Does calcium-enhanced phosphorus toxicity explain the absence of most Proteaceae species from calcareous habitats?

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BSc (Hons)

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School of Biological Sciences

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2018
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Patrick E. Hayes
12 February 2018
Abstract

The plant family Proteaceae represent an ecologically important component of the Australian flora, contributing towards south-western Australia’s exceptionally high biodiversity. Most Proteaceae are highly phosphorus (P) sensitive and occur exclusively on nutrient-impoveryed acidic soils (calcifuge), whilst few species also occur on young calcareous soils (soil-indifferent), higher in available calcium (Ca) and P. This general distribution pattern occurs along the Jurien Bay chronosequence in south-western Australia. The relatively high soil [P] of young calcareous soils is unlikely high enough to exclude P-sensitive species. Similarly, low soil micronutrient availability in calcareous soils is also unlikely to explain their distribution, as most Proteaceae possess carboxylate-releasing cluster roots, allowing them to access scarcely available micronutrients. Instead, I propose that higher soil [Ca] enhances P sensitivity in calcifuge Proteaceae. This is likely related to the cell-specific allocation of Ca and P, which must be tightly regulated to avoid the deleterious precipitation of Ca-phosphates. However, the physiological mechanisms of Ca-enhanced P toxicity remain unknown. My PhD project aimed to discover the physiological basis of Ca-enhanced P toxicity in the plant family Proteaceae and its role in their distribution.

First, I used quantitative X-ray microanalysis to assess leaf cell-specific [P] and [Ca] in 12 Proteaceae species from habitats of contrasting soil [P]. Species from extremely P-impoveryed habitats in south-western Australia preferentially allocated P to photosynthetic mesophyll cells, whilst those from P-richer habitats in Brazil and Chile showed no preferential allocation of P. The preferential allocation of P to photosynthetic mesophyll cells at low whole leaf [P] may contribute towards a higher photosynthetic P-use efficiency (PPUE). At higher P availability, a higher mesophyll [P] may also contribute to P sensitivity, by interfering with physiological zinc (Zn) availability, resulting in a P-enhanced Zn requirement (Chapter 2).

In order to assess changes in nutrition, biomass (Chapter 3), physiology, growth (Chapter 4), and leaf cell-specific nutrient concentrations (Chapter 5) during Ca-enhanced P toxicity, eight Proteaceae species were grown in hydroponics at a range of [P] and [Ca]. This included four calcifuge and four soil-indifferent species, from two genera, Banksia and Hakea. Nutrition and biomass responses are presented in Chapter 3. Calcium increased the severity of P-toxicity in
all species. Calcifuge Proteaceae were far more sensitive to Ca-enhanced P toxicity than soil-indifferent ones. Calcifuges shared these traits: low leaf [Zn], low Zn allocation to leaves, low leaf [Zn]:[P], low root:shoot ratio, and high seed P content, compared with soil-indifferent species. Calcifuge *Hakeas* generally had higher leaf [P] than soil-indifferent ones. These traits suggest that compared with calcifuges, soil-indifferent species are able to tolerate Ca-enhanced P toxicity through higher leaf [Zn] and/or lower leaf [P], thus compensating for a P-enhanced Zn requirement (Chapter 3).

In terms of physiological responses, I show that calcifuges only increased photosynthesis and stomatal conductance with increasing P supply under low Ca supply. Soil-indifferent species increased growth, photosynthesis and stomatal conductance with increasing P supply, almost regardless of Ca supply, whereas calcifuges showed a smaller response. Furthermore, reduced chlorophyll fluorescence in calcifuge *Hakeas* under high P and Ca supply was indicative of leaf damage, shown as leaf necrosis, whilst responses in calcifuge *Banksias* reflected reduced photosynthetic capacity, exhibited as leaf chlorosis (Chapter 4).

Leaf cell-specific [P] and [Ca] were assessed for four species grown in hydroponics, including two calcifuge and two soil-indifferent species. The palisade mesophyll [P] of calcifuges increased with increasing Ca supply, whilst soil-indifferent species showed only a minor increase. Therefore, soil-indifferent species were able to maintain a more stable palisade mesophyll [P], contributing to their greater tolerance of Ca-enhanced P toxicity (Chapter 5).

In conclusion, Ca-enhanced P toxicity can explain the exclusion of most Proteaceae from calcareous habitats. Calcifuges are more sensitive than soil-indifferent species. The proposed physiological mechanism for Ca-enhanced P toxicity is based on leaf cell-specific interactions of Ca and P. Despite no changes in whole leaf [P] with increasing Ca supply, leaf mesophyll [P] increased; this increase interfered with physiological [Zn] and/or precipitated with Ca, resulting in leaf chlorosis and necrosis. Therefore, I surmise that the higher soil [P] of young calcareous soils, in combination with higher soil [Ca] and low available [Zn], excludes calcifuge Proteaceae. Conversely, soil-indifferent Proteaceae can overcome this restriction, through several traits, increasing leaf [Zn] and/or decreasing leaf [P], thus compensating for a P-enhanced Zn requirement and/or reducing precipitation of Ca-phosphates.
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1. Introduction

2. Proteaceae species from P-impoverished habitats preferentially allocated P to photosynthetic cells

3. A mechanistic understanding of Ca-enhanced P toxicity

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5. Conclusions

Appendix  Additional publications during PhD


   Peppermint trees shift their phosphorus-acquisition strategy along a strong gradient of plant-available phosphorus by increasing their transpiration at very low phosphorus availability.


This thesis is in agreement with The University of Western Australia Doctor of Philosophy Rules for the content and format of a thesis (39-45) and is presented as a series of papers. Each chapter is formatted per the requirements of the journal to which it is published, submitted or to be submitted to, with remaining sections formatted to *New Phytologist*. 
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I consider myself exceptionally lucky to have had such truly amazing PhD supervisors, I could not have wished for better. Their inspiration, passion and guidance have been invaluable and certainly appreciated.

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Authorship Declaration: Co-authored Publications

This thesis contains work that has been published and/or prepared for publication.

Details of the work


Location in thesis

Chapter 2

Student contribution to work

P.E. Hayes designed the research in consultation with supervisors, Hans Lambers and Peta Clode. P.E. Hayes carried out sample collections in Australia and Chile. Rafael Oliveira organised sample collection in Brazil. P.E. Hayes analysed all cellular concentrations under the supervision of Peta Clode. P.E. Hayes performed all other sample analyses, statistical analyses and wrote the manuscript. All co-authors were involved in the discussion of results and provided editorial comments and suggestions to finalise the manuscript.

Details of the work

Hayes PE, Guilherme Pereira C, Clode PL, Lambers H. 2018. Calcium-enhanced phosphorus toxicity in calcifuge and soil-indifferent Proteaceae along the Jurien Bay chronosequence. (New Phytologist, under review)

Location in thesis

Chapter 3

Student contribution to work

P.E. Hayes and Caio Guilherme Pereira designed the research in consultation with supervisors, Hans Lambers and Peta Clode. P.E. Hayes and Caio Guilherme Pereira carried out the glasshouse experiment and the laboratory analyses. P.E. Hayes performed the
statistical analyses and wrote the manuscript. All co-authors were involved in the discussion of results and provided editorial comments and suggestions to finalise the manuscript. This manuscript was submitted with joint first co-authorship between P.E. Hayes and Caio Guilherme Pereira.

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Details of the work


Location in thesis

Chapter 4

Student contribution to work

P.E. Hayes and Caio Guilherme Pereira designed the research in consultation with supervisors, Hans Lambers and Peta Clode. P.E. Hayes and Caio Guilherme Pereira carried out the glasshouse experiment and the laboratory analyses. Caio Guilherme Pereira performed the statistical analyses and wrote the manuscript. All co-authors were involved in the discussion of results and provided editorial comments and suggestions to finalise the manuscript. This manuscript was submitted with joint first co-authorship between P.E. Hayes and Caio Guilherme Pereira.

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Chapter 5

Student contribution to work

P.E. Hayes designed the research in consultation with supervisors, Hans Lambers and Peta Clode. P.E. Hayes and Caio Guilherme Pereira carried out the glasshouse experiment. P.E.
Hayes analysed all cellular concentrations under the supervision of Peta Clode. P.E. Hayes performed all other sample analyses, statistical analyses and wrote the manuscript. All co-authors were involved in the discussion of results and provided editorial comments and suggestions to finalise the manuscript.

Patrick E. Hayes
12 February 2018

I, Hans Lambers, certify that the student statements regarding their contribution to each of the works listed above are correct

Coordinating supervisor signature
E/Prof Hans Lambers
12 February 2018
Chapter 1 | General Introduction

1. Proteaceae and their distribution

The Proteaceae are a Gondwanan plant family distributed mainly across the southern hemisphere (Weston, 2007). Many Proteaceae species are highly phosphorus (P) sensitive and are most abundant in severely P-impoverished regions, such as south-western Australia, where they represent an ecologically important component of the flora (Cowling & Lamont, 1998; George, 1998; Myers et al., 2000; Hopper & Gioia, 2004). There are over 650 Proteaceae species in south-western Australia, representing ~40% of all Proteaceae (Cowling & Lamont, 1998; George, 1998). However, very few of these species are found on young (<7,000 yr) calcareous soils of marine origin (Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/). Instead, most occur exclusively on old (>120,000 yr), nutrient-impoverished, acidic soils and are considered ‘calcifuge’ (Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/). The few Proteaceae species that grow on calcareous soils also occur on acidic soils and are therefore ‘soil-indifferent’. No Proteaceae occur exclusively on calcareous soils (‘calcicole’).

A well-documented example of Proteaceae distribution is along the Jurien Bay dune chronosequence in south-western Australia, ~200 km north of Perth (Laliberté et al., 2012; Hayes et al., 2014; Turner & Laliberté, 2015; Zemunik et al., 2015). Compared with older acidic soils, the young calcareous soils of this coastal chronosequence exhibit relatively high total and plant available [P] and calcium ([Ca]), high pH and, therefore, low micronutrient availability (Turner & Laliberté, 2015). There are two common Proteaceae genera found along this chronosequence, Banksia and Hakea, both of which include calcifuge and soil-indifferent species (Hayes et al., 2014; Zemunik et al., 2015). The ecophysiological mechanisms underpinning Proteaceae distribution in south-western Australia remain largely unexplored, despite Proteaceae representing an iconic and ecologically important component in the regions flora (Cowling & Lamont, 1998; Myers et al., 2000; Hopper & Gioia, 2004).
2. Sensitivity of Proteaceae to higher P supply

Many Proteaceae species are highly P sensitive, showing severe P-toxicity symptoms at even relatively low P supply (Groves & Keraitis, 1976; Shane et al., 2004b; Hawkins et al., 2008). These symptoms include, leaf chlorosis/necrosis, reduced biomass, and early leaf senescence (Grundon, 1972; Groves & Keraitis, 1976; Nichols & Beardsell, 1981; Webb & Loneragan, 1990; Lambers et al., 2002; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008). Species that are P sensitive show a low capacity to down-regulate their P-uptake system (Shane & Lambers, 2006; de Campos et al., 2013), along with a preferential allocation of P to mesophyll cells (Shane et al., 2004a; Hawkins et al., 2008; Lambers et al., 2015a). This disproportionately increases mesophyll [P], explaining the extreme P sensitivity of Proteaceae, even at relatively low P availability.

Phosphorus-toxicity symptoms are thought to result from the interaction of mesophyll P with zinc (Zn) and other micronutrients (Cakmak & Marschner, 1987; Lambers et al., 2002; Broadley et al., 2012), or from interference with leaf water relations (Bhatti & Loneragan, 1970). Little is known regarding the effects of P toxicity on water relations (Bhatti & Loneragan, 1970). However, the interaction of cellular P with Zn reduces the physiological [Zn], most likely through formation of sparingly soluble Zn-phosphates, resulting in an increased requirement for Zn, termed P-enhanced Zn-requirement (Cakmak & Marschner, 1987). This causes symptoms resembling Zn deficiency (e.g., leaf chlorosis) and is often misinterpreted as Zn deficiency, instead of P-toxicity (Broadley et al., 2012). Higher mesophyll [P] may also interfere with other micronutrients; however, the P/Zn interaction is likely to be of greatest importance to the Proteaceae, as they are known to operate at exceptionally low leaf [Zn] and are therefore particularly susceptible to reductions in its availability (Kuo et al., 1982; Denton et al., 2007; Hayes et al., 2014).

Extreme P sensitivity in the Proteaceae accounts for their general distribution; most abundant in severely-P impoverished regions (e.g., >650 sp. in south-western Australia and >330 sp. in South Africa; Weston, 2007; Sauquet et al., 2009; Lambers et al., 2015) and less abundant in P-richer ones (e.g., 33 sp. in Brazil and 7 sp. in Chile; Pate et al., 2001; Sauquet et al., 2009). However, it does not explain their calcifuge habit in south-western Australia. Although soil [P] is relatively greater in young calcareous soils of south-western Australia,
compared with older acidic soils (Turner & Laliberté, 2015), it is unlikely to be high enough to exclude Proteaceae species (Shane & Lambers, 2005b). However, these calcareous soils are also high in Ca, low in micronutrient availability and have a high pH (Turner & Laliberté, 2015).

Low soil P and micronutrient availability in calcareous soils often explains the calcifuge habits of other species (Tansley, 1917; Lee & Woolhouse, 1969; Tyler & Ström, 1995). However, this is unlikely to explain the exclusion of Proteaceae species, as they possess carboxylate releasing cluster roots, allowing them to access more P and micronutrients, as well as acidify the rhizosphere (Grierson & Attiwill, 1989; Shane & Lambers, 2005a,b). Therefore, the exclusion of Proteaceae species from calcareous habitats is likely different from that of other calcifuge species. I propose that the higher soil [Ca] of calcareous soils enhances P toxicity, which may be further exacerbated by reduced Zn availability and that this likely excludes most Proteaceae from calcareous habitats.

3. Phosphorus-toxicity symptoms are enhanced by Ca

Calcium can increase the severity of P toxicity, a phenomenon termed Ca-enhanced P toxicity. Grundon (1972) proposed that Ca may act alone in reducing growth in P-sensitive species, whilst Nichols and Beardsell (1981) speculated that Ca stimulates P uptake in *Grevillea* cv. ‘Poorinda Firebird’ (Proteaceae), increasing the severity of P toxicity, as shown for annual legumes, maize, and wheat (Robson *et al.*, 1970; McClure, 1972). Alternatively, Ca-enhanced P toxicity may be related to cell-specific allocation of Ca and P, which must be tightly regulated to avoid deleterious precipitation of Ca-phosphates (McLaughlin & Wimmer, 1999; White & Broadley, 2003; Conn & Gilliham, 2010). Calcium enhanced P-toxicity has been reported in only a few P-sensitive Proteaceae species; however, it has not been demonstrated across a range of species, nor has a clear explanation for the mechanism been described (Grundon, 1972; Nichols & Beardsell, 1981). In summary, the physiological mechanisms of Ca-enhanced P toxicity remain unknown, including the possible role of cell-specific Ca and P allocation.
4. **Cell-specific allocation of Ca and P**

Different cell types within leaves tend to accumulate different nutrients (Karley *et al.*, 2000; Conn & Gillham, 2010). This process of cell-specific nutrient allocation must be tightly regulated to maintain cellular processes and to avoid deleterious interactions among elements (Karley *et al.*, 2000). For example, Ca and P must be allocated to separate cell types within leaves to avoid the deleterious precipitation of Ca-phosphates, which reduces the availability of both nutrients and severely impacts cellular processes (McLaughlin & Wimmer, 1999; White & Broadley, 2003; Conn & Gillham, 2010).

Most eudicots are thought to allocate P to epidermis and bundle sheath cells, and Ca to mesophyll cells (Conn & Gillham, 2010). However, in contrast to most other eudicots, a few Proteaceae species from P-impoverished habitats in south-western Australia and South Africa preferentially allocate P to mesophyll cells and not to epidermis or bundle sheath cells (Shane *et al.*, 2004a; Hawkins *et al.*, 2008; Lambers *et al.*, 2015a). Proteaceae species from these severely P-impoverished regions have evolved several adaptations to use low amounts of leaf P far more efficiently than other plants, and this is reflected in their exceptionally high photosynthetic P-use efficiency (PPUE; Wright *et al.*, 2004; Denton *et al.*, 2007; Lambers *et al.*, 2012a, 2015a,b). Some of these adaptations include the replacement of phospholipids by sulfolipids and galactolipids (Lambers *et al.*, 2012b) and functioning at very low levels of ribosomal RNA (Sulpice *et al.*, 2014). In addition, I surmise that the preferential allocation of P to photosynthetic mesophyll cells – where it is needed in the greatest amount – may also contribute towards their exceptionally high PPUE (Stitt *et al.*, 2010; Lambers *et al.*, 2015b; Tsujii *et al.*, 2017). However, this leads to the question, is this a family-wide trait, or has it only evolved in species adapted to severely P-impoverished habitats?

Many studies have assessed cell-specific nutrient allocation in leaves of metal-accumulating and crop species (Treeby *et al.*, 1987; Zhao *et al.*, 2000; Küpper *et al.*, 2000, 2001; Mesjasz-Prybyłowicz & Pineda, 2001; Mesjasz-Przybyłowicz *et al.*, 2001; Ager *et al.*, 2002, 2003; Bidwell *et al.*, 2004; Fernando *et al.*, 2006, 2008, Vogel-Mikuš *et al.*, 2008, 2014; Conn & Gillham, 2010; Rios *et al.*, 2012; Regvar *et al.*, 2013; Villaforte Carvalho *et al.*, 2015). However, few studies have quantitatively assessed cellular [P], and no study has yet assessed it across a range of naturally occurring species, from strongly contrasting soil [P]. A
better understanding of P and Ca allocation within leaves will provide valuable insights into how species have evolved to survive under severely P-impoverished conditions, with important lessons for future crop breeding, to improve nutrient-use efficiency. More fundamentally, this knowledge will also improve our understanding of the factors influencing cell-specific nutrient allocation in plants. This will assist in understanding the physiological mechanisms of Ca-enhanced P toxicity in Proteaceae and its role in their distribution.

5. Outline of this thesis

Proteaceae species in south-western Australia are largely excluded from calcareous habitats, with most species found exclusively on severely P-impoverished acidic soils (Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/). I hypothesise that the calcifuge distribution of most Proteaceae species is due to Ca-enhanced P toxicity and that this is related to their cell-specific allocation of Ca and P. However, the ecophysiological reasons for this distribution remain largely unexplored, as do the physiological mechanisms underlying Ca-enhanced P toxicity.

The primary objective of this project was to discover the physiological basis for Ca-enhanced P toxicity in the plant family Proteaceae and its role in their distribution, but it also explored aspects of cell-specific nutrient allocation of far broader relevance.

First, leaf cell-specific P and Ca concentrations were assessed across a broad range of Proteaceae species from habitats of strongly contrasting soil [P], including species adapted to severely P-impoverished soils of south-western Australia and those found on P-richer soils in Brazil and Chile, in South America (Chapter 2). This was followed by two hydroponic experiments, in which four calcifuge (Banksia attenuata, B. menziesii, Hakea incrassata, and H. flabellifolia) and four soil-indifferent species (B. prionotes, B. sessilis, H. prostrata, and H. trifurcata) were grown at a range of Ca and P concentrations in order to study their responses to Ca-enhanced P toxicity (Chapters 3, 4, 5).

The key objectives were to:

• assess leaf cell-specific P and Ca allocation patterns across a range of Proteaceae species adapted to strongly contrasting soil [P] (Chapter 2);
• develop a mechanistic understanding of Ca-enhanced P toxicity by investigating changes in nutrition, biomass (Chapter 3), physiology, growth (Chapter 4), and leaf cell-specific nutrient allocation (Chapter 5) during Ca-enhanced P toxicity, across several Proteaceae species from south-western Australia;
• explore the role of Ca-enhanced P toxicity in the distribution of Proteaceae species by comparing and contrasting the responses of calcifuge and soil-indifferent Proteaceae to a range of external Ca and P supplies (Chapters 3, 4, 5).

