and bars represent standard errors (n = 5). Results were tested by two-way ANOVA and Tukey's multiple comparison with 95% confidence interval. The values for each organ were tested separately. Letters show the multiple comparison ($P < 0.05$).

Treatments also did not have a significant impact on stem fresh weight but overall, there was a significant difference in the stem fresh weight among the species (Fig. 15). There was a significant interaction between the species and treatments for root fresh weight, showing that species responded differently to the Pi treatments. Cluster root fresh weight was the only tissue that overall was significantly different among both species and treatments. All of the species tended to have lower cluster root fresh weight when exposed to higher Pi supplies (Fig. 15).

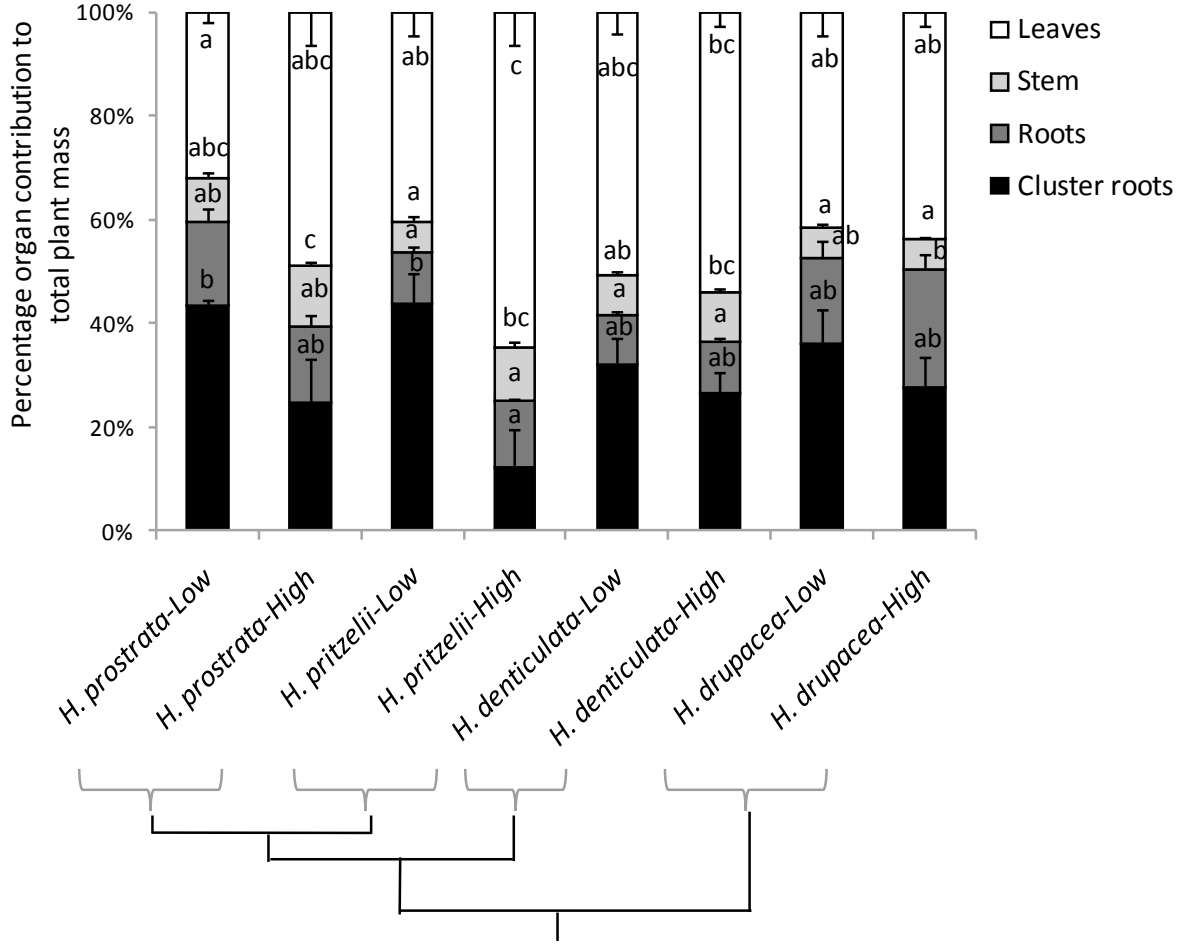

Fig. 16. Average percentage of leaves, stem, roots and cluster roots to whole-plant fresh weight in four *Hakea* species under low or high Pi supply. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Plants were harvested following development of necrotic lesions in plants treated with high Pi supply. Bars represent standard errors (n = 5). Results were tested by two-way ANOVA and Tukey's multiple comparison with 95% confidence interval. The values for each organ were tested separately. Letters show the multiple comparison ($P < 0.05$).

All of the species allocated relatively more resources to their leaves when they were treated with high concentrations of Pi (Fig. 16); however, the difference was small in *H. denticulata* and *H. drupacea*. Resource allocation to roots was diverse and did not follow a pattern and resource allocation to stems was similar between the treatments. All the species allocated more resources to their cluster roots when they were treated with lower Pi supplies (Fig. 16).

Cluster root numbers tended to be lower in all of the species when exposed to higher Pi supply (Fig. 17). *H. drupacea* tended to have a greater number of cluster roots when

exposed to either low or high Pi supplies compared to other species. The number of cluster roots was not significantly different between the most closely related species, but it was significantly affected by the treatments.