References


Chapter 2

Proteaceae from phosphorus-impoverished habitats preferentially allocate phosphorus to photosynthetic cells: An adaptation improving phosphorus-use efficiency

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Abstract
Plants allocate nutrients to specific leaf cell types: eudicots are thought to predominantly allocate phosphorus (P) to epidermal/bundle sheath cells. However, three Proteaceae species have been shown to preferentially allocate P to mesophyll cells instead. These Proteaceae species are highly adapted to P-impoverished habitats, with exceptionally high photosynthetic P-use efficiencies (PPUE). We hypothesized that preferential allocation of P to photosynthetic mesophyll cells is an important trait in species adapted to extremely P-impoverished habitats, contributing to their high PPUE. We used elemental X-ray mapping to determine leaf cell-specific nutrient concentrations for 12 Proteaceae species, from habitats of strongly contrasting soil P concentrations, in Australia, Brazil, and Chile. We found that only species from extremely P-impoverished habitats preferentially allocated P to photosynthetic mesophyll cells, suggesting it has evolved as an adaptation to extremely P-impoverished habitat and that it is not a family-wide trait. Our results highlight the possible role of soil P in driving the evolution of ecologically relevant nutrient allocation patterns and that these patterns cannot be generalized across families. Furthermore, preferential allocation of P to photosynthetic cells may provide new and exciting strategies to improve PPUE in crop species.

Key Words
Calcium accumulation, cell type-specific distribution, elemental analysis, scanning electron microscopy, X-ray microanalysis

1 | INTRODUCTION

Plants often acquire nutrients in excess of immediate demands and store them until required. This process of acquisition and storage must be tightly regulated, as particular elements can negatively interact with each other. For example, phosphorus (P) and calcium (Ca) must be stored separately to avoid the precipitation of calcium phosphate, which reduces the availability of both nutrients and severely impacts cellular processes (Conn & Gillham, 2010; White & Broady, 2003). Phosphorus is stored in leaves, as it is vital to many processes, such as carbon metabolism, energy transfer, and growth. However, leaves also accumulate Ca, which is transported via transpiration, from root to shoot, where it is accumulated in leaf cell vacuoles and is involved in cell structure and signalling (Gillham et al., 2011; White, 2001). Therefore, both P and Ca are accumulated in plant leaves, where their separation must be maintained to avoid the deleterious precipitation of calcium phosphate (Conn & Gillham, 2010; White & Broady, 2003). We still know very little about the patterns and processes of cell-specific nutrient allocation despite its obvious importance to overall plant nutrition and physiology.

The current model of cell-specific nutrient allocation is based on a recent review by Conn and Gillham (2010) and suggests that eudicots generally allocate P to epidemids (EP) and bundle sheath (BS) cells and Ca to mesophyll cells. However, in contrast to this model, it has been shown that some species of Proteaceae from severely P-impoverished habitats in south-western Australia (Lambers, Finnnegan, et al., 2015; Street, McCully, & Lambers, 2004) and South Africa (Hawkins, Hettrisch, Menenz-Przybylowski, Przybylowski, & Croner, 2008) preferentially allocate P to mesophyll cells and not to EP or BS cells.

The family Proteaceae are mainly found across the southern hemisphere and are most abundant in severely P-impoverished habitats, for example, south-western Australia (>680 sp.) and South Africa (>330 sp.).
sp.; Weston, 2007; Sauquet et al., 2009; Lambers, Clode, et al., 2015), with much lower abundances in regions of higher P fertility, for example, Chile (7 sp.) and Brazil (23 sp.); Paie, Verboom, & Galloway, 2001; Sauquet et al., 2009). Proteacea species that are abundant in P-improved habitats represent a major component of the vegetation and have key roles in ecosystem functioning (Cowling & Lamont, 1998; Lambers et al., 2012). To be abundant in such severely P-limited habitats, these Proteacea species must use the low amounts of available P far more efficiently, and this is reflected in their exceptionally high rates of photosynthetic P use efficiency (PPUE; Table 5); Wright et al., 2004; Denton, Vonekaarten, Frelinosor, & Lambers, 2007; Lambers, Bishop, Hopper, Laliberté, & Zúñiga-Feest, 2012; Lambers, Clode et al., 2015; Lambers, Finnegan et al., 2015). These exceptionally high PPUE values are achieved through a combination of adaptations, including replacement of phosphatase by sulfatase and galactofucanase (Lambers et al., 2012), and functioning at very low levels of ribosomal RNA (Sulpice et al., 2014). In addition, we surmise that the ability of some Proteacea to preferentially allocate P to photosynthetic mesophyll cells (where P is needed in the greatest amount), unlike other eudicots, may contribute to these species’ exceptionally high PPUE (Lambers, Finnegan et al., 2015; Sitt, Lunn, & Usadel, 2010; Tsuji, Oikawa, & Kiyazawa, 2017). However, this leads us to the question, Is this a family-wide trait? Is that, do Proteacea from relatively P-rich habitats also show a preferential allocation of P to mesophyll cells, or has this only evolved in species from severely P-improved habitats? Furthermore, does Ca allocation reflect the current model, or does this also differ?

To address the above questions, we used quantitative X-ray elemental analysis to determine leaf cell-specific nutrient concentrations and distributions for a phylogenetically diverse range of 12 Proteacea species, from three habitats of contrasting soil (P) in Australia, Brazil, and Chile (Weston & Barker, 2006). These habitats represent an extreme range in total soil (P), from the extremely P-improved southwestern Australia (Denton et al., 2007; Lambers, Brandreth, Raven, & Hooper, 2010) to the relatively P-rich soils of South America: Brazil (Lannes, Bustamante, Edwards, & Oide, 2016; Miotto, Wright, & Batalha, 2016) and Chile (Borie & Rubio, 2000; Delgado, Zúñiga-Feest, Almoneacida, Lambers, & Borí, 2013). Species from southwest Australia are highly adapted to their severely P-improved habitat, showing low whole leaf (P) compared with South American species (Lambers et al., 2010) and much higher PPUE (Table 5); Wright et al., 2004; Denton et al., 2007; Lambers et al., 2012).

Many studies have assessed cell-specific nutrients in leaves of metal-hyperaccumulating and crop species, from which the current model (Conn & Gillham, 2010) is largely based (Ager, Yrisa, Domínguez-Solís, Gotor, et al., 2002; Ager, Yrisa, Domínguez-Solís, López-Martín, et al., 2003; Bidwell, Crawford, Woodrow, Sommers-Knudsen, & Marshall, 2004; Fernando et al., 2006; Fernando et al., 2008; Köpper, Zhao, & McGrath, 1999; Köpper, Lombi, Zhao, & McGrath, 2000; Köpper, Lombi, Zhao, Machineer, & McGrath, 2001; Mesjasz-Przybylowicz & Pineda, 2001; Mesjasz-Przybylowicz, Ball, Przybylowicz, & Amargan, 1994; Mesjasz-Przybylowicz, Przybylowicz, Raina, & Pineda, 2001; Rybar et al., 2013; Rios et al., 2012; Treeby, van Steveninck, & de Vries, 1987; Villaforte, Carvalho et al., 2015; Vogel-Mikulski et al., 2008; Vogel-Mikulski, Porporato, & Pelicon, 2014; Zhao, Lombi, Breeden, & McGrath, 2000). However, few studies have quantitatively assessed cellular (P), and no study has yet assessed it across a wide range of naturally occurring species, covering an extreme range in soil (P). This is important, because species occurring in natural habitats with low concentrations of heavy metals are likely very different from metal-hyperaccumulating and crop species. Furthermore, although some studies have assessed individual Proteacea, this study, for the first time, has investigated patterns of cell-specific nutrient allocation across the broader Proteacea family, including species from extremely P-improved southwestern Australian habitats to the P-rich temperate rainforests of southern Chile. This knowledge will provide valuable insights into how native species have evolved to survive under P-improved conditions, with important lessons for future crop breeding to improve nutrient use efficiency and, more fundamentally, will improve our understanding of the factors influencing cell-specific nutrient allocation in plants.

We hypothesized that P would be preferentially allocated to photosynthetic cells in species from southwestern Australia, as an adaptation to their extremely P-improved habitat (Lambers, Clode, et al., 2015; Tsuji et al., 2017). We also hypothesized that this is not a family-wide trait, with South American species from P-rich habitats expected to show no preferential allocation of P to photosynthetic cells, as they have not had the same evolutionary pressures to increase their P use efficiency. We also hypothesized that Ca would accumulate in specific cell types, separate from P, and primarily along the transpiration pathway, because Ca must be tightly regulated within plant cells, separated from P to avoid precipitation, and is thought to move through leaves via transpiration (Conn & Gillham, 2010; Gilham et al., 2011; White & Bradley, 2003). Overall, this study aimed to provide a comprehensive assessment of cell-specific P and Ca allocation patterns in species adapted to habitats of strongly contrasting soil (P).

2 MATERIALS AND METHODS

2.1 Study areas and species selection

The three study areas (Australia, Brazil, and Chile) were selected because Proteacea species are present at all sites and because they cover a wide range in soil (P) (11 to 1.924 mg total P kg⁻¹; see Table 1 for full soil details). The study area in Australia was located in Juniper Bay, southwestern Australia, approximately 200 km north of Perth. The soils in this area were extremely P-improved, with soil (P) of 11 mg total P kg⁻¹. Three sites were identified in this study area: two on the 2-2.5 million year-old Bussandean stage of the Julien Bay dune chronosequence and one on the much older Pliocene slope (Hayes, Turner, Lambers, & Laliberté, 2014; Turner, Hayes, & Laliberté, 2017; Turner & Laliberté, 2015). All samples were collected within a 100 m area, at each site. The three sites were located within a 9 x 4 km area and were dominated by xerophytic bushland (Proteacea-rich shrubland). The study area in Brazil was located in Serra do Clau, southern Brazil, approximately 8 km north-east of Belo Horizonte. This area was intermediate in terms of total soil (P), with soil (P) of 135 mg total P kg⁻¹, placing it between that of sites in Australia and Chile. All samples were taken from a single site (<100 m). The area was dominated by cerrado vegetation. The study area in Chile was...
TABLE 1  Key soil chemical properties and vegetation types of the three study areas

<table>
<thead>
<tr>
<th>Study area</th>
<th>Vegetation type</th>
<th>Total P (mg kg⁻¹)</th>
<th>Olsen P (mg kg⁻¹)</th>
<th>Total N (g kg⁻¹)</th>
<th>Exchangeable Ca (mg kg⁻¹)</th>
<th>CEC (zmol kg⁻¹)</th>
<th>pH (H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>Kwongan</td>
<td>11 ± 0.5</td>
<td>0.18 ± 0.02</td>
<td>0.10 ± 0.00</td>
<td>183 ± 27</td>
<td>1.22 ± 0.17</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>Brazil</td>
<td>Corredo</td>
<td>133 ± 13</td>
<td>2.08 ± 0.03</td>
<td>1.8 ± 0.01</td>
<td>621 ± 208</td>
<td>3.08 ± 0.87</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Chile</td>
<td>Temperate rainforest</td>
<td>1,924 ± 83</td>
<td>0.69 ± 0.18</td>
<td>7.6 ± 0.06</td>
<td>49 ± 18</td>
<td>1.52 ± 0.12</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

Australia (Jureia Itatins, southern Brazil), Brazil (Serra do Cariri, south-eastern Brazil), and Chile (Puerto Montt, southern Chile). Olsen P, a measure of plant-available P. All values are mean ± standard error (n = 3–12).

CEC = cation exchange capacity.

located in Parque Kataliptu, Puerto Montt, southern Chile, approximately 1,000 km south of Santiago. All samples were taken from a single site (~100 m). The area had very P-rich young soil and was dominated by temperate rainforest, with soil [P] of 1,924 mg total P kg⁻¹. All sites consisted of soils with low pH (6.1, 5.4, and 4.6, Australia, Brazil and Chile, respectively). The main difference between sites was a clear and strong gradient in total soil [P], which, given the ability of these species to produce P-mining cluster nodules, is a good indicator of plant-available P for these species.

Twelve Proteaceae from eight genera were selected. These species were chosen as they are each locally abundant, and, together, they effectively cover a broad range of the Proteaceae phylogeny (Table 52; Weston, 2014). Each study area contained a different set of species. In Australia, seven species were selected: from four genera: Banksia (Banksia attenuata R.Br., Banksia menziesii R.Br., and Banksia prionotes Lindl.), Hakea (Hakea incrassata R.Br. and Hakea prostrata R.Br.), Persoonia (Persoonia montana Meisn.), and Petalophyllum (Petalophyllum macrostachyum R.Br.). A single species was selected from Brazil from the genus Roupala (Roupala montana Aubl.). In Chile, four species were selected, from three genera: Embothrium (Embothrium caximubum J.R. Forst. & G. Forst.), Gevuina (Gevuina oviflora Mol., and Lonchotis (Lonchostylis Termitina (Cav.) R.Br. and Lonchotis hirtula (Lam.) Diels ex Maidr.). Species from the same study area were not always closely related. For example, Lonchotis and Embothrium from Chile are more closely related to Hakea species from Australia than to other South American species (Weston, 2014). Two to three species were chosen for each of the Banksia, Hakea, and Lonchotis genera, allowing for investigation of within-genus variation.

2.2 Leaf sampling and nutrient analyses

Leaf samples were collected in January (Chile) and November (Australia, Brazil) 2014. Three healthy mature individuals were selected for each species in each study area. From each individual plant, only mature, undamaged, fully expanded, and sun-exposed leaves were sampled. A total of 36 samples were collected for whole-leaf nutrient analyses. All leaf material collected in Australia and Chile was immediately frozen in liquid nitrogen (N₂) before being freeze-dried and ground using a ball-mill grinder. Material from Brazil was collected and oven dried at 60°C, for 72 hr, before being ground using a ball mill grinder. All samples were then acid-digested using concentrated HNO₃/HClO₄ (9:1) and the concentration of P determined colorimetrically using malachite green method (Motomiya, Wakiimoto, & Toch, 1993). In all digests from Australia and Chile, the concentration of Ca was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). In all acid digests from Brazil, the concentration of Ca was determined by atomic absorption spectrometry (Labotario de Tecidos Vegetais, Escola Superior de Agricultura Luiz de Queiroz, ESALQ USP, Sao Paulo, Brazil).

2.3 Soil sampling and analyses

Soil samples were collected in January (Chile), June (Australia), and November (Brazil) 2015.

Samples were taken at 0–20 cm depth, after removal of the litter layer. Nine soil samples were collected in Australia, three representative samples from each of the three sites in Jureia Itatins, Brazil. Soil samples from Brazil (three) and Chile (12) were collected within 1 m of each individual plant. The soils in Brazil and Chile were collected from each individual because soil properties at these sites are less well known than those in Jureia Itatins (Hayes et al., 2014; Turner & Laliberté, 2015), and we aimed for an accurate estimate of soil properties at all sites. There was later found to be no significant difference (p < 0.05) among sites (Australia) or species (Brazil and Chile) within each study area, and, therefore, all soil properties were simply reported by study area (Australia, Brazil, or Chile). All samples were sieved (< 2 mm) to remove any large organic debris, before being homogenized and air-dried prior to chemical analysis. Samples from Australia and Brazil were analysed at the Smithsonian Tropical Research Institute (Panamá, Republic of Panama). Samples from Chile were analysed at the Laboratorio de Análisis de Suelos y Plantas, Universidad de Concepción (Udec, Chile) and at the Laboratorio Agropecuario (Nariño, Santiago, Chile). Total soil [P] was measured by ignition and acid digestion. Olsen P (soil P availability) was measured by sodium bicarbonate extraction and molybdate colourimetry. Total N was measured by dry combustion (Australia and Brazil) and by Kjeldahl digestion with sulphuric and salicylic acid, followed by colourimetric analysis (Chile). In samples from Australia and Brazil, the exchangeable Ca and other cations were determined by BaCl₂ (0.1 M) extraction and inductively coupled plasma optical emission spectrometry. In samples from Chile, the exchangeable Ca and other cations were determined using an ammonium acetate (1 M) extraction and atomic absorption spectrometry. Both types of cation extractions (BaCl₂ and ammonium acetate) yield similar results for exchangeable cations (Carter & Gregory, 2008). Soil pH was determined in a 1:2 soil-to-solution ratio in water using a glass electrode.

2.4 Leaf sampling for cell-specific element analysis and leaf anatomy

Leaf samples for cell-specific element analysis were collected from the same plants and at the same time as for leaf nutrient analysis, in January (Chile) and November (Australia, Brazil) 2014. From each
individual plant, only mature, undamaged, fully expanded, and sun-exposed leaves were sampled. Three to six samples were collected from one to two leaves, for each of the 36 individual plants. Upon removal of a leaf, small sections (≤2 × 2 mm) were consistently cut from either side of the midrib, mid-vein along the leaf, avoiding any large secondary veins. These sections were then mounted onto aluminum pins using optimal cutting temperature compound and plunged into liquid N until preparation in a cryomicrotome and analysis using cryo-scanning electron microscopy. With this, all samples were collected fresh in the field, were immediately plunged frozen in liquid N, and were always kept under cryo-conditions, such that all elements were preserved in situ.

Small (≤5 × 5 mm) leaf sections of the same plant leaves were also collected for anatomical imaging. These samples were immediately immersed in fixed, composed of 2.5% (v/v) glutaraldehyde + 1.6% (v/v) paraformaldehyde in 10 mM phosphate-buffered saline and left for 24 hr, before being stored at 4°C. Fixed leaves were then vibratome to produce transverse leaf sections of ~30–60 μm. These were then mounted in water on glass slides and imaged using bright-field and fluorescence (ultraviolet excitation) illumination on an Axioskop optical microscope (Zeiss, Oberkochen, Germany) fitted with an Axiocam digital camera (Zeiss).

2.5 | Cell-specific element analysis by X-ray microanalytical mapping

Transverse regions of frozen hydrated leaf samples were prepared by cryopressing a flat sample with a glass knife, followed by a diamond knife, at ~120°C in a cryomicrotome (Leica EM FC6 cryochamber integrated with Leica Ultracut EM UC6 microtome). Leaves were progressively microtomed flat, initially on a glass knife at 1 μm, 750 nm, and 300 nm steps and then finally with fine precision on a diamond knife at 250 and 100 nm steps. The petiole was then mounted on a custom-made stage mount, transferred under a nitrogen environment to a Leica MED2000 cryopreservation system, and sputter coated with 25–50 nm chromium, without sublimation. After coating, samples were transferred under vacuum to a Zeiss field emission scanning electron microscope fitted with a Leica VCT100 cryostage and stage, and an Oxford X-Max80 SDD X-ray detector interfaced to Oxford Instruments AztecEnergy software (Figure S1b). This preparation method and this fully integrated Oxford analytical system have been shown to be highly suitable for elemental analysis and quantification of biological samples (Huang, Cann, Oates, & McCully, 1994; Jin et al., 2017; Marshall, 2017; Marshall & Lodge, 2009; Marshall, Goodyear, & Crearther, 2012; Marshall & Xu, 1998; McCully, Cann, Huang, Miller, & Brink, 2010).

Samples were analyzed at ~150°C, 15 kV, and a 2 nA beam current (measured using a Faraday cup), in high-current mode. Prior to each map acquisition, the instrument was calibrated, and the beam current was measured using a pure copper standard. Elemental maps were acquired at a resolution of 512 pixels, for >3,000 frames with a dwell time of 10 μs per pixel. Drift correction and pulse pile-up correction were activated. Using the Oxford Instruments AztecEnergy software, quantitative numerical data were subsequently extracted from regions of interest drawn on the element maps (Figure S1c), with individual spectra from each pixel within the region of interest summed and processed to yield element concentration data. Summed spectra from regions of interest were quantified using the Aztec XPP model for matrix corrections using standard files included within the software package. Regions of interest represented various areas and individual cells of interest. Different cells were readily identified and classified, based on their leaf anatomy, cell appearance, and element levels. Only cells that were clearly identifiable and had a flat surface were analyzed, with airspaces, for example, avoided (Figure S1d). Because the central vacuole occupies most of the volume in a plant cell, it is assumed that the reported measurements typically reflect vacuolar concentrations. Different species possess different anatomy and combinations of cell types (Table S5; Figures S2–S6). Overall, the following six cell types were confidently identified and analyzed: epidermis (EP), hypodermis (HY), palisade mesophyll (PM), spongy mesophyll (SM), internal parenchyma (IP), and sclerenchyma (SC). In some species, some of these cell types accumulated Ca differently, with Ca not in all cells. In such cases, these were further subdivided, on the basis of their Ca. Calculating-accumulating cells were identified with the suffix "Ca": for example, HY.Ca, PM.Ca, SM.Ca, and IP.Ca. In total, 2,789 cells were analyzed, with 7–117 cells analyzed for each cell type within each species, across three individual plants (Table S2). The element maps for P are not presented because they did not pictorially reveal the distribution/variation in P, due to the consistently low levels of P; however, quantification of [P] from regions of interest was still readily achievable (Figure S1f–h).

Use of this method yielded significant advantages. By plunge-freezing samples directly in liquid N, we could prepare samples in the field, avoid loss or movement of elements, and analyse samples in a frozen fully hydrated state, thus measuring cellular concentrations exactly as they were in the field. This has significant advantages over alternative methods, where samples are freeze-dried or freeze-substituted, potentially affecting cell nutrient contents. This method is also appropriate for large-scale experiments, where samples can be stored (in liquid N) and analyzed over longer periods. These X-ray microanalytical data are also quantifiable, making the direct comparison of studies possible, including, for the first time, the direct comparison of a range of species in a single family from contrasting habitats. A limitation of this method is that it involves bulk samples, which means the analytical resolution is ~2 μm (sufficient for the analysis of individual plant cells: Marshall, 1982), and the detection limit is a few ppm (Romans & Dragoni, 2007). There was no freezing damage observed in any samples at the scale of these analyses. On occasion, surface frost was observed in some samples, particularly along the cuticle. This was unavoidable and occurs during transfer of the sample from the microscope to the preparation system, which is technically challenging. These areas were not analyzed, and thus, this had no impact on our resulting data. As such, our method of sample preparation is simple, suitable for the field, and the subsequent X-ray microanalysis allows for quantifiable analysis of a range of biologically relevant elements in fully hydrated cells.

2.6 | Statistics

Differences in [P] across cell types, between Australia and South America, were tested using general linear mixed-effect models, with
species and individual plants as the random effects (Pinheiro & Bates, 2000). Differences in nutrient concentrations across cell types, at the species level were tested using general linear mixed-effect models, with individual plants as the random effect (Pinheiro & Bates, 2000). The residuals of each model were visually inspected for heteroscedasticity. In the presence of heteroscedasticity, appropriate variance structures were specified if they significantly improved the model, based on likelihood ratio tests (Pinheiro & Bates, 2000). Data and statistical analyses were performed using the R software platform (R Core Team, 2016) and the nlme package (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2016). The effects package (Fox, 2003) was used to determine means and 95% confidence intervals, later used to define the differences among cell types and between Australia and South America.

3 | RESULTS

3.1 | Phosphorus

Species from severely P-impoveryished habitats of south-western Australia preferentially allocated P to their photosynthetic mesophyll cells (Figure 1a). These mesophyll cells (PM and SM) showed up to 6.3-fold greater [P] than non photosynthetic cells (EP, HY, IP, and SC). The mean cell [P] in Australian species ranged from 1.9 μmol g⁻¹ in SC to 12.6 μmol g⁻¹ in PM (Figure 1a). In contrast, South American species from relatively P-rich soils did not show a preferential allocation of P to photosynthetic cells. They instead showed similar and consistently low [P] across all cell types, ranging from 2.4 μmol g⁻¹ in the HY to 5.1 μmol g⁻¹ in the PM (Figure 1a). Hence, preferential allocation of P to photosynthetic mesophyll cells is not a Proteaceae family trait but appears restricted to those Proteaceae species from severely P-impoveryished habitats.

When comparing directly between Australian and South American species, the PM and SM cells of Australian species exhibited 2.5- and 1.7-fold greater mean [P], respectively, compared with that of the South American species (Figure 1b). The EP [P] was very low and showed no significant difference between Australian and South American species (Figure 1b).

At the species level, the highest [P] was 17.2 μmol g⁻¹ (PM, B. attenuata, Australia; Figure 2). Australian species all showed a clear allocation of P to photosynthetic PM and SM cells, with every species showing the highest [P] in PM, followed by SM (Figure 2). All other cell types in Australian species (EP, HY, IP, and SC) showed low [P] (<5 μmol g⁻¹; Figure 2). Collectively, all Australian species preferentially allocated P to PM and SM.

Two of the South American species (G. alpinum and L. hirsutum) did not show a significant difference in [P] among cell types (Figure 3). E. coccineum and L. ferruginea showed greater [P] in one photosynthetic cell type (SM), but it was only significantly greater than that in EP in E. coccineum and EP/PM/Ca in L. ferruginea. R. montana showed a greater [P] in both EP and PM but only when compared with that in HY and SM/Ca. Therefore, although there were some differences in [P] among cell types of South American species, none showed a preferential allocation of P to all photosynthetic cells (Figure 3).

On average, whole leaf [P] in Proteaceae species from south-western Australia (0.33 mg g⁻¹ DW) was approximately half that of species from South America (0.76 mg g⁻¹ DW; Brazil and Chile; Figure 4 and Table S4). Leaf [P] in Proteaceae species from south-western Australia ranged from 0.29 mg g⁻¹ DW in P. cuneata to 0.4 mg g⁻¹ DW in P. priodes (Figure 4 and Table S4). Whereas in South

![FIGURE 1](image_url)  
Leaf cell-specific phosphorus concentrations ([P]) comparing Proteaceae from P-impoveryished habitats in Australia with those from P-rich habitats in South America. (a) Mean cell-specific [P] of Australian and South American species, illustrating differences among cell types within each region. Different sets of cell types are shown, because of differences in leaf anatomy. (b) Mean cell [P] of the common cell types between Australian and South American species, illustrating differences between regions. Common cell types are those found across three or more species in both regions. The hypocotyls (HY) is not shown, because it is only found in one South American species. (a→b) This analysis includes all cells within each cell type, because [P] are not significantly different between Ca-accumulating and non Ca-accumulating cells, of the same cell type. Error bars show 95% confidence intervals from linear mixed-effect models. The different letters indicate significant differences (based on 95% confidence intervals) among cell types (a), or between regions (b). EP = epidermis; IP = internal parenchyma; PM = palisade mesophyll; SC = spongy mesophyll; SM = spongy mesophyll.
3.2 | Calcium

Calcium was accumulated within specific cells, generally separate from P-accumulating cells (except in Banksia) and was not always along the transpiration pathway. There was significant variation in the patterns of Ca accumulation among species, with no distinct shift between species that do and do not accumulate P in mesophyll cells (Figures 5–7). In most species, Ca accumulated in IP or SM, with some species also accumulating Ca in other cell types and/or regions (Figures 5–7). *P. cornata* and *P. macrostachya* showed Ca accumulation in specific cells of the IP (Figure 5). The Banksia and South American species all accumulated Ca in SM (Figures 6 and 7). Furthermore, South American species tended to accumulate Ca in SM cells close to veins, for example, *G. elatior* (Figure 7). *H. prostrata* and *H. incarnata* both accumulated Ca apoplastically around IP cells but did not significantly accumulate Ca in any specific cell type (Figure 5). *P. cornata*, *P. macrostachya*, and all South American species, except *P. montana*, also accumulated Ca apoplastically around adaxial EP cells (Figures 5 and 7). *L. ferreirina* and *L. hisunoto* accumulated Ca in PM as well as SM (Figure 7).

Both Cenotite species showed a unique pattern of Ca accumulation, in discrete alternating layers of PM. For example, the uppermost PM layer showed almost no Ca, whereas the second PM layer, immediately beneath it, showed accumulation of Ca up to an exceptionally high 1.413 mmol g⁻¹ in *L. ferreirina* (Figures 3 and 7). When four layers of PM were present, the layers of PM from adaxial showed low Ca, high Ca, low Ca, and high Ca. In summary, all species showed evidence of a tight regulation of Ca, allocating Ca within specific cells and/or regions. These were generally within the IP or SM and often associated with veins. Furthermore, only *L. ferreirina* and *L. hisunoto* showed accumulation of Ca in the PM, with a peculiar accumulation of Ca in alternating layers, not previously observed in any plant species.
FIGURE 3  Mean leaf cell-specific phosphorus ([P]) and calcium ([Ca]) concentrations across five Proteaceae species collected from relatively P-rich soils in South America. Most species were collected from Puerto Montt in southern Chile, except *Rhuspa montana*, which was collected in Serra do Cipó, southeastern Brazil. There are different cell types for each species because of differences in their anatomy. Concentrations are per unit fresh weight, from fully hydrated cells. Error bars show 95% confidence intervals from linear mixed-effect models. Within each panel, the different letters indicate significant differences among cell types, based on 95% confidence intervals. We did not present element maps for P, because they do not clearly show the distribution of P; however, P was accurately quantified from regions of interest (see Figure S1f–h). (P) = epidermis; PM = palisade mesophyll; PM.Ca = palisade mesophyll accumulating Ca; SM = spongy mesophyll; SM.Ca = spongy mesophyll accumulating Ca.

FIGURE 4  Whole leaf phosphorus ([P]) and calcium ([Ca]) concentrations. (a) Mean leaf P concentration for each species, with study area indicated by shading. (b) Total mean P concentration for Australian and South American (Brazil and Chile) Proteaceae species. (c) Mean leaf Ca concentration for each species, with study area indicated by shading. (d) Total mean Ca concentration for Australian and South American (Brazil and Chile) species. All concentrations are per unit dry weight and represent the entire leaf; therefore, values are not directly comparable with cell-specific concentrations, which are per unit fresh weight and represent individual cell types. Error bars show 95% confidence intervals from linear mixed-effect models. The different letters indicate significant differences (based on 95% confidence intervals) among species (a and c) or regions (b and d).
There was significant variation in patterns of Ca accumulation among species, including species of the same genus (Figure 6). For example, B. menziesii was the only Banksia species to accumulate Ca in BY (Figure 6), despite all Banksia species naturally co-occurring, interestingly, Ca did not always accumulate in every cell of a particular cell type (Figures 5–7). For example, P. comata accumulated Ca in IP but not in all cells of the IP (Figure 5), whereas L. ferruginea accumulated Ca within PM and SM but also not in all of these cells (Figure 7).

At the species level, the greatest mean [Ca] was 1.413 µmol g⁻¹ (PM, Ca, L. ferruginea, Chile) and the lowest was –2 µmol g⁻¹ (EP and PM, E. coccineum, Chile; Figure 3). The concentration of Ca within Ca-accumulating cell types of Australian species ranged from 127 µmol g⁻¹ (IP, Ca, P. macrostachya) to 348 µmol g⁻¹ (SM, Ca, B. prionotes; Figure 2). South American species showed a much larger range, from 97 µmol g⁻¹ (SM, Ca, L. hisitata) to 1,413 µmol g⁻¹ (PM, Ca, L. ferruginea; Figure 3). Leaf Ca obviously not only varied in location but also in concentration. Calcium-based crystals were observed in six of the 12 species (white arrows, Figures 5–7). They ranged from 2 to 40 µm in size and tended to be formed within cells that accumulated Ca, such as SM, Ca in Banksia species (Figure 6). These crystals were avoided when analysing cellular regions so as not to misrepresent cellular Ca levels.

Despite the exceptionally high [Ca] reported in PM, Ca cells of L. ferruginea (~1.413 µmol g⁻¹), we are confident that this Ca was not mineralised, in either a crystalline or an amorphous form, based on optical imaging, electron microscopy, and 3-D X-ray microscopy (microCT). Across all of these techniques and analyses, we found no evidence of crystalline or amorphous material in these high-Ca cells of the PM (Figure 57). The Ca is contained within a large vacuole and...
is isolated from the cytoplasm (Figure 57ab); it also has comparatively low water content and high S relative to the PM cells (60 ± 4 μmol S g⁻¹ in PM, Ca vs. 11 ± 4 μmol S g⁻¹ in PM; Figure 57cd). Furthermore, the Ca within these cells is only ~5.7 atomic wt%, which is considerably lower than what we would expect if these were pure calcium oxalate minerals (~31 atomic wt% or plant-based calcium oxalate minerals (~22 atomic wt% in B. attenuata and B. pinnatifida, n = 12). Therefore, although the Ca is very high by cellular standards, it is actually very low by mineral standards. Consistent with this, 3D X-ray microscopy scans revealed that the high-Ca cell layer in Lomatia is considerably less dense than that of typical Ca-based minerals present in leaves (Figure 57ef).

On average, whole leaf [Ca] in Proteaceae species from south-western Australia (4.90 mg g⁻¹ DW) were not significantly different to those from South America (6.99 mg g⁻¹ DW: Brazil and Chile; Figure 4 and Table 54). Leaf [Ca] in Proteaceae species from south-western Australia ranged from 2.6 mg g⁻¹ DW in H. prostrata to 6.7 mg g⁻¹ DW in B. menziesii (Figure 4 and Table 54). Whereas in South America, leaf [Ca] ranged from 2.60 mg g⁻¹ DW in E. cucullatum (Chile) to 12.47 mg g⁻¹ DW in L. jemaginata (Chile; Figure 4 and Table 54).

4 | DISCUSSION

4.1 | Phosphorus

Phosphorus was preferentially allocated to photosynthetic cells in Proteaceae from extremely P-impoorished habitats in south-western Australia. However, this is clearly not a family-wide trait, as it was not found in species from P-richer soils in Brazil and Chile. We, therefore, show that P distribution patterns vary within a single eudicot family, contrary to the model that eudicots follow a single general pattern (Conn & Gillham, 2010). The accumulation of P within photosynthetic cells contributes towards a high PPUE and may be linked to improved P-remobilization in these species (Lambers, Finnegan, et al., 2015) and is thus likely an adaptation to severely P-impoorished habitats.

The current model for P distribution in eudicots is that they typically accumulate P in EP and BS cells and exclude it from mesophyll cells (PM and SM) (Conn & Gillham, 2010). In contrast, Proteaceae from Australia showed a clear preferential allocation of P to these photosynthetic mesophyll cells, whereas species from South America showed much lower but consistent [P] across all cell types. Therefore,
none of the 12 species studied followed the current model of P allocation (Conn & Gillham, 2010). Furthermore, this trait appears to be under genetic control and is unlikely a phenotypic response to the environment: as the same preferential allocation of P is maintained under high P-supply in *R. prostrata* (Shane et al., 2004). Hence, patterns of P allocation vary within eudicots and even within a single family.

Preferential allocation of P to mesophyll cells has also been observed in Leucadendron "Safari Sunset," a Proteaceae from a severely P-impoveryed habitat in South Africa (Hawkins et al., 2008), as well as in two species from severely P-impoveryed south-western Australia (Lambers, Finnegan, et al., 2015; Shane et al., 2004). The consistent presence of this trait in Proteaceae from severely P-impoveryed habitats and its absence in Proteaceae from other P-
richer habitats suggests that it has evolved as an adaptation to severely P-poorer conditions (Hawkins et al., 2008; Lamberts, Finnegan, et al., 2015; Shane et al., 2004). Species from these exceptionally P-poorer habitats commonly show extremely high PPUE: 170 to 500 μmol CO₂ g⁻¹ leaf P⁻¹ s⁻¹ (Proteaceae from southwestern Australia; Denton et al., 2007; Lamberts et al., 2012), compared with 100 μmol CO₂ g⁻¹ leaf P⁻¹ s⁻¹ for species in a global comparison including different families (Wright et al., 2004), and compared with Proteaceae species from P-rich habitats in South America, 90 to 140 μmol CO₂ g⁻¹ leaf P⁻¹ s⁻¹ (Table S1; Franco, 1998; Wright et al., 2004; Franco et al., 2005). Such exceptionally high values of PPUE are achieved by maintaining rapid rates of photosynthesis at extremely low leaf P (Table S1). We surmise that by preferentially allocating P, these species are able to reduce their whole leaf P, while maintaining high P in photosynthetic cells and thus still able to achieve rapid rates of photosynthesis (Stitt et al., 2010). Therefore, the ability of Proteaceae from severely P-poorer habitats to preferentially allocate P to photosynthetic cells offers a partial explanation for their extremely high PPUE and may represent a critically important adaptation to surviving in a P-poorer limited habitat. Other traits that contribute to a high PPUE are replacement of phospholipids by sulfolipids and galactolipids (Lamberts et al., 2012) and functioning at very low levels of ribosomal RNA (Suberkropp, 2014).

Proteaceae from severely P-poorer southwestern Australia show whole leaf P [P] approximately half that of Proteaceae from P-rich parts of South America. Yet despite their extremely low whole leaf P, the Australian Proteaceae are still able to maintain rapid photosynthetic rates (10–22 μmol CO₂ m⁻² s⁻¹; Denton et al., 2007; Lamberts et al., 2012), even faster than from South America (8.7–14 μmol CO₂ m⁻² s⁻¹; Table S1; Franco, 1998; Wright et al., 2004; Franco et al., 2005). This can be partially explained by the ability of Australian Proteaceae to preferentially allocate P to photosynthetic cells and their ability to significantly reduce the [P] of non-photosynthetic cells. This results in Australian Proteaceae with photosynthetic cells that are ~6.5-fold greater in [P] than non-photosynthetic cells, reaching [P] that are up to ~2.5-fold greater than that of comparable photosynthetic cells in South American Proteaceae. However, these P-rich photosynthetic cells in the Australian Proteaceae only represent a small fraction of the whole leaf, with P-poor non-photosynthetic cells representing the larger proportion. Therefore, the exceptionally high [P] of photosynthetic cells in the Australian Proteaceae are effectively diluted by the extremely low [P] of the larger non-photosynthetic portion of the leaf. Therefore, although Proteaceae from south western Australia have exceptionally low whole leaf P, they are still able to maintain high [P] in their photosynthetic cells, and thus, they are able to achieve rapid rates of photosynthesis along with exceptionally high PPUE.

Outside of the Proteaceae, monocot species also preferentially allocate P to mesophyll cells (Coen & Gillittan, 2010). Interestingly, these monocot species also show high values of PPUE (Halstead & Lynch, 1996). With this, Proteaceae from P-poorer habitats, as well as monocot species, both preferentially allocate P to photosynthetic mesophyll cells and show high PPUE. Therefore, there is a growing body of support highlighting a link between allocation of P to photosynthetic cells and a high P use efficiency.

Increased capacity to resorb and reallocate P from senescing leaves is another trait common to Proteaceae from P-poorer habitats; this trait improves overall plant P-use efficiency by decreasing the plants' demand on external P. Proteaceae from P-poorer southwestern Australia are known to resorb up to ~90% of P from senescing leaves (Denton et al., 2007; Hayes et al., 2014; Wright et al., 2004), whereas Proteaceae from relatively P-rich habitats in South America show much lower levels of P resorption efficiency, ~13% (I. concinnum and Z. inflatum; Díaz et al., 2003; Lamberts et al., 2012). The ability to preferentially allocate P to mesophyll cells may allow for improved leaf P resorption, because, by allocating P to a specific region, this can allow for a more localized and efficient expression of phosphatase enzymes, required to breakdown organic P to inorganic P, which can then be more efficiently exported from the leaf, due to the close proximity of mesophyll cells to the phloem, through which P is exported. Therefore, we hypothesize that there is a link between the preferential allocation of P to mesophyll cells and increased P resorption.

Our results do not support the current model of cell-specific P allocation patterns in eudicot leaves; this discrepancy may be explained by the fact that most studies in this field have focused on metal-hyperaccumulating species and/or crop species (Ager, Ynsa, Donáno, Sollie, Gorton, et al., 2002; Ager, Ynsa, Donáno, Sollie, López-Marín, et al., 2003; Bidwell et al., 2004; Fernando et al., 2006; Fernando et al., 2008; Küpper et al., 1999; Küpper, Lombi, Zhao, & McGrath, 2000; Küpper, Lombi, Zhao, Wissmann, et al., 2001; Mesjasz-Prybylowicz & Pineda, 2001; Mesjasz-Prybylowicz et al., 1994; Repar et al., 2013; Ries et al., 2012; Treby et al., 1987; Villaforte Carvalho et al., 2015; Vogel-Mihal et al., 2006, 2014; Zhao et al., 2000). These species likely function very differently in their environment, compared with native species from nutrient-poor habitats. Therefore, our results are significant in broadening this field of research and adding to a more comprehensive understanding of P use in eudicots.