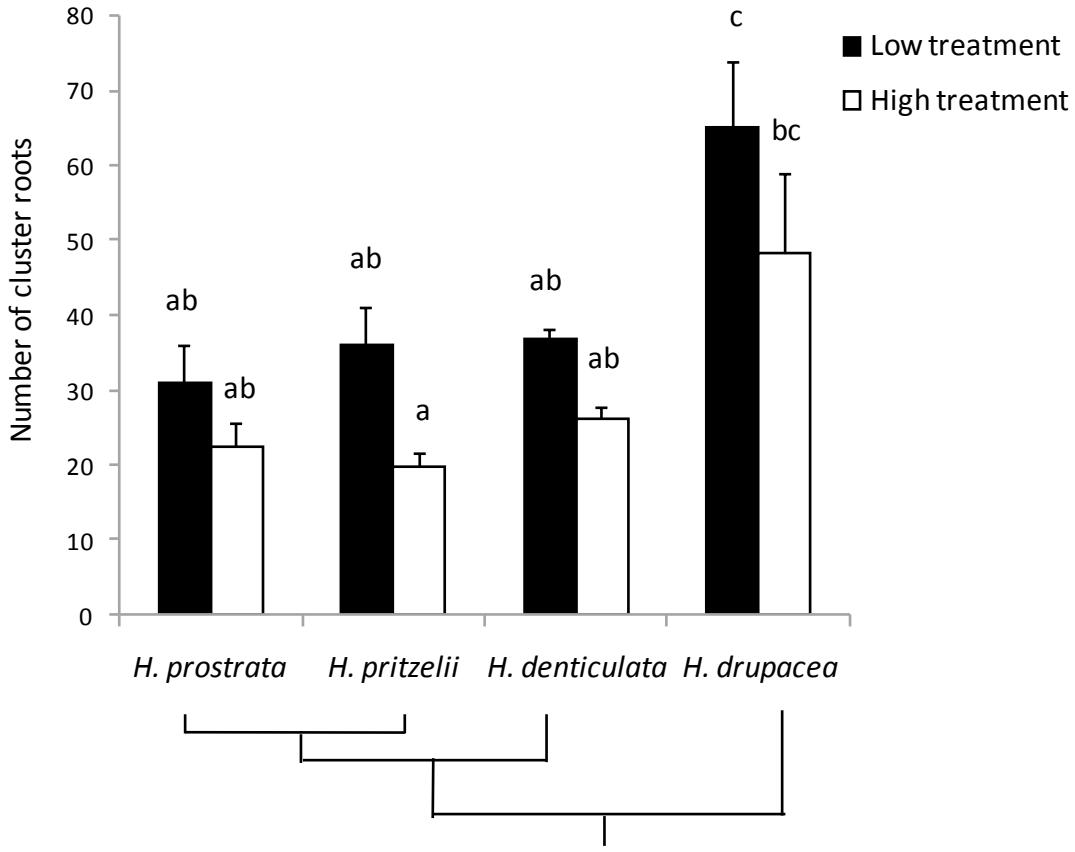

Fig. 17. Cluster root production in response to low and high Pi supply. Values represent means. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Plants were harvested following development of necrotic lesions in plants treated with high Pi supply. Values are means and bars are standard errors (n = 5). Results were tested by two-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Letters show the multiple comparison ($P < 0.05$). The number of cluster roots was counted to the highest possible accuracy.

3.10 Leaf photosynthetic pigment concentration response under low versus high Pi

All species tended to have less Chl a and Chl b in their leaves when treated with high levels of Pi (Fig. 17). Overall, there was a significant difference among the species and treatments in terms of Chl a and Chl b concentration. However, carotenoid concentrations were only significantly different among the species. Total photosynthetic pigment concentration of *H. drupacea* was significantly higher than in other species

under both low and high Pi supply. The Chl a to Chl b ratios are illustrated in Table 2. All species had lower Chl a/Chl b ratio following exposure to higher Pi supply except *H. drupacea*.