There is a clear need for more research into cell-type specific P distribution patterns across a more phylogenetically and ecologically diverse range of species. This will enhance our understanding of the factors underlying P distribution within plants, with significant implications for plant physiology, species distribution, and community functioning. By further investigation and improved understandings of the subcellular processes and mechanisms involved in P allocation and their link to improving whole-plant P-use efficiency, we will be able to improve crop breeding efforts, with the aim of decreasing society's demand for P in food production (Veneklaas et al., 2012).

4.2 Calcium

Calcium distribution patterns varied among species and, therefore, also did not reflect the notion of a single pattern in all eudicots (Coen & Gillittan, 2010). All species accumulated Ca within specific cell types, but these cell types differed among species, with no clear shift between species that did or did not allocate P to mesophyll cells. Calcium accumulating cells were not always along the transpiration pathway and were generally separate from P accumulating cells. Calcium typically accumulated within IP and SM; but, importantly, many species
also accumulated Ca within other cell types/regions. Variation in Ca accumulation strategies was even observed within a single genus, in co-occurring *Banikia* species.

The mechanisms underlying cell-specific accumulation of Ca and other elements are not fully understood (Conn et al., 2011; Conn & Gilliam, 2010; Gilliam et al., 2011; White & Broderick, 2003). Calcium primarily moves through leaves via the transpiration movement of water, from xylem to stomata (White, 2001; White & Broderick, 2003). Consequently, Ca is thought to accumulate primarily in cells along this path, such as SM, due to its greater apoplastic [Ca] ([Ca]_apop) (Gilliam et al., 2011; Karley, Leigh, & Sanders, 2000). The accumulation of Ca within SM (in *G. oerclantia* and *R. montana*) supports this model, as these areas are close to the xylem and typically show greater [Ca]_apop (Gilliam et al., 2011; Kerton, Newbury, Hand, & Pitchard, 2009; White, 2001). However, for cells to accumulate Ca within their vacuoles, they must also express both Ca²⁺-permeable ion channels, allowing Ca²⁺ to move down an electrochemical potential gradient into the cytosol, and tonoplast-localized transporters, allowing Ca²⁺ uptake against an electrochemical potential gradient (Ca²⁺-transporting P-type ATPases and Ca²⁺/H⁺ antiporters), into the vacuole, where it can accumulate to high concentrations (>1,000 umol g⁻¹; Conn et al., 2011; Mäser et al., 2001; McAlpin & Pittman, 2009; Shiokaki & Hirnchi, 2006).

We found that Ca accumulated in PM of *L. oerclantia* and HY of *B. marcelli*, these species. Ca accumulated in specific and distinct layers of cells, not located along the transpiration pathway, that is, not between the xylem and stomata. We, therefore, suggest that in these species, the transpiration pathway is not the dominant determinant of leaf cell-specific Ca accumulation. Instead, it is likely primarily driven by the differential expression of Ca²⁺ channels/transporters that function to accumulate Ca within vacuoles of specific cells, thus functioning to maintain [Ca]_apop and cytosolic [Ca] ([Ca]_cyt). This has also been demonstrated in Arabidopsis, where the preferential expression of a tonoplast Ca²⁺/H⁺ antiporter (CAX) in mesophyll cells is necessary for the preferential storage of Ca in these cells (Conn et al., 2011). In summary, the movement of water via transpiration is vital for the overall uptake and regulation of Ca in leaves (McLaughlin & Wiemer, 1999), but, on the basis of the patterns of Ca distribution, this is not invariably the primary mechanism underlying cell-specific accumulation of Ca in leaves. We suggest that the ability of specific cells to accumulate large amounts of Ca is primarily driven by a higher expression of Ca²⁺ channels and tonoplast Ca²⁺ transporters and is less reliant on the movement of water through leaves. This uncoupling of Ca²⁺ accumulation from water flow has also been demonstrated in other studies (Atkinson, Ruhl, & Mansfield, 1992; Kerton et al., 2009; Storey & Leigh, 2004). There is a need for further research into the differential expression of these transporters and channels within contrasting cell types, particularly in species such as *L. oerclantia*, where Ca accumulates preferentially in distinct layers of what appears to be the one cell type.

Exceptionally high [Ca] of ~1,413 umol g⁻¹ was observed in PM Ca cells of *L. marcelli*. At this concentration, it is highly unlikely that the Ca is in a readily soluble or ionized (Ca²⁺) form, due to the necessary cellular counterbalancing that would be required to maintain this level of Ca within the cell vacuole. However, as evidenced by a range of techniques, we are certain that this Ca is not mineralized, either in a crystalline or an amorphous form. On the basis of the evidence, we surmise that the Ca is bound to Ca-binding proteins within the vacuole (supported by higher [S], allowing for such high concentrations to be accumulated and stabilized. Further investigation of this unique phenomenon may yield exciting details regarding Ca regulation and accumulation in higher plants.

As hypothesized, almost all species allocated P and Ca to different cell types, thus avoiding precipitation of calcium phosphate. The only exception to this were the Australian *Banikia* species, which preferentially allocated both Ca and P to SM cells. Despite preferential allocation, [P] in these Ca-accumulating cells remained very low, at <10 umol g⁻¹. Maintenance of a low [P] may partly explain an ability to co-allocate these elements. The physiological implications of such P/Ca co-allocation are not yet known but might be relevant in understanding symptoms of P-toxicity, which are known to increase under higher Ca availability for many Proteaceae (Gurdon, 1972).

4.3 Concluding remarks

Our study shows that leaf cell specific distribution of P and Ca varies significantly among Proteaceae. This finding is in contrast with the current model and highlights the need for a better understanding of the underlying roles and mechanisms of cell-specific element accumulation within plants. The accumulation of P in photosynthetic cells of species from low-P habitats partially explains their very high PPUE. Further research is needed to determine if this pattern is found in other species, outside the Proteaceae, that are also adapted to severely P-impaired habitats. The link between accumulating P in photosynthetic cells and a high PPUE is highly applicable in improving P-use efficiency for crop species and thus reducing the P-demand of food production worldwide.

The extent to which cell-specific element accumulation varies among a range of ecologically and phylogenetically diverse species remains to be further investigated, as do the mechanisms underlying it. This knowledge will improve our understanding of the movement, accumulation, and overall regulation of essential elements within plants. An improved understanding of these processes will greatly benefit applied fields of research, such as the biofortification of our staple foods (Duyod, Tyerman, Leigh, & Gilliam, 2010; Pinto & Ferreira, 2015; Rios et al., 2012), phytoremediation/phytoextraction of contaminated soils (Shiokaki & Hirnchi, 2006), and improved nutrient-use efficiency in crop plants (Lamb, Cooke, et al., 2015; Veneklaas et al., 2012).

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AUTHOR CONTRIBUTION
P. E. H., H. L., and P. C. planned and designed the research; P. E. H., P. C., and R. S. O. performed the research; P. E. H., P. C., and H. L. analysed and interpreted data; and P. E. H., P. C., R. S. O., and H. L. wrote the paper.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Leaf phosphorus concentrations (P), photosynthesis per unit area and leaf photosynthetic P-use efficiency (PPUE) of Proteaceae from Australia (south-western Australia), Brazil (south-eastern Brazil) and Chile (southern Chile), as well as the world average from a global comparison, including different families.

<table>
<thead>
<tr>
<th>Study area</th>
<th>Leaf P (mg g(^{-1}) DW)</th>
<th>Photosynthesis (µmol CO(_2) m(^{-2}) s(^{-1}))</th>
<th>PPUE (µmol CO(_2) [g leaf P](^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>0.33 (0.01)(^{1})</td>
<td>10 - 22(^{2,3})</td>
<td>170 - 500(^{2,3})</td>
</tr>
<tr>
<td>Brazil</td>
<td>0.42 (0.04)(^{1})</td>
<td>9.9 - 14(^{4,5,6})</td>
<td>90 - 140(^{7})</td>
</tr>
<tr>
<td>Chile</td>
<td>0.84 (0.07)(^{1})</td>
<td>8.9 - 14(^{a})</td>
<td>90(^{7,8})</td>
</tr>
<tr>
<td>World</td>
<td>1.11(^{4})</td>
<td>11.5(^{4})</td>
<td>100(^{4})</td>
</tr>
</tbody>
</table>

Data sources: \(^{1}\)this study; \(^{2}\)Denton et al. (2007); \(^{3}\)Lambers et al. (2012b); \(^{4}\)Wright et al. (2004); \(^{5}\)Franco (1998); \(^{6}\)Franco et al. (2005); \(^{7}\)Lambers et al. (2012a).

\(^{1}\) Values calculated from the given leaf [P] and photosynthesis rates; \(^{2}\) Data only available for *Embothrium coccineum*.

Means (standard errors) are provided for leaf [P], from data collected in this study. Ranges are that of species-specific means. World data are mean values, derived from Wright et al. (2004). DW, dry weight.
<table>
<thead>
<tr>
<th>Species</th>
<th>Study area</th>
<th>Subfamily</th>
<th>Tribe</th>
<th>Subtribe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hakea incrassata</td>
<td>Australia</td>
<td>Grevilleoideae</td>
<td>Embothrieae</td>
<td>Hakeinae</td>
</tr>
<tr>
<td>Hakea prostrata</td>
<td>Australia</td>
<td>Grevilleoideae</td>
<td>Embothrieae</td>
<td>Hakeinae</td>
</tr>
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<td>Persoonioideae</td>
<td>Persoonieae</td>
<td></td>
</tr>
<tr>
<td>Petrophile macrostachya</td>
<td>Australia</td>
<td>Proteoideae</td>
<td>Petrophileae</td>
<td></td>
</tr>
<tr>
<td>Banksia attenuata</td>
<td>Australia</td>
<td>Grevilleoideae</td>
<td>Banksieae</td>
<td>Banksiinae</td>
</tr>
<tr>
<td>Banksia menziesii</td>
<td>Australia</td>
<td>Grevilleoideae</td>
<td>Banksieae</td>
<td>Banksiinae</td>
</tr>
<tr>
<td>Banksia prionotes</td>
<td>Australia</td>
<td>Grevilleoideae</td>
<td>Banksieae</td>
<td>Banksiinae</td>
</tr>
<tr>
<td>Embothrium coccineum</td>
<td>Chile</td>
<td>Grevilleoideae</td>
<td>Embothrieae</td>
<td>Embothriinae</td>
</tr>
<tr>
<td>Gevuina avellana</td>
<td>Chile</td>
<td>Grevilleoideae</td>
<td>Macadamieae</td>
<td>Gevuininae</td>
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<tr>
<td>Lomatia ferruginea</td>
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<td>Embothrieae</td>
<td>Lomatiinae</td>
</tr>
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<td>Lomatia hirsuta</td>
<td>Chile</td>
<td>Grevilleoideae</td>
<td>Embothrieae</td>
<td>Lomatiinae</td>
</tr>
<tr>
<td>Roupala montana</td>
<td>Brazil</td>
<td>Grevilleoideae</td>
<td>Roupaleae</td>
<td>Roupalinae</td>
</tr>
</tbody>
</table>

These species covered a broad range of the Proteaceae phylogeny. Phylogenetic classification based on Weston and Barker (2006). Australia (Jurien Bay, south-western Australia), Brazil (Serra do Cipó, south-eastern Brazil) and Chile (Puerto Montt, southern Chile.)
Table S3. Leaf anatomy and corresponding cell types analysed from X-ray element maps of each species, showing the total number of cells analysed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Anatomy</th>
<th>Cell type (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EP</td>
</tr>
<tr>
<td>Hakea incrassata</td>
<td>Isobilateral</td>
<td>47</td>
</tr>
<tr>
<td>Hakea prostrata</td>
<td>Isobilateral</td>
<td>43</td>
</tr>
<tr>
<td>Persoonia comata</td>
<td>Isobilateral</td>
<td>25</td>
</tr>
<tr>
<td>Petrophile macrostachya</td>
<td>Isobilateral</td>
<td>40</td>
</tr>
<tr>
<td>Banksia attenuata</td>
<td>Dorsiventral/stomatal crypt</td>
<td>38</td>
</tr>
<tr>
<td>Banksia menziesii</td>
<td>Dorsiventral/stomatal crypt</td>
<td>25</td>
</tr>
<tr>
<td>Banksia prionotes</td>
<td>Dorsiventral/stomatal crypt</td>
<td>38</td>
</tr>
<tr>
<td>Embathrium coccineum</td>
<td>Dorsiventral</td>
<td>58</td>
</tr>
<tr>
<td>Grevina avellana</td>
<td>Dorsiventral</td>
<td>75</td>
</tr>
<tr>
<td>Lomatia ferruginea</td>
<td>Dorsiventral</td>
<td>53</td>
</tr>
<tr>
<td>Lomatia hirsuta</td>
<td>Dorsiventral</td>
<td>87</td>
</tr>
<tr>
<td>Rosidae montana</td>
<td>Dorsiventral</td>
<td>50</td>
</tr>
</tbody>
</table>

Between seven and 117 cells were analysed per cell type for each species, across three individual plants. EP, epidermis; HY, hypodermis; HY.Ca, hypodermis with Ca; PM, palisade mesophyll; PM.Ca, palisade mesophyll with Ca; SM, spongy mesophyll; SM.Ca, spongy mesophyll with Ca; IP, internal parenchyma; IP.Ca, internal parenchyma with Ca; SC, sclerenchyma.
Table S4. Whole leaf phosphorus (P) and calcium (Ca) concentrations for the twelve Proteaceae species, indicating the study area in which they were collected

<table>
<thead>
<tr>
<th>Species</th>
<th>Study area</th>
<th>Leaf P (mg g⁻¹ DW)</th>
<th>Leaf Ca (mg g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hakea incrassata</em></td>
<td>Australia</td>
<td>0.31 (0.03)</td>
<td>3.37 (0.82)</td>
</tr>
<tr>
<td><em>Hakea prostrata</em></td>
<td>Australia</td>
<td>0.34 (0.03)</td>
<td>2.60 (0.82)</td>
</tr>
<tr>
<td><em>Persoonia comata</em></td>
<td>Australia</td>
<td>0.29 (0.03)</td>
<td>4.50 (0.84)</td>
</tr>
<tr>
<td><em>Petrophile macrostachya</em></td>
<td>Australia</td>
<td>0.35 (0.03)</td>
<td>6.00 (0.92)</td>
</tr>
<tr>
<td><em>Banksia attenuata</em></td>
<td>Australia</td>
<td>0.30 (0.03)</td>
<td>6.63 (0.98)</td>
</tr>
<tr>
<td><em>Banksia menziesii</em></td>
<td>Australia</td>
<td>0.30 (0.03)</td>
<td>6.70 (0.99)</td>
</tr>
<tr>
<td><em>Banksia prionotes</em></td>
<td>Australia</td>
<td>0.40 (0.04)</td>
<td>4.53 (0.84)</td>
</tr>
<tr>
<td><em>Embothrium coccineum</em></td>
<td>Chile</td>
<td>1.02 (0.09)</td>
<td>2.60 (0.81)</td>
</tr>
<tr>
<td><em>Gevuina avellana</em></td>
<td>Chile</td>
<td>0.71 (0.06)</td>
<td>6.13 (0.93)</td>
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<tr>
<td><em>Lomatia ferruginea</em></td>
<td>Chile</td>
<td>0.84 (0.07)</td>
<td>12.47 (4.40)</td>
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<tr>
<td><em>Lomatia hirsuta</em></td>
<td>Chile</td>
<td>0.79 (0.07)</td>
<td>8.13 (1.27)</td>
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<tr>
<td><em>Roupola montana</em></td>
<td>Brazil</td>
<td>0.42 (0.04)</td>
<td>2.62 (0.82)</td>
</tr>
</tbody>
</table>

Australia (Jurien Bay, south-western Australia), Brazil (Serra do Gipó, south-eastern Brazil) and Chile (Puerto Montt, southern Chile). DW, dry weight.
**Figure S1.** Details of method used to prepare and analyse samples for quantitative cell-specific elemental analysis. (a) Freshly cut transverse leaf section (~2x3 mm) placed on aluminium pin using optimal cutting compound (OCT), prior to plunge freezing in liquid nitrogen. (b) Secondary electron image of a cryo-planed sample in the scanning electron microscope prior to analysis. Note, samples were in a fully-hydrated state with no sublimation. (c) Qualitative X-ray map showing the oxygen (O) distribution and highlighted regions of interest (ROI) for a typical transverse leaf section. Regions of interest were drawn onto elemental maps using Oxford Instruments AZtec Energy software. Pixels within these ROI were summed and processed to yield quantitative numerical data for the determination of element concentrations from individual cells and areas of interest. Only cells that were clearly identifiable and with a flat surface were analysed. (d,e) All air spaces were avoided during data extraction, due to the importance of a flat surface for accurate quantitation. Some species showed very little air space, with numerous suitable ROI available (d), but other species showed large amounts of air space (e). (f-g) Matching qualitative X-ray maps showing the distribution of O and phosphorus (P) in a typical transverse leaf section, with a single ROI highlighted in the palisade mesophyll (PM). We did not present elemental maps for P in the main manuscript, because, as evident here, these maps do not clearly show the
Figure S1 (continued). Distribution of P. This was due to the consistently very low concentrations of P. However, P was present and could be readily quantified from regions of interest as evidenced by the example spectrum (h). (h) Extracted spectrum from the ROI shown in f-g, showing a clear P peak (shaded region). This shows that although the qualitative maps do not visually reflect differences in P due to the overall low P signal, the P was present and readily quantifiable. Scale bars: a, 1 mm; b, 200 μm; c-f, 50 μm.
Figure S2. Paired micrographs of vibratomed, transverse sections of leaves of *Hakea incrassata* *(a,b)* and *H. prostrata* *(c,d).* *(a,c)* Bright-field optical images indicating cell types. *(b,d)* Fluorescence images. Thickened cell walls, sclerenchyma, fibres and vascular tissues all fluoresce blue. Chlorophyll within photosynthetic palisade mesophyll (PM) cells fluoresce red. All leaves were isobilateral, with images capturing three quarters of the transverse section. EP, epidermis; PM, palisade mesophyll; IP, internal parenchyma; SC, sclerenchyma; V, vein. Scale bars: 100 μm.
Figure S3. Paired micrographs of vibratomed, transverse sections of leaves of *Persoonia comata* (a,b) and *Petrophile macrostachya* (c,d). (a,c) Bright-field optical images indicating cell types. (b,d) Fluorescence images. Thickened cell walls, sclerenchyma, fibres and vascular tissues all fluoresce blue. Chlorophyll within photosynthetic palisade mesophyll (PM) cells fluoresce red. All leaves were isobilateral, with images capturing most of the transverse section. EP, epidermis; PM, palisade mesophyll; IP, internal parenchyma; SC, sclerenchyma. Scale bars: 100 μm.
Figure S4. Paired micrographs of vibratomed, transverse sections of leaves of *Banksia attenuata* (a,b), *B. menziesii* (c,d), and *B. prionotes* (e,f). (a,c,e) Bright-field optical images indicating cell types. (b,d,f) Fluorescence images. Thickened cell walls, sclerenchyma, fibres and vascular tissues all fluoresce blue. Chlorophyll within photosynthetic palisade and spongy mesophyll (PM and SM) cells fluoresce red. All leaves were dorsiventral with stomatal crypts on the abaxial surface. Images capture upper part of transverse sections, with the adaxial surface at the top of each micrograph. EP, epidermis; HY, hypodermis; PM, palisade mesophyll; SM, spongy mesophyll; SC, sclerenchyma; V, vein. Scale bars: 100 μm.
Figure S5. Paired micrographs of vibratomed, transverse sections of leaves of *Embothrium coccineum* (a,b) and *Gevuina avellana* (c,d). (a,c) Bright-field optical images indicating cell types. (b,d) Fluorescence images. Thickened cell walls, sclerenchyma, fibres and vascular tissues all fluoresce blue. Chlorophyll within photosynthetic palisade mesophyll and spongy mesophyll (PM and SM) cells fluoresce red. All leaves were dorsiventral, with the adaxial surface at the top of each micrograph. EP, epidermis; PM, palisade mesophyll; SM, spongy mesophyll; V, vein. Scale bars: 100 μm.
Figure S6. Paired micrographs of vibratomed, transverse sections of leaves of *Lomatia ferruginea* (a,b), *Lomatia hirsuta* (c,d), and *Roupala montana* (e,f). (a,c,e) Bright-field optical images indicating cell types. (b,d,f) Fluorescence images. Thickened cell walls, sclerenchyma, fibres and vascular tissues all fluoresce blue. Chlorophyll within photosynthetic palisade mesophyll and spongy mesophyll (PM and SM) cells fluoresce red. All leaves were dorsiventral, with the adaxial surface at the top of each micrograph. EP, epidermis; HY, hypodermis; PM, palisade mesophyll; SM, spongy mesophyll; V, vein. Cell types with the suffix “Ca” are believed to accumulate Ca, based on distinct patterns identified from elemental maps. Scale bars: 100 μm.
Figure S7. Investigation of calcium (Ca)-rich palisade mesophyll (PM) cells within the South American species Lomatia ferruginea. This species shows exceptionally high Ca concentrations in alternating layers of the PM. (a) Bright-field optical image of a transverse leaf section of L. ferruginea, indicating cell types. (b) Secondary electron image of a cryo-planed, briefly sublimated transverse leaf section from L. ferruginea, indicating the major vacuole (V) present within the Ca-rich PM cells (PM.Ca), which is isolated from the cytoplasm (C), displays a fluid-like outline, and shows no crystalline structures within. The white arrow indicates cell wall and white arrow head indicates tonoplast. (c,d) Qualitative element maps showing Ca and sulfur (S) distributions in transverse leaf sections of L. ferruginea. Images capture entire leaf section, with the adaxial surface at the top. Qualitative element maps are corrected for peak overlaps and background subtraction. (e,f) Images capturing a single transverse slice from the 3-D X-ray microscopy scans of L.
Figure S7 (continued). *ferruginea* (e) and *Persoonia comata* (f). (f) The solid white regions indicate areas of high density, here they reveal mineralised forms of Ca, with no such structures or high densities evident in *L. ferruginea* (e). Images capture almost the full leaf section, with the adaxial surface at the top. EP, epidermis; PM, palisade mesophyll; PM.Ca, palisade mesophyll accumulating Ca; SM, spongy mesophyll; IP, internal parenchyma; V, vein. Scale bars: a,c-f, 100 μm; b, 20 μm.
Supporting Information: Materials and Methods