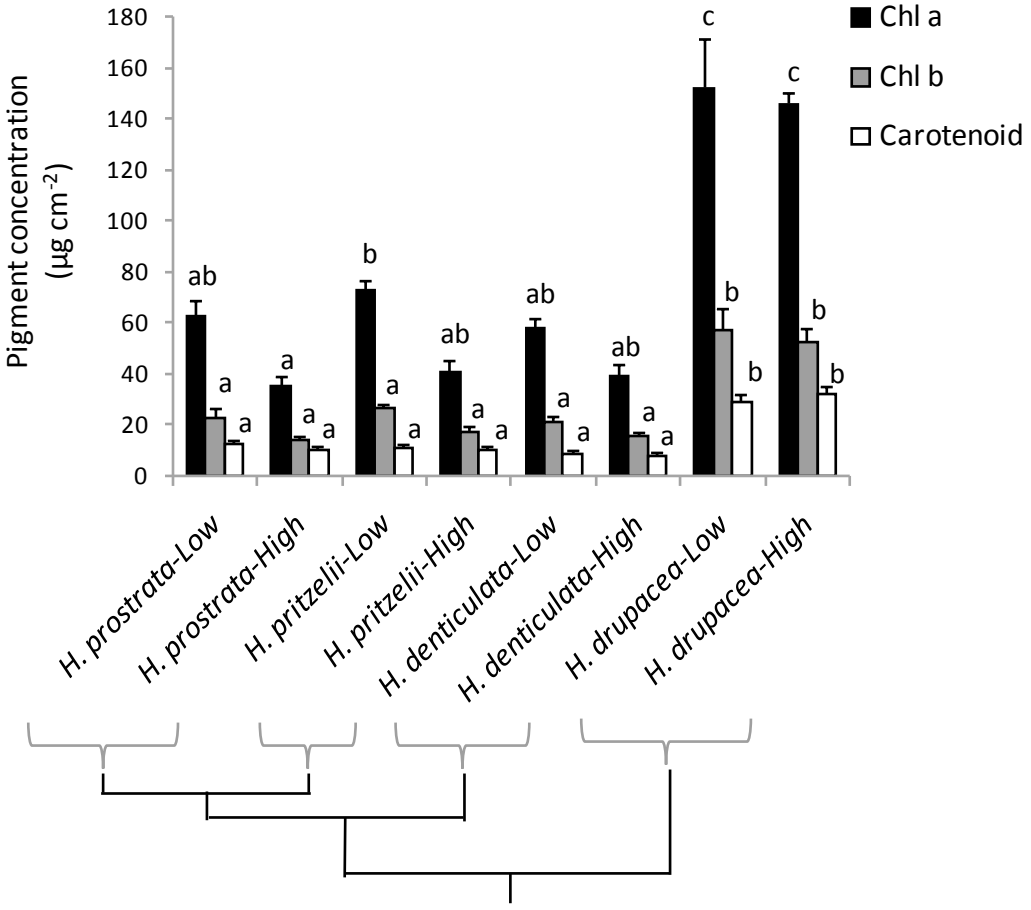

Fig. 18. Chl a, Chl b and carotenoid concentrations in leaves of four *Hakea* species under low and high Pi supplies. Species are ordered based on their phylogenetic relationships (see Methods). For growth conditions see Methods. Plants were harvested following development of necrotic lesions in plants treated with high Pi supply. Values represent means and bars are standard errors (n = 5). Results were tested by two-way ANOVA and Tukey's multiple comparison with 95% confidence interval. Pigments were tested separately among the species and letters show the multiple comparison ($P < 0.05$). *H. drupacea* leaves had spiky leaves with a thickness of approximately 1mm.

Table 2. Chlorophyll a to chlorophyll b ratios under low vs high Pi supply.

Species	Chl a / Chl b	
	Low Pi	High Pi
H. prostrata	2.76	2.48
H. pritzelii	2.75	2.39
H. denticulata	2.77	2.48
H. drupacea	2.70	2.88

4. Discussion

When plants were exposed to only low Pi supply (7.5 µmol Pi $week^{-1}$) the leaves showed some signs of necrosis. Following harvest, it was found that the Pi concentration in the tissues were at low levels. Consequently, this Pi supply and the similar Pi supply used in the next experiments (10 µmol Pi $week^{-1}$) were designated as a low Pi supply. Based on this finding the increasing Pi supply used in the experiments was indicated as a high Pi supply.

The experiments of this study were performed in sand and plants with larger root systems may have had more access to the applied chemicals; however, this does not necessarily mean that they had a higher ability to acquire the applied chemicals as this is also dependent on the ability of the species to regulate the uptake of the chemicals.

4.1 H. denticulata was the most P sensitive species and high P concentration in plant tissue is not likely to be the only factor responsible for P sensitivity in Hakea *species*

Under high Pi supply, *H. denticulata*, *H. pritzelii* and *H. prostrata* showed necrosis on their leaves that was associated with P toxicity. P toxicity has also been reported in a number of Australian species previously (Specht, 1963; Grundon, 1972; Groves and Keraitis, 1976; Handreck, 1997*b*). The combination of leaf Pi concentration, low Chl a and Chl b concentrations and leaf necrosis, a hallmark symptom of P toxicity, indicate that *H. denticulata* was the most sensitive species to Pi supply as this species developed toxicity symptoms at a lower rate of Pi supply (759 µmol Pi $week^{-1}$) than the other species. Surprisingly, the Pi concentration in the leaves was lower than that for all the other examined species. This interesting finding indicates that high tissue Pi concentration is not solely responsible for P sensitivity in *Hakea* species and it is likely that other factors such as the ability to esterify P into organic compounds (Schachtman *et al*., 1998) and remobilise P to various tissues have roles in determining the sensitivity of *Hakea* species. *H. pritzelii* was the second most sensitive species to P. This species developed necrosis in its leaves following exposure to 1.7 mmol Pi $week^{-1}$. At harvest its leaf Pi concentration was higher than *H. denticulata* showing that it was able to tolerate higher leaf Pi concentrations. *H. prostrata* was ranked third in terms of P sensitivity as this species developed necrosis in its leaves after exposure to 2.5 mmol Pi $week^{-1}$. In addition, at harvest this species had the highest concentration of Pi in its leaves showing that it had higher tolerance to tissue Pi than *H. denticulata* and *H.*

pritzelii. These findings are in contrast with another study that suggested the P sensitivity in *H. prostrata*, and some other Proteaceae species, is not due to increased susceptibility to tissue P concentrations as these species showed toxicity symptoms at leaf or shoot total P concentration of 0.27 to 14.1 mg P g^{-1} FW, whereas this range in crop species is 0.51 to 4.2 mg P g^{-1} FW (Shane *et al*., 2004*b*). However, except *H. prostrata*, none of the other species that were examined in the present study were included in the comparison that was made previously. Findings of the present study shows that some *Hakea* species (*H. denticulata* and *H. pritzelii*) are more sensitive to P accumulation in their tissues than others. Therefore, although increased susceptibility to tissue P concentration may not be the reason behind the P sensitivity in *H. prostrata* (Shane *et al*., 2004*b*), some other species of *Hakea* may be overly sensitive to lower tissue P concentrations. Thus, a combination of increased susceptibility to low tissue P concentrations and other factors that were mentioned in this section are likely to be responsible for P sensitivity in some *Hakea* species. Furthermore, *H. drupacea* was least sensitive to Pi supply among the tested species. This species did not show necrosis at harvest, despite being exposed to highest Pi supply. Interestingly, the leaves of *H. drupacea* had higher Pi concentrations than *H. denticulata*, but less than *H. prostrata*. Thus, not only was *H. drupacea* the least sensitive species to P, but it was also the most efficient species in regulating Pi accumulation and proficient in keeping Pi from leaves while being exposed to the highest Pi supply. This suggests that this species might be able to regulate its P uptake capacity. The ability to regulate Pi uptake has also been reported in a number of plants from different families (Shane *et al*., 2004*a*; Shane and Lambers, 2006; Delgado *et al*., 2013; de Campos *et al*., 2013).

It has been suggested that *H. drupacea* is less sensitive to P than *H. prostrata* (Handreck, 1997*a*), however, *H. pritzelii* and *H. denticulata* that are also phylogenetically closely-related to *H. prostrata* (Clopton, 2012) have not been tested for P sensitivity. Findings of the current study indicates that *H. denticulata*, *H. pritzelii*, *H. prostrata* and *H. drupacea* are the most to least P sensitive species respectively. Interestingly, the order of P tolerance in these species is not dependent on their phylogenetic relationships.

The leaf total P concentration of *H. prostrata* was approximately 3.9 μmol P g^{-1} FW in its natural habitat (Shane *et al*., 2004*b*). Leaf Pi concentration of *H. prostrata* plants that developed P toxicity symptoms (necrosis) was 129.8 μmol Pi g^{-1} FW showing that their

leaf total P concentration was higher. This finding is in agreement with previous studies that reported P toxicity symptoms in *H. prostrata* at leaf total P concentration of about 86 μmol P g^{-1} FW and higher (Shane *et al*., 2004*a*; Shane *et al*., 2004*b*). In terms of leaf total P concentration based on dry weight, it was reported that *H. prostrata* showed P toxicity symptoms at about 10 mg P g^{-1} DW (Shane *et al*., 2004a; Shane *et al*., 2004*b*), while *Banksia attenuata* and *B. menziesii* showed signs of P toxicity at leaf total P concentration of 10 and 13 mg P g^{-1} DW respectively (de Campos *et al*., 2013). Conversely, *Acacia truncata* and *A. xanthina* did not develop P toxicity symptoms even at leaf total P concentrations of 19 and 21 mg P g^{-1} DW respectively (de Campos *et al*., 2013). Thus, it can be concluded that *H. prostrata* and *B. attenuata* have similar levels of tolerance to leaf P concentration. In addition, *H. prostrata* is somewhat more sensitive to leaf P accumulation than *B. menziesii*, *A. truncata* and *A. xanthina*. Given that *H. denticulata* and *H. pritzelii* are more sensitive to leaf Pi accumulation than *H. prostrata*, based on the findings of the current study, it is likely that these two species are also more sensitive to tissue P concentration than *B. menziesii*, *A. truncata* and *A. xanthina* and *B. attenuata*.

Previously *H. prostrata* showed P toxicity symptoms that led to necrosis in the leaves when exposed to 0.7 and 1.4 mmol Pi $week^{-1}$ in a hydroponics system (Shane *et al*., 2004*a*). The development of P toxicity symptoms by *H. prostrata* at higher levels of external P in the current study (2.5 mmol Pi $week^{-1}$) may be partially due to the difference in the growth environment. In the current experiment plants were grown in sand and it is highly likely that the plants will not be able to access all the P available in the nutrient solution. Another consideration is the application dose. In the previous study, plants were supplied with P on a daily basis (Shane *et al*., 2004*a*) which gives the plants the chance to acquire lower amounts of P over a short time interval, while in the current experiment, when plants were exposed to both low and high P, Pi was applied twice a week, requiring plants to respond to higher amounts of P at once. However, these comparisons might be somewhat biased as the *H. prostrata* plants that showed toxicity symptoms at lower P supplies (0.7 and 1.4 mmol Pi $week^{-1}$) than used here were constantly treated with the same P supplies (Shane *et al*., 2004*a*), while in the current study plants were exposed to increasing levels of Pi. Symptoms of P toxicity in leaves of *H. prostrata* were also reported at supplies of 2.1 mmol P $week^{-1}$ (300 μmol P d^{-1}) (Shane *et al*., 2004*b*), that is somewhat more similar to the findings of the current study.

4.2 Patterns of Pi accumulation and allocation to the leaves in a time-course of 12 weeks showed the ability of Hakea *species to regulate Pi allocation*

The Pi concentration in the leaves of all the species treated with low Pi (10 µmol Pi $week^{-1}$) supply was low and constant during the time-course experiment. Showing that plants were able to acquire Pi and allocate it to their leaves and the low Pi in their leaves is likely due to the low Pi supply. The four species also showed a somewhat similar pattern to one another when exposed to high Pi supplies. Previously it was suggested that *H. prostrata* was able to sense the P treatments and respond accordingly by regulating root and cluster root production and early leaf senescence (Shane *et al*., 2004*a*). The current experiment suggests that all the species tested were able to sense increases in Pi supply and initially down-regulate their Pi accumulation and allocation to the leaves as they still showed low concentrations of Pi after exposure to 150 µmol Pi $week^{-1}$. However, following exposure to 506 µmol Pi $week^{-1}$, the available P exceeded the threshold beyond which the plants were able to effectively regulate Pi accumulation and allocation to the leaves. This resulted in a large increase in leaf Pi concentration for all species that was more pronounced at higher doses of Pi.

At the highest external Pi supply, *H. prostrata*, *H. pritzelii* and *H. denticulata* showed inability to down-regulate their Pi accumulation and allocation to the leaves that may be due to inability of these species to down-regulate their Pi uptake capacity. This finding is somewhat consistent with a previous study that suggested an inability to down-regulate P uptake in *H. prostrata* (Shane *et al*., 2004*a*). Inability to down-regulate P uptake has also been reported in *B. grandis* (Lambers *et al*., 2002), *B. attenuata* and *B. menziesii* that are also Australian native species (de Campos *et al*., 2013). Conversely, *H. drupacea* was somewhat able to down-regulate its Pi accumulation and allocation to the leaves. This finding suggests that *H. drupacea* may be able to down-regulate its Pi uptake. This trait was previously reported in *A. truncata*, *A. xanthina* (de Campos *et al*., 2013), *Triticum aestivum*, *Medicago truncatula* and *Lupinus albus* (Shane *et al*., 2004*a*). In addition, the ability to regulate P uptake has also been suggested in other members of Proteaceae, *Grevillea crithmifolia* (Shane and Lambers, 2006) and *Embothrium coccineum* (Delgado *et al*., 2013).

Following the exposure to 1.7 mmol Pi $week^{-1}$, *H. prostrata* plants had the highest leaf Pi concentration, followed by *H. pritzelii*, *H. denticulata* and *H. drupacea*. This pattern was similar to the phylogenetic relationships among these species, suggesting that the Pi

accumulation and allocation patterns in leaves were likely to be dependent on the phylogenetic relationships among the species while exposed to high levels of Pi and phylogeny can be used to predict Pi allocation patterns in plants. The findings of this experiment showed that *H. prostrata* was the most efficient species in Pi accumulation under high Pi supply as it had the highest Pi concentration in its leaves at the end of twelve weeks. In addition, *H. drupacea* is the most efficient species in down-regulating Pi accumulation and allocation to the leaves under high Pi supply among the examined species.