Analysis of calcium rich palisade mesophyll in *Lomatia ferruginea*

Optical images were obtained as described in the main text. Cryo-SEM images were prepared as described in the main text, except, the sample surface was etched for 18 minutes at -100°C and 10^{-7} mBar, before coating with 10 nm chromium. Images were then taken at -150°C and 5 kV. To check for the presence of biominerals, leaves were scanned in 3-dimensions using X-ray microscopy. Fixed pieces of leaf were quickly superglued to aluminum pins and placed in a BEEM cylinder filled with fixative. All scans were performed on an Xradia 520 X-ray Microscope. An X-ray energy of 60 kV and a power of 4.5 W was used. A total of 1601 projections were collected over a full 360° range. Exposure time was set to obtain ~10000 counts per frame. XMRD reconstructor was used for image reconstruction.
Chapter 3

Calcium-enhanced phosphorus toxicity in calcifuge and soil-indifferent Proteaceae along the Jurien Bay chronosequence

Summary

- Many Proteaceae are highly phosphorus (P)-sensitive and occur exclusively on old nutrient-impoverished acidic soils (calcifuge), whilst a few also occur on young calcareous soils (soil-indifferent), higher in available calcium (Ca) and P. Calcium increases the severity of P-toxicity symptoms, but its underlying mechanisms are unknown. We propose that Ca-enhanced P-toxicity explains the calcifuge habit of most Proteaceae.

- Four calcifuge and four soil-indifferent Proteaceae from south-western Australia were grown in hydroponics, at a range of P and Ca concentrations.

- Calcium increased the severity of P-toxicity symptoms in all species. Calcifuge Proteaceae were far more sensitive to Ca-enhanced P toxicity than soil-indifferent ones. Calcifuges shared these traits: low leaf zinc concentration ([Zn]), low Zn allocation to leaves, low leaf [Zn]:[P], low root:shoot ratio, and high seed P content, compared with soil-indifferent species.

- This is the first demonstration of Ca-enhanced P toxicity across multiple species. Calcium-enhanced P toxicity provides an explanation for the calcifuge habit of most Proteaceae and is critical to the management of this iconic Australian family. This study represents a major advancement in our understanding of the physiological mechanisms of P toxicity and its role in the distribution of Proteaceae.
Introduction

Phosphorus (P) toxicity has been studied in a range of plant species, including crops and P-sensitive species from severely P-impoverished habitats (Asher & Loneragan, 1967; Bhatti & Loneragan, 1970; Robson et al., 1970; McClure, 1972; Grundon, 1972; Groves & Keraitis, 1976; Nichols & Beardsell, 1981; Loneragan et al., 1982; Shane et al., 2004a; Hawkins et al., 2008; de Campos et al., 2013). Typical P-toxicity symptoms include leaf chlorosis/necrosis, reduced biomass, and early leaf senescence (Grundon, 1972; Groves & Keraitis, 1976; Nichols & Beardsell, 1981; Webb & Loneragan, 1990; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008). Species that are P sensitive show a low capacity to down-regulate P-uptake, along with a preferential allocation of P to mesophyll cells (Shane et al., 2004a,b; Hawkins et al., 2008; Hayes et al. 2018; Chapter 2). This disproportionately increases mesophyll [P], explaining their sensitivity to P, even at relatively low P availability (Shane et al., 2004a,b; Hawkins et al., 2008; Lambers et al., 2015; Hayes et al. 2018).

Symptoms of P toxicity are thought to result from interference of P with leaf water relations (Bhatti & Loneragan, 1970), or interaction of high mesophyll [P] with zinc (Zn) and other micronutrients (Cakmak & Marschner, 1987; Lambers et al., 2002; Broadley et al., 2012). The interaction of cellular P with Zn reduces the physiological [Zn], most likely through precipitation of sparingly soluble Zn-phosphates, resulting in a P-enhanced Zn requirement (Cakmak & Marschner, 1987). This causes symptoms resembling micronutrient deficiency (e.g., leaf chlorosis) and these are often misinterpreted as micronutrient-deficiency, instead of P toxicity (Broadley et al., 2012).

Other nutrients influence the severity of P toxicity (Robson et al., 1970; McClure, 1972; Grundon, 1972; Nichols & Beardsell, 1981). Potassium (K) and nitrogen (N) generally alleviate the severity of P toxicity, whilst calcium (Ca) increases it (Ca-enhanced P toxicity). Grundon (1972) proposed that Ca may act alone in reducing growth in P-sensitive species, whilst Nichols & Beardsell (1981) speculated that Ca stimulates P uptake in Grevillea cv. ‘Poorinda Firebird’ (Proteaceae), increasing the severity of P toxicity, as shown for annual legumes, maize, and wheat (Robson et al., 1970; McClure, 1972). Alternatively, Ca-enhanced P toxicity may be related to cell-specific allocation of Ca and P, which must be regulated to avoid deleterious precipitation of Ca-phosphates (McLaughlin & Wimmer, 1999; White & Broadley,
2003; Conn & Gillham, 2010). In summary, the physiological mechanisms of Ca-enhanced P toxicity remain unknown.

Many Proteaceae are highly P sensitive and are most abundant in severely P-impoverished regions, such as south-western Australia and South Africa (Weston, 2007). Over 650 Proteaceae occur in south-western Australia, representing almost 40% of all Proteaceae and contributing significantly to the region’s biodiversity (Cowling & Lamont, 1998; George, 1998; Myers et al., 2000; Hopper & Gioia, 2004). However, very few of the Proteaceae in south-western Australia occur on the young (<7,000 yr) calcareous dunes of the Jurien Bay chronosequence on the Swan Coastal Plain; most occur on the much older (>120,000 yr), nutrient-impoverished, acidic dunes and are considered calcifuge (Hayes et al., 2014; Zemunik et al., 2015). The few Proteaceae that do occur on younger calcareous dunes are also found on acidic dunes and are therefore considered soil-indifferent. The ecophysiological mechanisms underpinning this distribution remain largely unexplored, despite the Proteaceae representing an iconic component of the flora, contributing towards south-western Australia’s exceptionally high biodiversity (Pate & Beard, 1984; Myers et al., 2000; Hopper & Gioia, 2004; Lambers, 2014).

The young calcareous dunes of the Jurien Bay chronosequence exhibit a relatively high total and plant-available soil P concentration, compared with the much older acidic dunes (McArthur & Bettenay, 1974; McArthur, 2004; Hayes et al., 2014; Turner & Laliberté, 2015; Turner et al., 2018). However, this [P] is unlikely to be high enough to exclude P-sensitive Proteaceae (Shane & Lambers, 2005). Importantly, these calcareous dunes also show very high Ca concentrations, which may enhance P-sensitivity (Grundon, 1972; Nichols & Beardsell, 1981). Furthermore, if the symptoms of P toxicity are due to P-enhanced Zn requirements, then they would be further exacerbated under calcareous conditions, where soil Zn availability is also low (Cakmak & Marschner, 1987). However, this low micronutrient availability alone does not explain the calcifuge habit of Proteaceae, because, they possess carboxylate-releasing cluster roots, allowing them to access more micronutrients than other species (Tansley, 1917; Shane & Lambers, 2005). We surmise that Ca-enhanced P toxicity, along with reduced micronutrient availability, explains why most Proteaceae are excluded from calcareous habitats. This leads to the question: are soil-indifferent Proteaceae less sensitive to Ca-enhanced P toxicity than calcifuges, and what underlying traits account for this?
We grew eight Proteaceae in hydroponics at a range of P and Ca concentrations. This included four calcifuge and four soil-indifferent species. These species occur along the Jurien Bay dune chronosequence, and represent two major Proteaceae genera in south-western Australia, Banksia and Hakea. The aim of this research was to: 1) study the effects of Ca on P-toxicity symptoms across eight Proteaceae, 2) assess differences in P sensitivity between calcifuge and soil-indifferent species, and 3) compare traits of calcifuge and soil-indifferent species, to explain differences in their distribution.

First, we hypothesised that the severity of P-toxicity symptoms would increase with increasing Ca supply under high-P treatment, with only minor symptoms under low-P treatment (Ca-enhanced P toxicity). Second, we hypothesised that calcifuge Proteaceae would be more sensitive to Ca-enhanced P toxicity than soil-indifferent ones, thus accounting for their distribution. Finally, a comparison of calcifuge and soil-indifferent Proteaceae would allow us to discover why soil-indifferent species tolerate increased Ca and P supplies, providing a mechanistic understanding of this important phenomenon and its role in the calcifuge habit of most Proteaceae.

**Materials and Methods**

**Species selection**

We selected eight species from two Proteaceae genera, Banksia and Hakea, along the Jurien Bay chronosequence, located on the Swan Coastal Plain in south-western Australia, approximately 200 km north of Perth (Laliberté et al., 2012; Hayes et al., 2014; Zemunik et al., 2015). We selected four calcifuge species (Banksia attenuata R.Br., B. menziesii R.Br., Hakea incrassata R.Br. and H. flabellifolia Meisn.) and four soil-indifferent species (B. prionotes Lindl., B. sessilis (Knight) A.R.Mast & K.R.Thiele, H. prostrata R.Br. and H. trifurcata (Sm.) R.Br.). Species distributions were identified through published information and personal observations of their occurrence along the Jurien Bay chronosequence, over a number of years (Dixon, 2011; Hayes et al., 2014; Zemunik et al., 2015).
Plant growth

Plants were grown from seed and transferred to hydroponics. Seeds were collected in November 2013, from several populations along the Jurien Bay chronosequence (Zemunik et al., 2015). Seeds from calcifuge species were collected from at least two populations on acidic dunes, whilst seeds from soil-indifferent species were collected from at least two populations on both acidic, and calcareous dunes (at least two from each).

This study was divided into two hydroponics experiments. *Hakea* species were grown between February and September 2014, and *Banksia* species between June 2014 and March 2015. Seeds were sterilised and germinated on moist filter paper in Petri dishes, until the primary root and cotyledons had emerged (15°C, 12 h : 12 h, light : dark). Seedlings were then transferred by placing the single initial root through floating plastic mesh into trays containing 3 l of continuously-aerated nutrient solution. As plants became larger, the strength of the nutrient solution (pH 5.8) was increased, from ‘25% growth’ to ‘100% growth’ (see Table S1 for concentrations) and the solution was replenished more frequently, from once to three times per week. After ten (*Banksia*) or six (*Hakea*) weeks of growth, seedlings were transferred to 4.5 l pots and placed in a glasshouse.

Two plants were transferred to each 4.5 l pot, with each plant held in place by a grey-foam disk. Each pot contained 4 l continuously-aerated nutrient solution at a constant temperature, maintained at 18°C by placing pots in a root-cooling tank. During acclimation in the glasshouse, the strength of the nutrient solution was increased from ‘25% growth’ to ‘50% growth’ for *Banksias* and kept at ‘25% growth’ for *Hakeas*. During growth and acclimation, P and Ca were always supplied at 0.1 µM and 10 µM, respectively.

After six (*Banksia*) or eight (*Hakea*) weeks of acclimation in the glasshouse, 64 (*Banksia*) or 80 (*Hakea*) plants of uniform size were selected for each species and exposed to eight treatments (n = 8 – 10 plants per treatment). During the treatment period, plants were supplied with ‘50% basal’ (*Banksia*) or ‘25% to 33% basal’ (*Hakea*) nutrient solution (see Table S1 for concentrations). This was supplemented with different P (0.1, 10 µM; supplied as KH₂PO₄) and Ca (0, 0.1, 0.6, 6 mM; supplied as CaCl₂) concentrations (eight treatments in total). All nutrient solutions were replenished three times per week. Despite no additional Ca being added under the 0 mM Ca treatment, there would be a very low
background level of Ca in the deionised water. Plants were grown under treatment in a
temperature-controlled glasshouse; at a mean temperature, of 21°C *(Banksia)* and 17°C
(*Hakea)*.

*Harvest and plant biomass*

Plants were harvested after 11-weeks of treatment. During harvest plants were separated
into immature leaves (IL; soft expanding leaves and shoots), mature leaves (ML; fully-
expanded leaves), stems (ST), cluster roots (CR), and non-cluster roots (NCR). All plant parts
were weighed fresh (FW), then rinsed in deionised water before being oven dried (70°C, 72
h) and weighed (DW).

*Plant nutrient analyses*

Mature leaf, stem, and non-cluster root nutrient concentrations were analysed from at least
five plants per species/treatment combination (*n* ≥ 5). Dried subsamples of leaves, stems, and
non-cluster roots were ground in a ball-mill grinder using plastic vials and yttria-stabilised
zirconia ceramic beads. Samples were then digested in concentrated nitric acid under heat
and analysed for Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn, using inductively-coupled plasma
optical-emission spectrometry (ICP-OES; National Measurements Institute, Perth, WA,
Australia) on an axially-configured Varian Vista Pro (Varian Australia Pty Ltd, Mulgrave,
Victoria, Australia).

*Seed nutrient analyses*

A subset of seeds collected for each species was analysed to determine seed mass, [P] and P
content. Four lots of 5 – 30 seeds (~0.2 – 2 g each) were randomly selected for each species.
Each lot of seeds was first counted, then oven dried (70°C, 72 h), before being weighed and
ground, as described above. Samples were then digested in concentrated HNO₃:HClO₄ (3:1)
and the concentration of P determined colourimetrically using the malachite green method
(Motomizu *et al.*, 1983).
Statistics

Percentage of mean total DW (%$dw$) was calculated as the percentage of species-specific mean total DW (Eqn 1):

$$%dw = 100\frac{dw}{dw_m}$$

where $dw$ is the total DW per plant, and $dw_m$ is the mean total DW for that species in all treatments. Root:shoot ratios were calculated based on total aboveground DW (including IL, ML, and ST) and belowground DW (NCR and CR). Leaf Zn allocation (%$Zn$) was calculated as a percentage of total Zn allocated to the leaves, (Eqn 2):

$$%Zn = \frac{ML_c \times L_m}{(ML_c \times L_m) + (ST_c \times ST_m) + (NCR_c \times R_m)}$$

where $ML_c$ is the ML [Zn], $L_m$ is the total leaf DW, $ST_c$ is the ST [Zn], $ST_m$ is the ST DW, $NCR_c$ is the NCR [Zn] and $R_m$ is the total root DW.

Differences in biomass, root:shoot ratios, leaf nutrient concentrations, leaf Zn allocation, and seed [P], across Ca treatments, P treatments and distribution types, including their interactions, were tested using general linear mixed-effect models, with species set as the random effect (Pinheiro & Bates, 2000). Within each species, differences in biomass and leaf nutrient concentrations, across Ca and P treatments, including their interaction, were tested using generalised least squares models (Pinheiro & Bates, 2000). Within soil-indifferent species, we also tested for differences between plants grown from seeds collected in acidic versus calcareous habitats (habitat type). Differences in total DW and leaf nutrient concentrations (Zn, P and Ca), across Ca treatments, P treatments and habitat types, including their interactions, were tested using generalised least squares models (Pinheiro & Bates, 2000). Based on post hoc Tukey tests, there were no consistent differences between habitat types, and, therefore, all analyses of soil-indifferent species include equal representation of both habitat types. The relationships between leaf [P] and leaf [Zn], [Fe], and [Mn], were inspected through regression analysis. The models were selected based on Akaike Information Criterion (AIC), and the model’s parameters (P-values, and standardised regression coefficient) were presented whenever significant ($P < 0.05$).
The residuals of each model were visually inspected for heteroscedasticity. In the presence of heteroscedasticity, appropriate variance structures were specified if they significantly improved the model, based on AIC and Bayesian Information Criterion (BIC) values (Pinheiro & Bates, 2000). Statistical analyses were performed using the R software platform (R Core Team, 2017) and the nlme package (Pinheiro et al., 2016). The effects package (Fox, 2003) was used to determine means and 95% confidence intervals (CI), later used to define differences among treatments and distribution types.

Results

Visual symptoms

Visual symptoms were most severe under the high-P (10 μM) / high-Ca (6 mM) treatment, but almost completely absent under the high-P (10 μM) / low-Ca (0 mM) treatment (Fig. 1). Under the high-P / high-Ca treatment, all species showed severe signs of necrosis and chlorosis, indicating severe nutrient toxicity and/or deficiency (Fig. 1). In contrast, under the same high-P treatment, but with no Ca supplied, all species showed healthy, green leaves, with no signs of nutrient toxicity (Fig. 1). Under low-P (0.1 μM) treatment all species showed less severe visual symptoms, again, increasing in severity with increasing Ca supply. In summary, all plants appeared healthiest under high P supply, as long as little to no Ca was supplied.

Calcifuges showed more severe visual symptoms and at lower Ca supply than soil-indifferent species (Fig. 1). In calcifuge Banksias, the main visual leaf symptoms were severe chlorosis, mild leaf tip necrosis, and red blotching. In contrast, soil-indifferent Banksias showed only minor chlorosis (Fig. 1). Calcifuge Hakeas showed the most severe visual leaf symptoms: severe and extensive leaf necrosis, significant red blotching, and minor leaf chlorosis. Soil-indifferent Hakeas, however, showed only minor leaf chlorosis (Fig. 1).
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**Fig. 1** Visual symptoms of four representative Proteaceae species from two genera (*Banksia* and *Hakea*), under the lowest and highest phosphorus (P) and calcium (Ca) treatment combinations. Calcifuge (CF) species do not naturally occur on calcareous soils, whilst soil-indifferent (SI) species occur on both calcareous and acidic soils. Single leaf insets illustrate typical leaf symptoms under the highest Ca treatments (insets not to scale). 0.1 μM P and 10 μM P, indicate the two P treatments. 0 mM Ca and 6 mM Ca, indicate the lowest and highest Ca treatments. Scale bars are 10 cm.
Biomass response

A direct comparison of standardised total biomass between calcifuge and soil-indifferent species showed that percentage of mean total biomass changed among treatments and distribution-types (Fig. 2a). The effect of Ca treatment on total biomass depended on P treatment in both Banksias and Hakeas (Ca treatment x P treatment interaction: $P < 0.01$; Fig. 2a). Thus, total biomass decreased with increasing Ca supply under high-P treatment, whilst there was no significant difference under low-P treatment (Fig. 2a). Furthermore, biomass tended to be greatest under high-P treatment, except under high Ca supply, where the biomass was reduced to levels similar to that under low-P treatment (Fig. 2a).