4.3 Interactions between root and cluster root production and Pi accumulation and P allocation patterns under low Pi supply with respect to tissue P concentration and introduction of a new P-efficient model

An increase in root production is a mechanism used by Australian plants to increase P uptake (Handreck, 1997*b*); however, some plants, such as those in Proteaceae family, are also capable of more specialised root responses such as cluster root production (Purnell, 1960). Proteaceae are adapted to nutrient-impoverished soils (Pate *et al*., 2001). However, different species of Proteaceae that are phylogenetically closely related (Clopton, 2012) have never been compared to one another in terms of efficiency in P acquisition and allocation to different tissues and root and cluster root production in order to acquire P. *H. pritzelii* was one of the species with the lowest root and cluster root biomass and was ranked second last in number of cluster roots. Despite the low root and cluster root biomass, surprisingly, this species tended to have a greater concentration of Pi in its leaves than the other species. This illustrates that *H. pritzelii* was the most efficient species in harvesting Pi per unit of root and cluster root biomass. Moreover, *H. pritzelii* had the highest Po concentration in its leaves and therefore was the most efficient species in converting Pi to Po in its leaves. As *H. pritzelii* had low cluster root biomass and numbers, the reason for the higher Pi accumulation efficiency of this species was not clear. This finding shows that *H. pritzelii* can be used as a new model plant to further study and understand the possible mechanisms for P uptake and allocation to the leaves under low available P.

Although *H. megalosperma* had high tissue biomass, lack of a significant difference between root and cluster root biomass of this species and *H. prostrata* indicates the ability of *H. prostrata* in root and cluster root production. Showing that *H. prostrata* was able to allocate as much resources to cluster root development as its larger relative.

The higher number of cluster roots in *H. megalosperma* compared to *H. prostrata*, in the absence of a significant difference in cluster root biomass between these two species, may indicate the production of larger cluster roots by *H. prostrata*. These findings emphasise the ability of *H. prostrata* to sense and respond to low P levels in its environment that has been suggested previously (Shane *et al*., 2004*a*). In the current study the response appeared as formation of more roots and cluster roots, presumably to maximise the acquisition of available P.

These two species also had similar leaf Pi concentrations that tended to be greater than for the other species except *H. pritzelii*. This observation may indicate a positive relationship between root and cluster root production and leaf Pi concentration in these species. Exudation of carboxylates from cluster roots is likely to aid P acquisition especially in nutrient-impoverished soils (Shane *et al*., 2003), also larger root systems can explore more of the growing environment and therefore can have access to more nutrients (Handreck 1997*b*). In addition, *H. prostrata* and *H. megalosperma* had similar concentrations of Po in their leaves, making these two species equally efficient in Pi to Po conversion. Even though *H. prostrata* and *H. megalosperma* tended to have greater Pi concentrations in their leaves compared to the other species, except *H. pritzelii*, similar levels of Po in their leaves than other species, except *H. pritzelii*, indicates that these two species are not only similar in their Pi accumulation ability, Pi allocation patterns and Pi to Po conversion but they also show slight inability in Pi to Po conversion in comparison to the other species. Conversely, *H. denticulata*, *H. amplexicaulis*, *H. ruscifolia* and *H. drupacea* had similar Pi accumulation and also leaf Pi allocation patterns. They also had similar patterns of Pi to Po conversion in their leaves. Overall, these seven species showed diverse Pi accumulation and allocation patterns that were not dependent on their phylogenetic relationships. They were also diverse in their ability to convert Pi to Po that was independent of their phylogenetic relationships.

In general, all the species had low concentrations of Pi in their roots and cluster roots, indicating that these *Hakea* species do not keep Pi in their roots and cluster roots following acquisition. These results are in agreement with previous studies that showed low levels of P in roots of *H. prostrata* plants following exposure to 1 μM P day^{-1} in a hydroponics system (Shane *et al*., 2003). Po concentrations in the roots of species were higher than Po concentrations in the cluster roots showing that these plants use their

cluster roots solely as a Pi acquisition tool and they minimise the investment of P in their cluster roots. In addition, higher levels of Po in roots compared to cluster roots is an indication of plants P investment in the roots.

4.4 Interactions between root production and Pi acquisition and P allocation patterns under low Pi supply with respect to P content

In comparing the Pi mining and P allocation ability of the species at the whole tissue level, *H. megalosperma* had the highest Pi and Po content in the leaves. The higher capacity of this species to mine Pi and allocate it to its leaves was probably due to the larger size of its root system. The higher Pi content in the whole leaves of *H. megalosperma* was likely due to its greater leaf fresh weight, since it has more tissue to store the Pi in. Furthermore, the higher Po content in the leaves of *H. megalosperma* was likely due to their higher Pi content, as there was more Pi available to convert to Po. Therefore, this species could be used for agricultural and decontamination purposes to mine the extra Pi out of the fields that are contaminated due to high levels of Pi fertilisation. Subsequently, this plant could be used as a source of P (fertiliser) in fields with low levels of P. Other tested species in this study illustrated similar range of Pi and Po content in their whole leaf tissues under low Pi supply.

All of the species had similar Pi and Po content in the roots and cluster roots except *H. megalosperma*, which had somewhat higher Po content in its roots and cluster roots. Thus, there was a higher P investment in whole roots and cluster roots of this species that may be due to higher root and cluster root fresh weight of this species.