The effect of distribution-type on total biomass depended on P treatment in both Banksias and Hakeas (distribution-type x P treatment interaction: $P < 0.001$; Fig. 2a). Thus, total biomass in soil-indifferent species tended to be greater than that in calcifuges under high P-treatment but was lower under low-P treatment. Consequently, soil-indifferent species showed a much greater biomass response than calcifuges (Figs 2a, S1). For example, percent mean total biomass in soil-indifferent species showed a range of 153% (Banksias) and 111% (Hakeas), compared with that in calcifuge species, 83% (Banksias and Hakeas; Fig 2a).

In general, Banksias had a higher root:shoot ratio than Hakeas, especially under low-P treatment (Fig. 2b). A direct comparison of root:shoot ratios between calcifuge and soil-indifferent species revealed that soil-indifferent species generally had a higher root:shoot ratio (Fig. 2b). The effect of distribution-type on root:shoot ratio depended on P treatment (distribution-type x P treatment interaction: $P < 0.0001$; Fig 2b). This means that under low-P treatment soil-indifferent species had a much higher root:shoot ratio than calcifuges, while under high-P treatment this difference was reduced. In summary, root:shoot ratios were highest in soil-indifferent species, and generally higher in Banksias, with the greatest differences under low-P treatment.
Fig. 2 (a) Standardised total biomass and (b) root:shoot ratios of calcifuge and soil-indifferent *Banksia* and *Hakea* species, across different phosphorus (P) and calcium (Ca) treatments. (a) Standardised total biomass was calculated as the percentage of species-specific mean total dry weight (DW) (Eqn 1) and the dashed line indicates values at 100% of the mean. (b) Root:shoot ratios were calculated from dry weights. Bar heights show means and error bars show 95% confidence intervals (CI) from linear mixed effect models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within each distribution type (calcifuge and soil-indifferent), based on 95% CI. 0.1 μM P and 10 μM P, indicate the two P treatments.
Nutrient concentrations

Zinc

All soil-indifferent species, except *H. prostrata*, tended to have higher leaf [Zn] than calcifuges did (Fig. 3a). Soil-indifferent *Banksias* had significantly higher leaf [Zn] than calcifuges (*P* = 0.01; Fig. 3b), with this difference dependent on P treatment (distribution-type x P treatment interaction: *P* < 0.001; Fig. 3b). Soil-indifferent *Banksias* showed high leaf [Zn], up to ~10-fold greater than calcifuges and up to ~3.7-fold greater than that considered adequate for crop species (Fig. 3b; Bloom & Epstein, 2005). In contrast, calcifuge *Banksias* showed exceptionally low leaf [Zn], consistently below half that considered adequate for crops (Fig. 3; Bloom & Epstein, 2005). Soil-indifferent *Banksias* showed a large range in leaf [Zn], 16.5 – 73.8 µg g⁻¹, whilst, calcifuges showed no significant difference in leaf [Zn], from 6.5 – 10.1 µg g⁻¹ (Fig. 3b). Soil-indifferent *Banksia* leaf [Zn] also decreased with increasing P and Ca concentrations, with those in the high-P / higher-Ca treatments (0.6 and 6 mM) showing leaf [Zn] < 20 µg g⁻¹, the level considered adequate for crop species (Fig. 3b; Bloom & Epstein, 2005). The soil-indifferent species *B. sessilis*, showed the highest leaf [Zn], 96 µg g⁻¹, and the calcifuge species *B. menziesii* the lowest, 5.6 µg g⁻¹ (Fig. 3a).
Fig. 3 Mature leaf zinc concentrations ([Zn]) of (a) each individual species, and (b) calcifuge (CF) and soil-indifferent (SI) Banksia species, across different phosphorus (P) and calcium (Ca) treatments. Bar heights show means and error bars show 95% confidence intervals (CI) from (a) generalised least squares models and (b) linear mixed effect models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within (a) each species and (b) each distribution type (CF and SI), based on 95% CI. The dashed line represents the leaf [Zn] considered adequate for crop species (Bloom & Epstein, 2005). Hakea species are not shown in (b), because there was a significant difference between the two soil-indifferent Hakea species, as shown in (a). 0.1 μM P and 10 μM P, indicate the two P treatments.
The two soil-indifferent *Hakea* species showed contrasting leaf [Zn]; *H. trifurcata* showed consistently high leaf [Zn] (32 – 86 µg g⁻¹), and *H. prostrata* consistently low leaf [Zn] (6.3 – 26 µg g⁻¹; Fig. 3a). Due to this variation between soil-indifferent *Hakea* species it was not appropriate to directly compare soil-indifferent with calcifuge species, and hence *Hakea* species are only presented at the species level (Fig. 3a). *Hakea trifurcata* showed high leaf [Zn]; up to ~8.9-fold greater than that of calcifuge *Hakeas*, up to ~9.3-fold greater than that of *H. prostrata* and up to ~4.3-fold greater than that considered adequate for crops (Bloom & Epstein, 2005). The other *Hakeas* (calcifuge *Hakeas* and *H. prostrata*) all showed low leaf [Zn], 6.3 – 31 µg g⁻¹ (Fig. 3a; Bloom & Epstein, 2005).

Soil-indifferent *Banksias* allocated a greater percentage of Zn to their leaves (13%, 9%; *B. prionotes, B. sessilis*, respectively), than calcifuge *Banksias* did (4.8%, 5.4%; *B. attenuata, B. menziesii*, respectively; Fig. 4a). Similarly, soil-indifferent *Hakeas* tended to show a greater allocation of Zn to their leaves, than calcifuges (Fig. 4a). *Hakea trifurcata* showed by far the greatest allocation of Zn to leaves, with 30% of total Zn allocated to leaves (Fig. 4a). In contrast, calcifuge *Hakeas* showed only 8% and 11% (*H. flabellifolia, H. incrassata*, respectively), whilst *H. prostrata*, a soil-indifferent species, also showed similarly low allocation of Zn to leaves, at 13%. Therefore, leaf Zn allocation was generally greater in soil-indifferent species than in calcifuges and was lowest in calcifuge *Banksias*.

There was a strong correlation between leaf [Zn] and [P] in *Banksias* and in the soil-indifferent *H. trifurcata*, under high-P treatment (P < 0.001; Fig. 4b). Both *Banksia* distribution types had similar slopes, except soil-indifferent *Banksias* had higher leaf [Zn]:[P], compared with calcifuges. Also, soil-indifferent *Banksias* reached a much higher leaf [P] (10.6 mg g⁻¹), than calcifuges (5.8 mg g⁻¹; Fig. 4b). The soil-indifferent *H. trifurcata* was the only *Hakea* to show a significant correlation between leaf [Zn] and [P], with the other soil-indifferent species (*H. prostrata*), and calcifuges showing no correlation. *Hakea trifurcata* showed a similarly high proportion of leaf [Zn]:[P], as soil-indifferent *Banksias*. Data from the low-P treatment are not shown, because there was only a small range in leaf [P] under this treatment.
Fig. 4 (a) Zinc (Zn) allocation to leaves of calcifuge and soil-indifferent Banksia and Hakea species. Allocation was calculated as the percentage of total Zn allocated to leaves (Eqn 2). Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models, with all treatments included. Different letters indicate significant differences across species within each genus, based on 95% CI. (b) Changes in leaf zinc (Zn) concentration with leaf phosphorus (P) concentration for Banksia and Hakea species grown under high P treatment (10 μM). Each line represents the line of best fit for each distribution type and/or species, with grey regions indicating the 95% confidence range, derived from a linear model. The two soil-indifferent Hakea species are separated because they show contrasting patterns. Only significant relationships (P ≤ 0.05) are shown. β = regression coefficients.
Phosphorus

Leaf [P] was invariably greater under high-P treatment than under low-P treatment (P < 0.001; Figs 5a, S2). There was generally no change in leaf [P] across Ca treatments (Fig. 5a, S2). *Hakeas* had higher leaf [P] (0.13 – 7.14 mg g⁻¹) than *Banksias* (0.09 – 3.49 mg g⁻¹; Fig. 5a, S2). Calcifuge *Banksias* had the lowest leaf [P] (Fig. 5a), with *B. menziesii* the overall lowest (0.09 mg g⁻¹; Fig. S2). Calcifuge *Hakeas* had the highest leaf [P] (Fig. 5a), with *H. incrassata* the overall highest (7.8 mg g⁻¹; Fig. S2).

There was no significant difference in leaf [P] between calcifuge and soil-indifferent *Banksias* (P = 0.67; Fig. 5a). In contrast, calcifuge *Hakeas* had higher leaf [P], compared with soil-indifferent *Hakeas* (Fig. 5a). Furthermore, the effect of distribution-type on leaf [P] in *Hakeas* depended strongly on P treatment (distribution-type x P treatment interaction: P = 0.015; Fig.5a). This interaction reflects the greater difference between calcifuge and soil-indifferent *Hakeas* under low-P treatment than under high-P treatment (Fig. 5a).

Calcifuges showed a higher seed P content than soil-indifferent species (Fig. 5b, Table S2), mainly driven by higher seed mass (data not shown). Under low-P treatment, seed P content accounted for more than 100% of the whole plant P content (Table S2). Such high values can be explained by loss of plant material during the 6-month growing period, including loss of cotyledons, roots and senesced leaves, all of which contain some P.
Fig. 5 (a) Mature leaf phosphorus concentrations ([P]) of calcifuge and soil-indifferent Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments. (b) Seed phosphorus (P) content of naturally-occurring calcifuge and soil-indifferent Banksia and Hakea species. Bar heights show means and error bars show 95% confidence intervals (CI) from (a) linear mixed effect models and (b) generalised least squares models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within each distribution type (calcifuge and soil-indifferent) and (b) within each genus, based on 95% CI. 0.1 μM P and 10 μM P, indicate the two P treatments.
Calcium

Leaf [Ca] increased with increasing Ca supply ($P < 0.001$; Fig. 6). Hakeas showed slightly higher leaf [Ca] (5.9 – 27.5 mg g$^{-1}$) than Banksias (3.1 – 20.7 mg g$^{-1}$). Leaf [Ca] in Banksias mainly changed with Ca treatment ($P < 0.001$; Fig. 6). However, under low-P treatment, leaf [Ca] also varied between Banksia distribution-types, with soil-indifferent Banksias showing higher leaf [Ca] than calcifuges (Fig. 6). However, this difference is explained almost solely by a single species, B. sessilis (Fig. S3). In Hakeas, leaf [Ca] did not depend on P treatments ($P = 0.847$) or distribution-type ($P = 0.938$; Fig. 6). In summary, P treatment and distribution-type had little effect on leaf [Ca] in either Banksias or Hakeas, with Ca treatment being the primary determinant of leaf [Ca].

Fig. 6 Mature leaf calcium concentrations ([Ca]) of calcifuge and soil-indifferent Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments. Bar heights show means and error bars show 95% confidence intervals (CI) from linear mixed effect models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within each distribution type (calcifuge and soil-indifferent), based on 95% CI. The dashed line represents the leaf [Ca] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P, indicate the two P treatments.
**Iron, manganese and magnesium**

All species showed leaf [Fe] well below the 100 μg g⁻¹ level considered adequate for crops (Figs S4, S5; Bloom & Epstein, 2005). Leaf [Fe] was generally higher in *Banksias* (17 – 60 μg g⁻¹) than in *Hakeas* (18 – 29 μg g⁻¹). Soil-indifferent *Banksias* tended to show higher leaf [Fe] than calcifuges, but this was not significant (Fig. S4). *Hakeas* showed no difference between distribution types (Fig. S4). Both genera showed no consistent trends across treatments (Fig. S4).

There were significant correlations between leaf [Fe] and [P] under high-P treatment: *Banksia* (CF: $P < 0.001$; SI: $P < 0.01$), and *Hakea* (CF: $P < 0.01$; SI: $P < 0.001$; Fig. S6). Soil-indifferent species generally had a higher leaf [Fe] relative to [P]. The patterns between leaf [Fe] and [P] were similar to those observed for leaf [Zn] and [P] (Fig. 7).

All species generally showed leaf [Mn] at or above the 50 μg g⁻¹ level considered adequate for crops (Figs S7, S8; Bloom & Epstein, 2005). Like leaf [Fe], leaf [Mn] was generally higher in *Banksias* (66 – 297 μg g⁻¹) than in *Hakeas* (42 – 108 μg g⁻¹). Unlike leaf [Fe], leaf [Mn] in soil-indifferent *Banksias* showed a similar trend to leaf [Zn] (Fig. 4), generally higher than in calcifuges, and declining with higher P and Ca treatments (Fig. S7). However, *Banksia* leaf [Mn] showed less of a difference between distribution types than leaf [Zn] (Figs 4, S7). *Hakeas* again showed no difference between distribution types and no consistent trends across treatments (Fig. S7).

There was a significant correlation between leaf [Mn] and [P] under high-P treatment, for both distribution types of *Banksia* ($P < 0.001$) and only calcifuge *Hakeas* ($P = 0.004$; Fig. S6). There was no clear difference between distribution types.

*Banksia* leaf [Mg] gradually decreased with increasing Ca supply (Fig. S9). *Hakeas*, however, showed a sudden decrease in leaf [Mg] between the 0 and 0.1 mM Ca, after which it remained low for all other Ca treatments (0.1 – 6 mM; Fig. S9).

**Intraspecific variation within soil-indifferent species**

In terms of total plant dry weight, leaf [Zn], [P] and [Ca], there was no significant difference within soil-indifferent species, regardless of the habitat in which seeds were collected, acidic or calcareous (Fig. S10). The only minor exception to this was total dry weight in *B. prionotes*,

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showing a significant difference in the 0.6 mM Ca treatment (Fig. S10). In summary, we found no evidence of ecotypic differentiation between populations of soil-indifferent species from acidic versus calcareous habitats.

Discussion

Calcium enhanced the severity of P-toxicity symptoms

The severity of P-toxicity symptoms increased with increasing Ca supply under high-P treatment, with only minor symptoms under low-P treatment. The P-toxicity symptoms were primarily leaf chlorosis, necrosis, and reduced biomass, consistent with P-toxicity symptoms in other P-sensitive Proteaceae, and crops (Grundon, 1972; Groves & Keraitis, 1976; Nichols & Beardsell, 1981; Webb & Loneragan, 1990; Lambers et al., 2002; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008; de Campos et al., 2013).

As expected, we observed the most severe symptoms of P toxicity under high-P / high-Ca treatment. However, one of the most striking results was the lack of symptoms in plants grown under high-P / low-Ca treatment, even though this P level is typically toxic to these species (Shane et al., 2004a; de Campos et al., 2013). Also, there were only minor symptoms in plants grown under low-P / high-Ca treatment, suggesting that Ca was not the sole cause (Jefferies & Willis, 1964; Grundon, 1972). In summary, Ca significantly enhanced P-toxicity symptoms.

Phosphorus sensitivity is primarily caused by a low capacity to down-regulate P-uptake, along with a preferential allocation of P to mesophyll cells (Shane et al., 2004a,b; Hawkins et al., 2008; Lambers et al., 2015). However, the role of Ca in enhancing P toxicity remains unknown. Based on a study of Grevillea cv. ‘Poorinda Firebird’ (Proteaceae), Nichols & Beardsell (1981) speculated that Ca stimulates P uptake, enhancing P sensitivity. However, we found no evidence for increased P uptake and no change in leaf [P] with increasing Ca supply. This is perhaps not surprising, considering P-sensitive Proteaceae have little capacity for down-regulation of their P-uptake (Shane et al., 2004b; Shane & Lambers, 2006; de Campos et al., 2013). The observed Ca-enhanced P toxicity is therefore not due to Ca acting
alone and is not due to Ca-stimulated P uptake. We surmise that this Ca and P interaction is due to leaf cell-specific allocation of Ca and P.

Whole leaf [P] did not increase with increasing Ca supply, but the [P] in specific cells may have. Proteaceae from P-impoverished habitats preferentially allocate P to photosynthetic mesophyll cells, with up to ~6.5-fold greater [P] than that in non-photosynthetic cells, contributing to their very high photosynthetic P-use efficiency (Lambers et al., 2015; Hayes et al., 2018; Chapter 2). Under natural conditions, these Proteaceae separate the cell-specific allocation of Ca and P, avoiding the deleterious precipitation of Ca-phosphates (McLaughlin & Wimmer, 1999; White & Broadley, 2003); P is allocated to mesophyll cells and Ca to other cells (Hayes et al., 2018; Chapter 2). Under low Ca supply, these other cells may also accumulate excess P (P.E. Hayes et al., unpublished; Chapter 5). However, under high Ca supply the [Ca] would increase in these cells and excess P would then be displaced to the mesophyll, increasing the mesophyll [P] (P.E. Hayes et al., unpublished; Chapter 5). Therefore, we surmise that an increase in Ca supply effectively leads to an increase in mesophyll cell [P], with no increase in total leaf [P].

An increase in mesophyll cell [P], concomitant with a decrease in other cells, would account for Ca-enhanced P-toxicity symptoms, and for the lack of an increase in whole leaf [P]. Leaf chlorosis likely results from higher [P] interfering with the physiological availability of Zn and other micronutrients, i.e. P-enhanced Zn/micronutrient requirement (Chapman & Vanselow, 1937; Boawn & Leggett, 1964; Cakmak & Marschner, 1987; Loneragan & Webb, 1993). Leaf necrosis may result from precipitation of Ca-phosphates, when separation is not maintained. In summary, for the first time, there is a testable mechanism for the phenomenon of Ca-enhanced P toxicity.

As Ca was supplied in the form of CaCl$_2$, we cannot definitively exclude the possibility that chloride (Cl$^-$) may be a contributing factor in this experiment; however, the maximum 12 mM [Cl$^-$] used in this experiment is below the level considered to be toxic and is not expected to cause any significant reduction in biomass (Munns & Tester, 2008). Indeed, there was no change in biomass under low P supply, despite increased Cl$^-$ supply. Furthermore, two other studies that supplied excess Ca as CaSO$_4$ (Nichols and Beardsell, 1981) and Ca(NO$_3)_2$ (Grundon, 1972), both reported similar Ca-enhanced P-toxicity symptoms as those reported here. Therefore, we believe it is extremely unlikely for high Cl$^-$ supply to play a role in the results of this study.
Calcium-enhanced P toxicity was more severe in calcifuge than in soil-indifferent Proteaceae

Calcifuge Proteaceae were far more sensitive to Ca-enhanced P toxicity than soil-indifferent ones, evidenced by visual P-toxicity symptoms and reduced biomass. Calcifuges were more sensitive to higher Ca and P availability, thus likely accounting for their inability to inhabit calcareous soils (Pate & Beard, 1984; Cowling & Lamont, 1998; Lambers, 2014; Zemunik et al., 2015). In contrast, the ability of soil-indifferent species to tolerate higher Ca and P availability may explain their ability to survive in both calcareous and acidic soils (Thiele & Ladiges, 1994; Bennett & Attiwill, 1997; Dixon, 2011; Zemunik et al., 2015). This is the first study to demonstrate differences between calcifuge and soil-indifferent Proteaceae.

Common traits in calcifuge versus soil-indifferent Proteaceae

We identified five traits that differed between calcifuge and soil-indifferent species. All calcifuges showed low leaf [Zn], low allocation of Zn to leaves, low leaf [Zn]:[P], low root:shoot ratio, and high seed P content, compared with soil-indifferent species. These traits are likely important for differences in Proteaceae distribution and understanding the physiological basis for Ca-enhanced P toxicity.