4.5 Growth analysis of Hakea *species under low Pi supply and growth comparison under low versus high Pi supply*

The growth response of various tissues in seven different *Hakea* species following exposure to 7.5 μmol Pi $week^{-1}$ was very diverse and not related to the phylogenetic relationships among the species. While *H. pritzelii*, which tended to have lower leaf biomass, tended to have greater Pi concentration in the leaves, the finding of a moderate, but not low, Pi concentration in the leaves of *H. megalosperma*, which had a high leaf biomass, showed that it is unlikely that there was a negative relationship between the leaf biomass and Pi concentration in the leaves. Greater Pi concentration in the leaves of *H. pritzelii* might confirm the idea that some plants may be capable of sensing the available Pi levels through their roots and regulate the shoot growth

accordingly and before the Pi concentration in the leaves decreases to low levels (Veneklaas *et al*., 2012) as the species that tended to have greater P concentration in its leaves, tended to have lower leaf fresh weights. More than half of the plant biomass in *H. prostrata*, *H. pritzelii* and *H. denticulata* was allocated to cluster roots, indicating the importance of cluster roots in these species under low Pi supply.

In terms of low (10 μmol Pi $week^{-1}$) versus high Pi supply, there was no clear and uniform pattern in leaf, stem and root fresh weight response to treatments across all the species. Higher trends for leaf fresh weight in *H. prostrata* provided with high Pi supply was consistent with previous findings (Shane *et al*., 2004*b*). In the current study this pattern was also observed in *H. drupacea*. Conversely, *H. pritzelii* and *H. denticulata* showed the opposite trend, with leaf fresh weights tending to be lower after exposure to high Pi supply, indicating a different response of leaf growth in these two species compared to *H. prostrata* and *H. drupacea*. However, the mass ratio results illustrate that all the species tended to allocate somewhat more resources to their leaves when exposed to high Pi supply. This finding is in contrast with another study that reported a lower shoot biomass in *A. truncata*, *A. xanthina* and *B. menziesii* following exposure to high levels of P supply (de Campos *et al*., 2013), showing that in different species shoot growth responds differently to high external P supply.

Cluster root growth tended to be lower in all of the species when they were treated with higher levels of Pi. This is in agreement with the previous studies on *H. prostrata* (Shane *et al*., 2004*b*). In addition, the cluster root mass ratio and fresh weight show that all four species tended to allocate less resources to their cluster roots when provided with a higher Pi supply. This finding was expected as the main role of cluster roots is to aid P acquisition and uptake under low P conditions (Shane and Lambers, 2005) and plants do not need to rely on cluster roots for P uptake when there is enough P available to their roots. The number of cluster roots tended to be lower under the high Pi supply than under the low (10 μmol Pi $week^{-1}$) Pi supply for all the four tested species. Thus, cluster root production in *H. pritzelii*, *H. denticulata* and *H. drupacea* responded to P supply in generally the same way as was previously found for *H. prostrata* (Shane *et al*., 2003). Furthermore, the somewhat greater number of cluster roots in *H. drupacea* under both low and high Pi supplies than in the other three species suggested a greater capacity for Pi acquisition in this species. This is compatible for the greater leaf fresh weight found for *H. drupacea* than the other species, as P has major impacts on plant

growth (Lambers *et al*., 2006). The difference in the pattern of cluster root production in *H. drupacea* in response to Pi supply in comparison to the other three species may be partially due to the phylogenetic distance between this species and the other three species included in this experiment.

4.6 Pigment production of Hakea *species under low Pi supply*

Chl a, Chl b and carotenoids have major roles in photosynthesis (Chappelle *et al*., 1992). Their concentrations are representative of the photosynthesis level and capacity of the plants (Chappelle *et al*., 1992). There is a strong correlation between the concentration of these pigments and the physiological conditions of the plants (Chappelle *et al*., 1992).The content of pigments in the leaves can change due to abiotic and biotic factors (Bacci *et al*., 1998). In this study the concentration of the pigments in the leaves of species was diverse and independent of their phylogenetic relationships at low Pi (7.5 µmol Pi $week^{-1}$) supply. Chl a is the key pigment (Lichtenthaler, 1987), which explains why all the examined species had higher concentrations of Chl a than Chl b. Lack of a significant difference in the concentration of Chl a among the species suggests that the photosynthetic capacity per area of the species was not significantly different from one another under low Pi supply. However, since *H. megalosperma* tended to have greater leaf fresh weights than other species, this species is likely to have greater absolute photosynthetic capacity among the examined species. A higher Chl a/Chl b ratio may indicate a higher photosynthetic capacity for a plant (Chappelle *et al.*, 1992). Thus, *H. drupacea* and *H. denticulata* were likely to have had the highest and lowest photosynthetic capacity, respectively, when exposed to low Pi supplies.

4.7 Pigment production responses of Hakea *species to low versus high Pi supply*

Lower trends in the concentrations of Chl a and Chl b in plants given high Pi supplies may be a further indication that the P levels in the leaves are reaching toxic levels and therefore affecting the photosynthestic potential of the plants. A decreased Chl a/Chl b ratio may indicate lower photosynthetic potential (Chappelle *et al.*, 1992). The lower Chl a/Chl b ratio in *H. prostrata*, *H. pritzelii* and *H. denticulata* following exposure to higher Pi supply suggests that the photosynthetic potential of these species was likely to be decreased. However, *H. drupacea* had a slight increase in Chl a/Chl b ratio following exposure to higher Pi supply. This may indicate that this species was not affected as intensely as the other three species following exposure to high Pi supply. This is

consistent with results that showed *H. drupacea* illustrated the lowest levels of P toxicity symptoms on its leaves.

Greater pigment concentrations in *H. drupacea* under both low (10 µmol Pi $week^{-1}$) and high Pi supplies compared to the other species is consistent with the greater leaf fresh weight in this species, suggesting a positive impact of increased photosynthesis on growth. In the first experiment and when species were only exposed to low Pi supply (7.5 µmol Pi $week^{-1}$), *H. drupacea* did not show greater pigment concentrations than other species. This could be due to slight differences in the growth conditions between experiments. In addition, the pigment concentrations in plants following exposure to 10 µmol Pi $week^{-1}$ were higher than those following exposure to 7.5 µmol Pi $week^{-1}$. This is likely due to slight differences in the growth conditions in the experiments as plants exposed to 10 µmol Pi $week^{-1}$ were healthier. Furthermore, plants receiving 10 µmol Pi $week^{-1}$ were under this Pi supply for several weeks, whereas plants in the first experiment were exposed to 7.5 µmol Pi $week^{-1}$ only for one week so these plants were exposed to less available Pi and plats with more P supply may have higher photosynthesis rate as shown previously (Shane *et al*., 2004*b*).