Leaf Zn

The leaf [Zn] of calcifuge species was extremely low, well below the level considered adequate for crops (<20 μg g⁻¹; Bloom & Epstein, 2005) and often below that of soil-indifferent species. Leaf [Zn] of similarly low levels have been reported for other Proteaceae from P-impoverished habitats (Kuo et al., 1982; Denton et al., 2007; Hayes et al., 2014). These Proteaceae employ strategies to reduce their demand for P and increase photosynthetic P-use efficiency (Lambers et al., 2012, 2015; Sulpice et al., 2014). One of these strategies is to function at low leaf protein levels, thereby reducing P-demanding protein synthesis (Sulpice et al., 2014). Many proteins require Zn as a structural component and/or cofactor and thus a reduced protein level in Proteaceae may partly account for their exceptionally low leaf [Zn] (Robson,
1993; Broadley et al., 2007, 2012; Caldelas & Weiss, 2017). A low leaf protein concentration is advantageous for a high P-use efficiency (Sulpice et al., 2014), but it may limit a species’ ability to inhabit P richer calcareous soils, because high P availability would reduce the internal physiological availability of Zn, which is sparingly available in calcareous soils. This can therefore result in a P enhanced Zn requirement, possibly leading to Zn deficiency.

Three of the four soil indifferent species had higher leaf [Zn] than the calcifuges. This is likely important in compensating for a P enhanced Zn requirement (Cakmak & Marschner, 1987). A higher leaf [Zn] would allow for adequate physiological [Zn] to be maintained, even at higher leaf (cell) [P].

The reduced leaf [Zn] of most soil indifferent species under high P treatment compared with low P treatment may be explained by a lower root:shoot ratio (reducing the amount of roots for Zn uptake) and increased biomass (effectively diluting [Zn]) (Cakmak & Marschner, 1987; Loneragan & Webb, 1993). This decline in total leaf [Zn] under high P treatment likely induces P toxicity symptoms, as it dramatically reduces the amount of Zn taken up and translocated to the leaves. However, it does not explain the observed Ca enhanced P toxicity, as leaf [Zn] did not change with increasing Ca supply. This is likely because, under high P treatment the mesophyll [P] may increase with increasing Ca supply, reducing the physiological Zn availability, leading to Zn deficiency symptoms, with no change in total leaf [Zn].

In summary, the low leaf [Zn] typical of Proteaceae from P impoverished habitats may reflect low leaf protein concentrations but renders these species susceptible to Zn deficiency in P richer habitats or those with extremely low Zn availability. This limits the distribution of such species to P impoverished habitats, where leaf (cell) [P] is low enough to not interfere with the extremely low leaf [Zn]. Therefore, the ability of certain soil indifferent Proteaceae to maintain higher leaf [Zn] may allow them to inhabit a wider range of habitats, because their higher leaf [Zn] compensates for P enhanced Zn requirements.

Leaf Zn allocation
Calcifuges allocated an extremely low percentage of total plant Zn to leaves, suggesting a low capacity to translocate Zn to leaves (~5-11%). In contrast, three of the four soil indifferent species allocated a greater percentage of Zn to leaves (~9-30%), contributing to their higher
leaf [Zn]. Most Zn was allocated to roots > stem > leaves. In summary, soil-indifferent species allocated more Zn to leaves, contributing to their higher leaf [Zn], which likely plays a role in their ability to tolerate P-enhanced Zn requirements. Calcifuges, however, allocated very little Zn to leaves, contributing to their low leaf [Zn] and low ability to tolerate P-enhanced Zn requirement.

Leaf [Zn]:[P]
Calcifuges had lower leaf [Zn]:[P] than most soil-indifferent species. Both Banksia distribution types showed a positive correlation between leaf [Zn] and [P], but soil-indifferent Banksias always had higher leaf [Zn]:[P]. Of the Hakeas, only the soil-indifferent H. trifurcata showed a significant positive correlation between leaf [Zn] and [P]. It also had consistently higher leaf [Zn]:[P], which would increase the physiological [Zn], avoiding Zn-deficiency symptoms (Boawn & Leggett, 1964; Cakmak & Marschner, 1987). In summary, the ability of soil-indifferent species to maintain higher leaf [Zn]:[P] and to increase leaf [Zn] with increasing leaf [P] may help maintain a higher physiological [Zn].

Root:shoot ratio
Root:shoot biomass ratios were generally lower in calcifuge than in soil-indifferent species. A lower root:shoot ratio in calcifuge species can effectively reduce leaf [Zn]. This occurs because the greater relative shoot biomass in calcifuge species can dilute shoot [Zn], whilst the reduced investment in root biomass can reduce the relative amount of roots available for Zn absorption. For example, both root:shoot ratio and leaf [Zn] were reduced under high-P treatment. Furthermore, the difference in both root:shoot ratio and leaf [Zn] between distribution types, was greatest under low-P treatment and lowest under high-P treatment. Therefore, P supply strongly influenced the root:shoot ratio, which influenced leaf [Zn]. In summary, root:shoot ratios were generally lower in calcifuges and this may be linked to their lower leaf [Zn], and increased susceptibility to P-enhanced Zn requirements.

Seed P content
Calcifuges had higher seed P content than soil-indifferent species, primarily related to their up to ~25-fold greater seed mass. The high seed P content of calcifuges is advantageous in P-
impoverished habitats, as it reduces seedling reliance on external P; however, in P-richer habitats this can lead to higher leaf [P] in seedlings, inhibiting the formation of cluster roots (Shane et al., 2003). This was evident in calcifuge seedlings, which showed very few cluster roots (Fig. S1). This would reduce the ability of calcifuge seedlings to acquire Zn/micronutrients from calcareous soil, and likely contributes towards their exclusion from calcareous habitats, where the availability of Zn/micronutrients is very low. In contrast, soil-indifferent seedlings, with their lower seed P content formed cluster roots at an earlier stage and this would allow them to access more soil Zn/micronutrients (Fig. S1). In summary, the large seed P content of calcifugues provides an advantage in P-impoverished habitats, but delays seedling cluster-root formation, reducing their capacity to acquire Zn/micronutrients. This may therefore partly explain the inability of calcifugues to establish in calcareous habitats. Conversely, the low seed P content of soil-indifferent seedlings promotes cluster-root formation, allowing them to acquire more Zn/micronutrients earlier, thus contributing towards their ability to establish in calcareous habitats.

Strategies of soil-indifferent Banksia and Hakea species

Chlorosis was the main visual symptom in Banksias and the main difference between Banksia distribution types were the five common traits described above. Therefore, the ability of soil-indifferent Banksias to tolerate Ca-enhanced P toxicity appears mainly related to maintaining higher leaf [Zn], thus enabling them to meet P-enhanced Zn requirements.

Hakeas exhibited severe and extensive necrosis, significant red blotching, and only minor chlorosis. The two soil-indifferent Hakeas varied in their traits, with H. trifurcata sharing the five common traits, whilst H. prostrata did not. Soil-indifferent Hakeas generally had lower leaf [P] than calcifugues, indicating a somewhat greater capacity to down-regulate P uptake. This, along with higher leaf [Zn] in H. trifurcata may explain why soil-indifferent Hakeas better tolerate Ca-enhanced P toxicity. Furthermore, the greater severity of necrosis and red blotching in Hakeas may indicate cell damage, possibly due to precipitation of Ca-phosphates, as opposed to chlorosis in Banksias, likely associated with P-enhanced Zn requirements.
Concluding remarks

Calcium increased the severity of P-toxicity symptoms, providing the first clear evidence for Ca-enhanced P toxicity across multiple species. Calcifuges were far more sensitive to P toxicity than soil-indifferent species. The phenomenon of Ca-enhanced P toxicity provides an explanation for why calcifuge Proteaceae are excluded from calcareous habitats and why soil-indifferent ones are not. We surmise that it is the higher soil [P] of young calcareous soils, in combination with higher soil [Ca] and low available [Zn] that restricts most Proteaceae from establishing in calcareous habitats, and that soil-indifferent Proteaceae can overcome this restriction through several traits, which increase leaf [Zn] and compensate for P-enhanced Zn requirement.

The comparison of calcifuge and soil-indifferent species provides the first testable mechanism of Ca-enhanced P toxicity. We propose that Ca-enhanced P toxicity is due to the leaf cell-specific interactions of Ca and P, resulting in higher leaf mesophyll [P] under increasing Ca supply, thus interfering with the physiological [Zn] and/or precipitating with Ca. In addition, species that show no increase in leaf [Zn], yet appear soil-indifferent, such as H. prostrata, may instead show differences in their ability to regulate cell-specific [Ca] and [P]. These proposed mechanisms are under investigation through cell-specific nutrient analyses.

This study will assist in the management of Proteaceae in the restoration of disturbed areas and in the horticultural industry, as it advances our understanding of the environmental factors impacting on the distribution of Proteaceae and highlights the importance of considering P, Ca, and micronutrients in the nutrition of P-sensitive plants (Bunn & Dixon, 1992; Enright & Lamont, 1992; Fuss et al., 1992; Stephenson, 2005; Cross & Lambers, 2017). This research advances our fundamental understanding of plant mineral nutrition and highlights the importance of considering interactions between nutrients. This study represents a major advancement in our understanding of Ca-enhanced P toxicity, particularly in relation to the distribution of Proteaceae.
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References


Chapter 3 | Supporting Information

**Fig. S1** Leaf, stem, cluster root and non-cluster root dry weights for each species.

**Fig. S2** Mature leaf phosphorus concentrations for each species.

**Fig. S3** Mature leaf calcium concentrations for each species.

**Fig. S4** Mature leaf iron concentrations, comparing distribution types.

**Fig. S5** Mature leaf iron concentrations for each species.

**Fig. S6** Scatter plot showing changes in mature leaf iron and manganese with leaf phosphorus, comparing distribution types.

**Fig. S7** Mature leaf manganese concentrations, comparing distribution types.

**Fig. S8** Mature leaf manganese concentrations for each species.

**Fig. S9** Mature leaf magnesium concentrations for each species.

**Fig. S10** Set of figures demonstrating a lack of intraspecific variation between habitat types of soil-indifferent species, showing total dry weights, leaf zinc, leaf phosphorus, and leaf calcium.

**Table S1** Concentrations of elements used in the different nutrient solutions.

**Table S2** Seed phosphorus (P) content, range of total plant P content, and range of percentage total plant P content that could be accounted for by seed P content in each species.
**Fig. S1** Leaf, stem, cluster root and non-cluster root dry weights of calcifuge (CF) and soil-indifferent (SI) *Banksia* and *Hakea* species, grown under different phosphorus (P) and calcium (Ca) treatments. Error bars show 95% confidence intervals (CI) from generalised least squares models for each species and tissue type. Different letters indicate significant differences within each species, for above- and belowground dry weights, based on 95% CI. 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S2 Mature leaf phosphorus concentrations ([P]) of each *Banksia* and *Hakea* species, across different phosphorus (P) and calcium (Ca) treatments. Calcifuge (CF) and soil-indifferent (SI) distribution types are indicated. Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models. Different letters indicate significant differences within each species, based on 95% CI. 0.1 µM P and 10 µM P indicate the two P treatments.
Fig. S3 Mature leaf calcium concentrations ([Ca]) of each Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments. Calcifuge (CF) and soil-indifferent (SI) distribution types are indicated. Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models. Different letters indicate significant differences within each species, based on 95% CI. The dashed line represents the leaf [Ca] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S4 Mature leaf iron concentrations ([Fe]) of calcifuge and soil-indifferent *Banksia* and *Hakea* species, across different phosphorus (P) and calcium (Ca) treatments. Bar heights show means and error bars show 95% confidence intervals (CI) from linear mixed effect models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within each distribution type (calcifuge and soil-indifferent) across all treatments, based on 95% CI. The dashed line represents the leaf [Fe] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S5 Mature leaf iron concentrations ([Fe]) of each Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments. Calcifuge (CF) and soil-indifferent (SI) distribution types are indicated. Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models. Different letters indicate significant differences within each species, based on 95% CI. The dashed line represents the leaf [Fe] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S6 Changes in leaf iron ([Fe]) and manganese ([Mn]) concentration with leaf phosphorus concentration ([P]) for Banksia and Hakea species grown under high-P treatment (10 μM). Each line represents the line of best fit for each distribution type, with grey regions indicating the 95% confidence range, derived from linear models. Only significant relationships (P ≤ 0.05) are shown. \( \beta \) = regression coefficients.
Fig. 57 Mature leaf manganese concentrations ([Mn]) of calcifuge and soil-indifferent *Banksia* and *Hakea* species, across different phosphorus (P) and calcium (Ca) treatments. Bar heights show means and error bars show 95% confidence intervals (CI) from linear mixed effect models. Means where the 95% CI do not overlap are considered significantly different. Different letters indicate significant differences within each distribution type (calcifuge and soil-indifferent) across all treatments, based on 95% CI. The dashed line represents the leaf [Mn] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S8 Mature leaf manganese concentrations ([Mn]) of each *Banksia* and *Hakea* species, across different phosphorus (P) and calcium (Ca) treatments. Calcifuge (CF) and soil-indifferent (SI) distribution types are indicated. Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models. Different letters indicate significant differences within each species, based on 95% CI. The dashed line represents the leaf [Mn] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 µM P and 10 µM P indicate the two P treatments.
Fig. 59 Mature leaf magnesium concentrations ([Mg]) of each Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments. Calcifuge (CF) and soil-indifferent (SI) distribution types are indicated. Bar heights show means and error bars show 95% confidence intervals (CI) from generalised least squares models. Different letters indicate significant differences within each species, based on 95% CI. The dashed line represents the leaf [Mg] considered adequate for crop species (Bloom & Epstein, 2005). 0.1 μM P and 10 μM P indicate the two P treatments.
Fig. S10 Intraspecific variation between habitat types of soil-indifferent (SI) Banksia and Hakea species, across different phosphorus (P) and calcium (Ca) treatments, showing total dry weights (DW), leaf zinc concentration ([Zn]), leaf P concentration ([P]), and leaf Ca concentration ([Ca]). Comparisons are made between plants grown from seeds collected on acidic versus calcareous habitats. Points indicate means and bars show 95% confidence intervals (CI) from generalised least squares models. No significant difference was found between habitat types, except Banksia prionotes total DW. Significant differences between habitat types are based on post hoc Tukey tests ($P < 0.05$). 0.1 μM P and 10 μM P indicate the two P treatments. *, $P < 0.05$; ** $P < 0.01$. 

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**Table S1** Concentrations of elements used in the different nutrient solutions. Recipes based on Shane et al. (2004).

<table>
<thead>
<tr>
<th>Nutrient solution</th>
<th>PO$_4^{3-}$</th>
<th>Ca$^{2+}$</th>
<th>NO$_3^-$</th>
<th>K$^+$</th>
<th>SO$_4^{2-}$</th>
<th>Mg$^{2+}$</th>
<th>Fe-EDTA</th>
<th>Mn$^{2+}$</th>
<th>Zn$^{2+}$</th>
<th>Cu$^{2+}$</th>
<th>H$_3$BO$_3$</th>
<th>Mo$^{4+}$</th>
<th>SiO$_2^{5-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% growth</td>
<td>0.1</td>
<td>10</td>
<td>50</td>
<td>50</td>
<td>9</td>
<td>9</td>
<td>2.5</td>
<td>0.06</td>
<td>0.025</td>
<td>0.018</td>
<td>0.6</td>
<td>0.075</td>
<td>50</td>
</tr>
<tr>
<td>100% growth</td>
<td>0.1</td>
<td>10</td>
<td>200</td>
<td>200</td>
<td>36</td>
<td>36</td>
<td>10</td>
<td>0.24</td>
<td>0.1</td>
<td>0.018</td>
<td>2.4</td>
<td>0.3</td>
<td>50</td>
</tr>
<tr>
<td>25% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
<td>50</td>
<td>50</td>
<td>9</td>
<td>9</td>
<td>2.5</td>
<td>0.06</td>
<td>0.025</td>
<td>0.018</td>
<td>0.6</td>
<td>0.075</td>
<td>50</td>
</tr>
<tr>
<td>33% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
<td>66.7</td>
<td>66.7</td>
<td>12</td>
<td>12</td>
<td>3.3</td>
<td>0.08</td>
<td>0.33</td>
<td>0.018</td>
<td>0.8</td>
<td>0.1</td>
<td>50</td>
</tr>
<tr>
<td>50% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
<td>100</td>
<td>100</td>
<td>18</td>
<td>18</td>
<td>5</td>
<td>0.12</td>
<td>0.05</td>
<td>0.018</td>
<td>1.2</td>
<td>0.15</td>
<td>50</td>
</tr>
</tbody>
</table>

‘Growth’ nutrient solutions were used during pre-treatment growth and acclimation.

‘Basal’ nutrient solutions were used during the treatment period. See methods section for further details.
Table S2 Seed phosphorus (P) content, range of total plant P content, and range of percentage total plant P content that could be accounted for by seed P content in each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution type</th>
<th>Seed P content (mg P seed(^{-1}))</th>
<th>Total plant P content (mg P plant(^{-1}))</th>
<th>Total plant P content accounted for by seed P content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Banksia attenuata</em></td>
<td>Calcifuge</td>
<td>1.08 (0.02)</td>
<td>0.83 (0.17) – 14 (2.2)</td>
<td>7.9 - 130</td>
</tr>
<tr>
<td><em>Banksia menziesii</em></td>
<td>Calcifuge</td>
<td>1.04 (0.02)</td>
<td>0.52 (0.1) – 19 (4.9)</td>
<td>5.6 - 200</td>
</tr>
<tr>
<td><em>Hakea flabellifolia</em></td>
<td>Calcifuge</td>
<td>2.38 (0.09)</td>
<td>1.29 (0.2) – 13 (2.5)</td>
<td>18.9 - 185</td>
</tr>
<tr>
<td><em>Hakea incrassata</em></td>
<td>Calcifuge</td>
<td>1.26 (0.18)</td>
<td>0.56 (0.1) – 11 (2.5)</td>
<td>11.1 - 223</td>
</tr>
<tr>
<td><em>Banksia prionotes</em></td>
<td>Soil-indifferent</td>
<td>0.49 (0.01)</td>
<td>0.21 (0.02) – 16 (1.7)</td>
<td>3.1 - 233</td>
</tr>
<tr>
<td><em>Banksia sessilis</em></td>
<td>Soil-indifferent</td>
<td>0.084 (0.003)</td>
<td>0.06 (0.01) – 4.9 (1.5)</td>
<td>1.7 - 140</td>
</tr>
<tr>
<td><em>Hakea prostrata</em></td>
<td>Soil-indifferent</td>
<td>0.47 (0.02)</td>
<td>0.29 (0.02) – 13 (0.82)</td>
<td>3.5 - 159</td>
</tr>
<tr>
<td><em>Hakea trifurcata</em></td>
<td>Soil-indifferent</td>
<td>0.15 (0.007)</td>
<td>0.12 (0.01) – 9.5 (1.2)</td>
<td>1.6 - 128</td>
</tr>
</tbody>
</table>

Seed P content and total plant P content are means (SE), \(n = 4 – 5\). Total plant P content indicates the range of treatment level means.

The percentage of total plant P content accounted for by seed P content is determined from the mean seed P content and the range of total plant P content. Values above 100% can be explained by loss of plant material during the six-month growing period, including loss of cotyledons, roots and senesced leaves, all of which would contain some P.
Chapter 4

Physiological aspects of calcium-enhanced phosphorus toxicity in Proteaceae

Summary

• Proteaceae species differ in their phosphorus (P)-sensitivity and this might be an important factor explaining their natural distribution. Most Proteaceae occur exclusively on P-impoverished, acidic soils (calcifuge), but some also occur on young, calcareous soils (soil-indifferent), where soil P-availability is higher. The mechanism allowing soil-indifferent species to grow on these P-rich, calcareous soils is unclear, especially considering that high calcium (Ca) concentrations enhance P-toxicity symptoms. We surmise that this is due to differences in their physiological responses to high P and Ca supplies.