4.8 Conclusion

H. denticulata was the most sensitive species to high Pi supply. It developed leaf necrosis at a lower Pi supply, and had the lowest leaf Pi concentration among the four examined species at this Pi supply. *H. pritzelii* was the second most sensitive species to increasing supplies of Pi. *H. drupacea* was the species with the highest ability to down-regulate Pi allocation to the leaves at high Pi supply. It has been suggested that *H. prostrata* is likely to be able to sense and respond to P application (Shane *et al*., 2004*a*). This study showed that not only *H. prostrata* but also *H. pritzelii*, *H. denticulata* and *H. drupacea* are capable of sensing and regulating Pi allocation to the leaves to some extent with increases in Pi supply. All of these species to some extent were able to maintain a constant leaf Pi concentration until the Pi supply was increased to 150 µmol Pi $week^{-1}$. Once this threshold Pi supply was exceeded, there was a large increase in the leaf Pi concentration. This illustrated the inability of all the species examined to regulate Pi accumulation when supplied with 500 µmol Pi $week^{-1}$ or more. The patterns of Pi allocation to the leaves when supplied with high Pi was somewhat related to the phylogenetic relationships among the species. Higher Pi concentrations in leaves of *H. prostrata* showed that this species was the most efficient species in Pi allocation at high

Pi supply. Conversely, the low Pi concentration in leaves of *H. drupacea* showed that this species was the most efficient species in restricting Pi accumulation in the leaves.

This study showed that the Pi acquisition and allocation patterns of *H. prostrata*, *H. pritzelii*, *H. denticulata*, *H. amplexicaulis*, *H. megalosperma*, *H. ruscifolia* and *H. drupacea* upon low Pi supply was not related to the phylogenetic relationships among the species. In addition, *H. pritzelii* was the most efficient species in Pi accumulation and allocation to the leaves with respect to leaf Pi concentration when exposed to low Pi supply. *H. pritzelii* was also the most efficient species in converting Pi to Po in the leaves at low Pi supply and with respect to P concentration in the tissues. This suggests that *H. pritzelii* may be even more efficient than *H. prostrata* in Pi uptake at low Pi supplies. Therefore, more studies should be done on *H. pritzelii* as this species has the potential to be used as a model to improve the Pi acquisition efficiency of other plants including crops in environments with deficient levels of available P. I propose that *H. pritzelii*, the most efficient species in Pi accumulation under low Pi supply, *H. prostrata*, the most efficient species in Pi accumulation and allocation to the leaves under high Pi supply, and *H. drupacea*, the most efficient species in regulating Pi accumulation and allocation to the leaves under high Pi supply, are the three species that could be used in combination with one another, to reveal the complete mechanism of P uptake in *Hakea* species that in the long term may result in the production of P-efficient crops that may help minimise the need of P fertiliser.

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Appendices

Tables associated with statistical analysis used in each figure

Table 3. Summary of statistical analysis (one-way ANOVA) for figure 6.

Figure	*P*
6	Leaves: <0.001 Stems: 0.002 Roots: 0.003 Cluster roots: 0.009

Table 4. Summary of statistical analysis (one-way ANOVA) for figure 7.

Figure	*P*
7	Leaves: 0.012 Stems: 0.915 Roots: 0.022 Cluster roots: 0.053

Table 5. Summary of statistical analysis (one-way ANOVA) for figure 8.

Figure	*P*
8	Cluster roots: <0.001

Table 6. Summary of statistical analysis (one-way ANOVA) for figure 9 (Chl a: Chlorophyll a, Chl b: Chlorophyll b, Car: Carotenoids).

Figure	*P*
9	Chl a: 0.051 Chl b: 0.032 Car: <0.001

Table 7. Summary of statistical analysis (one-way ANOVA) for Pi concentrations in figure 10.

Figure	*P*
10	Leaves: 0.023 Roots: 0.004 Cluster roots: 0.031

Table 8. Summary of statistical analysis (one-way ANOVA) for Po concentrations in figure 10.

Figure	*P*
10	Leaves: 0.004 Roots: 0.379 Cluster roots: 0.488

Table 9. Summary of statistical analysis (one-way ANOVA) for Pi contents in figure 11.

Figure	*P*
11	Leaves: 0.002 Roots: 0.391 Cluster roots: 0.003

Table 10. Summary of statistical analysis used (one-way ANOVA) Po contents in figure 11.

Figure	*P*
11	Leaves: <0.001 Roots: 0.033 Cluster roots: 0.001

Table 11. Summary of statistical analysis (two-way ANOVA) in figure 14.

Figure	*P* (Species)	*P* (Pi treatment)	*P* (Interaction)
14	Leaves: <0.001	Leaves: <0.001	Leaves: <0.001

Table 12. Summary of statistical analysis (two-way ANOVA) in figure 15 (CR: Cluster roots).

Figure	*P* (Species)	*P* (Pi treatment)	*P* (Interaction)
15	Leaves: <0.001 Stems: <0.001 Roots: <0.001 CR: 0.004	Leaves: 0.729 Stems: 0.965 Roots: 0.774 CR: 0.005	Leaves: 0.087 Stems: 0.710 Roots: 0.008 CR: 0.592

Table 13. Summary of statistical analysis (two-way ANOVA) in figure 16 (CR: Cluster roots).

Figure	*P* (Species)	*P* (Pi treatment)	*P* (Interaction)
16	Leaves: 0.017 Stem: <0.001 Roots: <0.001 CR: 0.719	Leaves: <0.001 Stem: <0.001 Roots: 0.120 CR: <0.001	Leaves: 0.053 Stem: 0.024 Roots: 0.212 CR: 0.130

Table 14. Summary of statistical analysis (two-way ANOVA) in figure 17 (CR: Cluster roots).

Figure	*P* (Species)	*P* (Pi treatment)	*P* (Interaction)
17	CR: <0.001	CR: 0.002	CR: 0.851

Table 15. Summary of statistical analysis (two-way ANOVA) in figure 17 (Chl a: Chlorophyll a, Chl b: Chlorophyll b, Car: Carotenoids).

Figure	*P* (Species)	*P* (Pi treatment)	*P* (Interaction)
18	Chl a: <0.001 Chl b: <0.001 Car: <0.001	Chl a: <0.001 Chl b: 0.009 Car: 0.799	Chl a: 0.366 Chl b: 0.898 Car: 0.171

PHT gene sequence analysis in H. prostrata

Phosphate transporter genes play major roles in Pi acquisition from the environment and also in Pi translocation within the plants (Smith *et al*., 2003). Therefore, it is important to study the number of these genes that are expressed in roots and shoots of *H. prostrata* as this information may be directly related to the ability and efficiency of *Hakea* species in Pi acquisition and P allocation within the plant. Several *PHT1* transcript sequences from *H. prostrata* were previously obtained from direct cloning experiments or extracted from a database of assembled RNAseq reads from shoot and root tissues (Pontre, 2011). The RNAseq reads were reassembled (Jost *et al*., unpublished) using the Soap DeNovo platform (http://www.onekp.com) and probed with several of the known *HpPHT1* sequences using basic local search alignment tool (BLAST in Geneious 6.1.5, Biomatters Ltd.). A second database of assembled *H. prostrata* RNAseq data was constructed with Trinity (Jost *et al*., unpublished), allowing the *PHT* genes sequences from *H. prostrata* to be matched to their likely orthologs in *Arabidopsis thaliana.* The results from two databases were combined and compared resulting in identification of fifteen *PHT* sequences in *H. prostrata* (Table 1.). Six *PHT* mRNA sequences belonged to the *PHT1* family, while one and three sequences were identified from the *PHT2* and *PHT3* families, respectively. In addition, five *PHT* sequences did not have orthologs in *Arabidopsis* and their gene family could not be identified. Three of these mRNA sequences were extracted from the shoot database showing that they were expressed in leaves, while the other two were present in roots.

Table 16. Fifteen *PHT* sequences were identified in *H. prostrata* using two databases constructed from transcriptome sequences. Results from Trinity database were obtained from (Jost *et al*., unpublished).

Phosphate transporter name	Identifier		Arabidopsis ortholog
	SOAP denovo database	Trinity database	
HpPHT1;3/HpPHT1;5	2103871-roots	comp3147_c0_seq1/comp3147_c0_seq2	*PHT1;3/PHT1;5*
HpPHT1;4	2006891-leaves/2006890 leaves/2104014 roots	comp537_c0_seq1	*PHT1;4*
HpPHT1;7	None	comp72421_c0_seq1	*PHT1;7*
HpPHT1;8	None	comp67853_c0_seq1	*PHT1;8*
HpPHT1;9	None	comp31552_c0_seq1	*PHT1;9*
HpPHT2;1	None	comp2845_c0_seq1	*PHT2;1*
HpPHT3;1(2)	None	comp_327_c0_seq3	*PHT3;1*
HpPHT3;2	None	comp_25886_c0_seq1	*PHT3;2*
HpPHT3;3	None	comp_19366_c0_seq1	*PHT3;3*
Hp2000848-leaves	2000848-leaves	None	None
Hp2005506-leaves/Hp2012485-roots	2005506-leaves/2012485-roots	None	None
Hp 2012485-roots/Hp2005506-leaves	2012485-roots/2005506-leaves	None	None
Hp2090243-leaves	2090243-leaves	None	None
Hp2018344-roots	2018344-roots	None	None

Arabidopsis contains nine genes of the *PHT1* family in its genome (Smith *et al*., 2003; Nussaume *et al*., 2011). Transcripts from this family were mainly present in roots (Daram *et al*., 1999; Nussaume *et al*., 2011) and were mainly responsible for both Pi uptake and translocation in the plants (Smith *et al*., 2003). *H. prostrata* is very efficient in Pi uptake and shows P toxicity symptoms at lower P supplies than some other species (Shane and Lambers, 2005). As shown in the current study, it is the most efficient of the species tested in Pi acquisition under high Pi supply. Thus, it is likely that *H. prostrata* and its phylogenetically closely-related relatives (Clopton, 2012) have greater numbers of *PHT1* genes or these genes are more abundantly expressed in these species. In the current study, the presence of only six *PHT1* genes was confirmed in *H. prostrata*. This illustrates the lack of available information in regards to *H. prostrata*, showing that the databases that were used in this study probably did not contain all the mRNAs associated with PHTs. It is likely that there are other *PHT1* genes in *H. prostrata* but were not detected in the tissue from which the RNA was extracted and used for constructing the database.

The mRNA sequence analysis also confirmed the expression of PHT2;1 transporters in *H. prostrata* (Table 1). Previously, presence of PHT2;1 transporters in *Arabidopsis* has been reported (Daram *et al*., 1999), this transporter is likely to be different from PHT1 family in terms of structure, in Pi affinity and mechanism of transport and is suggested to be partially responsible for Pi translocation in shoots (Daram *et al*., 1999). In addition to PHT2;1, some members of PHT1 family are also responsible for Pi remobilisation within the shoot (Smith *et al*., 2003). There is not that much information available about the PHT3 family. It is proposed that these transporters are mitochondrial Pi transporters (Rausch and Bucher, 2002) and three members of this family are present in *Arabidopsis* (Rausch and Bucher, 2002). Orthologs for all three were found in *H. prostrata* in the current gene analysis. As this analysis mainly focused on detection of PHT1, PHT2 and PHT3 families in *H. prostrata*, there are two possible explanations for the five sequences that were extracted but whose gene family could not be identified. These sequences may belong to the members of PHT1 family where orthologs have not been identified in *H. prostrata*, such as PHT1;1, PHT1;2 and PHT1;6, or these sequences may belong to other families of Pi transporters that have not been included in this study.

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