• We analysed plant growth, gas-exchange and chlorophyll fluorescence of two soil-indifferent and four calcifuge Hakea and Banksia (Proteaceae) species from south-western Australia across a range of P and Ca concentrations in nutrient solutions.

• Calcium-enhanced P toxicity was observed in all species, but to different extents depending on distribution type and genus. Increasing [P] had a positive effect on growth and photosynthesis of soil-indifferent species. Calcifuges were far more sensitive to P-toxicity. In calcifuge Hakeas, this was attributed to higher leaf [P], and in calcifuge Banksias to lower leaf [Zn]. Moreover, the response of calcifuge species to higher P supply strongly depended on Ca supply.

• Differences in physiological responses of soil-indifferent and calcifuge Proteaceae species to high P/Ca supplies were associated with these species' ability to regulate nutrient uptake, particularly that of P and Zn, as well as with their nutrient-allocation pattern at the cellular level.
Introduction

Phosphorus (P) is a plant nutrient frequently limiting plant productivity in a range of terrestrial ecosystems (Vitousek et al., 2010; Veneklaas et al., 2012; Lambers et al., 2015). Extensive research on P limitation of plant productivity has been conducted in multiple habitats, but there is limited knowledge on P toxicity, even though this may be significant in explaining the distribution of species in severely P-impoveryished environments. Phosphorus-toxicity symptoms may include growth inhibition, early leaf senescence and chlorotic/necrotic regions on leaves (Bhatti & Loneragan, 1970; Shane et al., 2004a,b). While these symptoms are well documented, the underlying physiological mechanisms remain poorly understood. High P supply may interfere with leaf water relations (Bhatti & Loneragan, 1970) and with the zinc (Zn) nutrition (Boawn & Leggett, 1964; Cakmak & Marschner, 1987; Loneragan et al., 1979; Loneragan & Webb, 1993). However, a clear mechanism of P toxicity is still lacking.

Most Proteaceae are particularly sensitive to high soil P availability (Ozanne & Specht, 1981; Lambers et al., 2002; Parks et al., 2000; Shane et al., 2004a; Handreck, 1997), mainly due to their low capacity to down-regulate P uptake (Shane et al., 2004a,b; de Campos et al., 2013). In fact, species adapted to severely P-povertyished environments may develop P-toxicity symptoms even at relatively low P supplies (Ozanne & Specht, 1981; Shane et al., 2004a). This might be a defining factor explaining these species' exclusion from P-rich dunes in south-western Australia (Zemunik et al., 2015), characterised by soils with high P and calcium (Ca) concentrations, as well as high soil pH (McArthur et al., 1991; Laliberté et al., 2012; Turner & Laliberté, 2015). These factors may play a role in these species' response to high P supply, as elevated pH can prevent the formation of cluster-roots, and P-toxicity symptoms are aggrivated at high Ca supplies (Grundon, 1972), i.e. Ca-enhanced P toxicity. However, some Proteaceae are soil-indifferent, occurring on both acidic dunes, where calcifuge species occur, and calcareous ones (Dixon, 2011; Hayes et al., 2014; Zemunik et al., 2015). The mechanism that allows these soil-indifferent species to cope with calcareous soils is unclear, especially since high soil [Ca] enhances P-toxicity symptoms.

High Ca supply may inhibit growth (Grundon, 1972) or increase P uptake (Leggett et al., 1965; Hyde, 1966; Edwards, 1968; Robson et al., 1970). In both cases, Ca-enhanced P toxicity is thought to result from high Ca supplies leading to higher total leaf [P]. However,
this is not the case for some Proteaceae (P.E. Hayes et al., unpublished; Chapter 3), which show enhanced P-toxicity symptoms with increasing Ca supplies without differences in leaf [P], leading us to hypothesise that differences in physiological responses to increasing P and Ca supplies may play a role in the expression of Ca-enhanced P toxicity.

Low P supply primarily affects leaf expansion (Chiera et al., 2002; Assuero et al., 2004; Kavanová et al., 2006; Hawkesford et al., 2011; Dissanayaka et al., 2018), reducing photosynthesis due to low sink demand (Pieters et al., 2001). Limiting P supply also reduces total leaf [P] and orthophosphate (Pi) pools (Veneklaas et al., 2012). Since Pi is required for the synthesis of intermediates of the Calvin-Benson cycle (Dietz & Foyer, 1986; Foyer & Spencer, 1986; Giersch & Robinson, 1987; Ellsworth et al., 2015) and the export of triose-P to the cytosol, there is a relationship between P supply and photosynthesis. However, few studies focused on this relationship at toxic P levels. In addition, there is strong evidence that this relationship may depend on Ca supply, which can impact P-uptake rates (Legget et al., 1965; Hyde, 1966; Edwards, 1968; Robson et al., 1970), the activity of key Calvin-Benson cycle enzymes (fructose bisphosphatase and sedoheptulose bisphosphatase; Portis & Heldt, 1976), and stomatal functioning (De Silva et al., 1985). Thus far, no studies have examined the interaction between these nutrients at the physiological level.

We grew six Proteaceae species (two soil-indifferent and four calcifuge) that naturally occur along the Jurien Bay chronosequence in south-western Australia (Hayes et al., 2014; Zemunik et al., 2015) in hydroponic solutions with different P and Ca supplies. The aims of this study were to: 1.) analyse the physiological responses (plant growth, gas exchange, chlorophyll fluorescence) of Proteaceae species to a range of P and Ca supplies; 2.) compare the expression of Ca-enhanced P toxicity in soil-indifferent and calcifuge species; 3.) determine if differences in carbon metabolism explains why soil-indifferent and calcifuge species are distributed differently along the Jurien Bay chronosequence.

We hypothesised that photosynthetic responses to varying P and Ca supplies depend on both genus and distribution type. Second, that soil-indifferent species are far less sensitive to Ca-enhanced P toxicity than calcifuges, being either unaffected by high P and Ca supply or only affected at extremely high concentrations. Finally, we hypothesised that P-toxicity symptoms would, in conjunction with photosynthetic responses, provide clues for how these
nutrients interact in leaves, allowing us to propose a mechanism for Ca-enhanced P toxicity effects on physiological processes.

**Material and Methods**

*Species selection*

We selected six Proteaceae species from two genera, *Hakea* and *Banksia*, which naturally occur along the Jurien Bay chronosequence (Hayes *et al.*, 2014; Zemunik *et al.*, 2015). This chronosequence is located in south-western Australia, approximately 200 km north of Perth, and consists of a series of overlapping dune systems deposited during periods of high sea level over ~2 million years, from the Early Pleistocene and Late Pliocene to the present (Laliberté *et al.*, 2012; Turner & Laliberté, 2015). These dune systems, formed through the deposition of carbonate-rich material (McArthur *et al.*, 1991), increase in age with distance from the coast. With time, carbonate is progressively leached from the upper horizons and soil pH, [Ca] and [P] gradually decline along the coastal-inland gradient (Turner & Laliberté, 2015).

Most Proteaceae that occur along the chronosequence occur exclusively on older, acidic dunes, where soil [Ca] and [P] are much lower than on calcareous dunes. These species are referred to as calcifuge (Hayes *et al.*, 2014; Zemunik *et al.*, 2015). However, a few Proteaceae species occur on both young, calcareous dunes and on older, acidic dunes, further inland (Dixon, 2011), being referred to as soil-indifferent. In this study, we selected four calcifuge (*Hakea flabellifolia* Meisn., *H. incrassata* R.Br., *Banksia menziesii* R.Br. and *B. attenuata* R.Br.) and two soil-indifferent species (*H. prostrata* R.Br. and *B. prionotes* Lindl.).

*Plant growth*

Plants were grown from seeds collected in November, 2013 from populations along the Jurien Bay chronosequence (Turner & Laliberté, 2015) and immediately transferred to hydroponics. Seeds from calcifuge species (*H. flabellifolia*, *H. incrassata*, *B. menziesii*, and *B. attenuata*) were collected from two or more populations on acidic soils. Seeds from soil-indifferent
species (*H. prostrata* and *B. prionotes*) were collected from populations on both acidic and calcareous soils (at least two from each soil-type).

This study was divided into two separate experiments. The *Hakeas* were grown between February and September 2014, and the *Banksias* between June 2014 and March 2015. Seeds were sterilised with 1% (w/v) sodium hypochlorite - an additional sterilisation with 70% (v/v) ethanol was performed for *Banksias* - and sown on moist filter paper in 20 cm Petri dishes until the primary root and cotyledons emerged (15°C, 12 h of light). The seedlings were then transferred by placing the single initial root through floating plastic mesh into trays containing 3 l of continuously-aerated nutrient solution. The strength of the nutrient solution (pH 5.8) was increased, from ‘25% growth’ to ‘100% growth’ (Table S1), as the plants became larger. The complete nutrient solution was replenished 1-3 times per week, depending on seedling size. Temperature was continuously increased (15°C to 21°C) to acclimate plants, and after six (*Hakea*) or ten (*Banksia*) weeks of initial establishment, the seedlings were transferred to 4.5 l pots and moved to the glasshouse. In total, 320 *Hakea* and 256 *Banksia* plants (80 and 64 per *Hakea* and *Banksia* species, respectively) of uniform size were selected for the experiment (n= 8-10 plants per species per treatment).

Plants were transferred in pairs to 4.5 l pots, with each plant held in place by a grey foam disk, creating a light-tight seal around the base of the stem. Each pot contained 4 l of continuously-aerated nutrient solution, maintained at 18°C by a root-cooling tank. During acclimation, the nutrient solution was kept at ‘25% growth’ (*Hakea*) or increased from ‘25% growth’ to ‘50% growth’ (*Banksia*). During growth and acclimation, P and Ca were continuously supplied at 0.1 μM and 10 μM, respectively (Table S1 for concentrations). Plants were maintained in the glasshouse during acclimation for eight (*Hakea*) or six (*Banksia*) weeks, after which the treatment began.

During the treatment, plants were supplied with either ‘25% to 33% basal’ or ‘50% basal’ nutrient solution (*Hakea* and *Banksia*, respectively; Table S1 for concentrations). This solution was supplemented with P (0.1, 10 μM; supplied as KH₂PO₄) and Ca (0, 0.1, 0.6, 6 mM; supplied as CaCl₂), with eight treatments in total. The complete nutrient solutions with the treatments were replenished three times per week. Plants were grown under treatment for 11 weeks in a temperature-controlled glasshouse at a mean temperature of 17°C (*Hakea*) and 21°C (*Banksia*), with root temperatures maintained at 18°C.
Plants were harvested after 11 weeks. During harvest, plants were separated into subsamples: mature leaves (ML; fully-expanded leaves), immature leaves (IL; soft expanding leaves and shoots), stems (ST), cluster roots (CR) and non-cluster roots (NCR). All sub-samples were weighed fresh (FW), rinsed in deionised water, oven-dried (70°C, 72 h) and weighed again dry (DW).

**Nutrient analyses**

Mature leaves, stems and non-cluster root samples of five individual plants per species/treatment combination were analysed for total [P], [Ca] and [Zn]. The dried samples were ground (2010 Geno/Grinder, SPEX SamplePrep, Metuchen, USA) using 5 ml plastic vials and yttria-stabilised zirconium ceramic beads. The finely-ground tissue samples were then acid-digested using concentrated nitric acid under heat, and analysed for total nutrient concentration using inductively coupled plasma optical-emission spectrometry (ICP; National Measurements Institute, Perth, Australia) on an axially configured Varian Vista Pro (Varian Australia Pty Ltd., Mulgrave, Australia).

**Gas-exchange measurements**

Light-saturated photosynthesis ($A_{\text{sat}}$) and leaf dark respiration ($R_{\text{dark}}$) rates were measured on mature, fully-expanded leaves of >four individuals for each species/treatment combination using an infra-red gas analyser (LI-6400XT; LI-COR BioSciences, Lincoln, USA) equipped with a light source (LI-6400-02B). Photosynthetic measurements were carried out at 2500 µmol photons m$^{-2}$ s$^{-1}$, which was saturating, but not damaging, to all species. Leaves were exposed to 400 µmol CO$_2$ mol$^{-1}$ air during measurements (using the built-in LI-6400XT CO$_2$ controller). All photosynthetic measurements were performed on leaves that had been exposed to at least 2 h of daytime illumination, whilst the respiration measurements were made 4 h after sunset. Measurements were made on >two leaves per individual plant, and the results averaged for each individual.
Chlorophyll fluorescence measurements

Leaf chlorophyll fluorescence was measured using a LI-6400XT (LI-COR BioSciences, Lincoln, USA). The variable over maximal fluorescence (Fv/Fm) measurements were carried out on plants dark-acclimated for more than 4 h. Reaction centre closure was achieved by applying a 0.8 s pulse of saturating light. Fv/Fm was calculated as the variable over the maximum fluorescence (Genty et al., 1989).

Statistical analyses

Differences in plant growth, dry matter content (DMC), light-saturated photosynthesis ($A_{sat}$), leaf dark respiration ($R_{dark}$), stomatal conductance ($g_s$), intercellular CO$_2$ concentration ($C_i$) and variable over maximal fluorescence (Fv/Fm) among P and Ca treatments for Hakea and Banksia species with similar distribution types were tested using generalised least squares (GLS) models. The residuals of the models were screened for normality through formal tests (Shapiro-Wilk and Anderson-Darling) and inspected for heteroscedasticity, visually and through Fligner-Killeen test, with appropriate variance structures being specified to the models when necessary. Model selection was based on Akaike's Information Criterion corrected for finite sample size ($AIC_c$; Tables S2-S8) and the Tukey HSD post-hoc test was used to determine differences among treatment combinations (McCulloch & Neuhaus, 2005). The relationship between $A_{sat}$ and leaf [Ca], and between leaf [Zn] and leaf [P], was inspected through regression analysis. These models were selected based on Akaike's Information Criterion corrected for finite sample sizes ($AIC_c$), and the model's parameters ($P$-values, $R^2$ and $\beta$; standardised regression coefficients) were presented whenever significant ($P<0.05$). All the statistical analyses were performed with the R software platform (Fox, 2003; Pinheiro et al., 2017; R Development Core Team).
Results

Plant growth and tissue quality

The soil-indifferent *Hakea* and *Banksia* showed an increase in growth with increasing P supply, except at the highest Ca supply ($P<0.001$; Fig. 1a). There was also a significant effect of Ca treatment on plant growth ($P<0.001$ and $P=0.037$ for *Hakea* and *Banksia*, respectively; Fig. 1a), with increasing Ca supply negatively affecting the production of biomass. The calcifuge *Banksias* presented a similar pattern, with increased growth in response to higher P supply ($P=0.029$; Fig. 1a), but only under low-Ca conditions (0 and 0.1 mM). The significant effect of Ca on the growth of calcifuge *Banksias* ($P<0.001$; Fig. 1a), likewise, indicated a decrease in plant growth with increasing Ca supply, particularly at the high-P treatment. The calcifuge *Hakeas*, in contrast, showed no increase in growth with the increasing P supply. Rather, these either showed similar (0 and 0.1 mM Ca) or decreased growth (0.6 and 6 mM Ca) with the additional P. Calcifuge *Hakeas* also showed the slowest growth rates of all.
Fig. 1 Mean (± SE, n= 11-20) values of plant growth (as mass increase; (a)) and dry matter content (b) for soil-indifferent and calcifuge species (*Hakea* and *Banksia*) across different treatments of phosphorus (P) and calcium (Ca) supply. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.

In terms of dry matter content (Fig. 1b), soil-indifferent *Hakea* and *Banksia* both showed an increase in DMC with increasing P supply (*P*<0.001; Fig. 1b). There was also a significant effect of Ca treatment on DMC of *Banksia* (*P*<0.001; Fig. 1b), with increasing Ca concentration leading to higher DMC, a trend that was not found in *Hakea* (*P*=0.178). The calcifuge species, in contrast, showed no difference in DMC with increasing P or Ca supply.
**Photosynthesis, stomatal conductance and intercellular CO₂ concentration**

The soil-indifferent *Hakea* showed faster photosynthetic rates with increasing P supply ($P<0.001$; Fig. 2a), except at 6 mM Ca. There was also a significant effect of the Ca treatment ($P<0.001$; Fig. 2a), with increasing Ca concentration negatively affecting rates of plants in the high-P treatment, but not under low-P condition. The soil-indifferent *Banksia*, similarly, showed faster photosynthetic rates with increasing P supply ($P<0.001$; Fig. 2a), regardless of Ca treatment. In these plants, however, the increasing Ca supply did not affect photosynthetic rates, with plants within each P treatment showing similar rates across Ca treatments (Fig. 2a). The calcifuge *Banksias* presented a similar pattern, with increased photosynthetic rates in response to higher P availability ($P=0.018$; Fig. 2a). However, the positive effect of P supply on photosynthetic rates was observed only in low-Ca conditions (0 and 0.1 mM). There was also a significant effect of the Ca treatment on photosynthesis ($P<0.001$), shown by the decrease in assimilation rates at very high Ca concentrations, distinctly in the high-P treatment. The calcifuge *Hakeas*, in contrast, showed no increase in photosynthetic rates with increasing P supply ($P<0.001$; Fig. 2a). In fact, these plants either showed similar (0 mM Ca) or decreased photosynthetic rates (0.1, 0.6 and 6 mM Ca) with increasing P. There was also a distinct difference between plants from contrasting P treatments in regards to their response to Ca supplies: in the low-P treatment, the photosynthetic rates only decreased at the highest Ca supply (6 mM); whilst under the high-P treatment, the photosynthetic rates decreased with even the smallest amount of Ca (0.1 mM), reaching extremely low levels as the Ca availability increased.
Fig. 2 Mean (± SE, n= 10-14) values of light-saturated rate of CO$_2$ assimilation ($A_{sat}$; (a)), stomatal conductance ($g_s$; (b)), and intercellular CO$_2$ concentration ($C_i$; (c)) for soil-indifferent and calcifuge species (Hakea and Banksia) across different treatments of phosphorus (P) and calcium (Ca) supply. All saturated photosynthetic measurements were performed at 2500 µmol photons m$^{-2}$ s$^{-1}$ and 400 µmol CO$_2$ mol$^{-1}$. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.
The soil-indifferent *Hakea* showed higher stomatal conductance with increased P supply (*P*<0.001; Fig. 2b), except in high-Ca treatments (0.6 and 6 mM). There was also a significant effect of the Ca treatment (*P*<0.001; Fig. 2b) on the conductance of these plants, with increasing Ca supply negatively affecting conductance rates of plants in the high-P treatment, but not in low-P condition. The soil-indifferent *Banksia*, likewise, showed higher stomatal conductance with increased P supply (*P*<0.001; Fig. 2b), except in the highest Ca treatment (6 mM). In these plants, the Ca treatment had no effect on stomatal conductance (*P*=0.085; Fig. 2b), with plants within each of the P treatments showing consistent stomatal conductances, regardless of Ca supply. In calcifuge *Hakeas* and *Banksias*, there were no differences in stomatal conductance with increasing P supply (Fig. 2b), with the exception of *Hakeas* growing at 0.6 mM Ca. However, there was a significant effect of Ca treatment on the conductance of these species (*P*<0.001; Fig. 2b), with increasing Ca supply reducing stomatal conductance under the high-P treatment, especially in *Hakeas*, but not in plants in low-P conditions.

In terms of leaf intercellular CO₂ concentration (Ci; Fig. 2c), soil-indifferent *Hakea* showed no difference in Ci with increasing P supply, except at the highest Ca treatment, in which Ci declined with the high P availability. In these plants, the Ca treatment did not affect Ci (*P*=0.900; Fig. 2c). In soil-indifferent *Banksia*, on the other hand, Ci decreased with increasing P concentration at the lower Ca treatments (0 and 0.1 mM) and, like in soil-indifferent *Hakea*, there was no significant effect of Ca treatment (*P*=0.227; Fig. 2c) on Ci. The calcifuge *Hakeas* showed no difference in Ci with increasing P supply, except at the highest Ca treatment (6 mM), when Ci increased with higher P availability. Unlike soil-indifferent species, there was a significant effect of the Ca treatment (*P*=0.021; Fig. 2c) on Ci, with this parameter steadily increasing with higher Ca supplies at the high P treatment, but not under low-P conditions. In calcifuge *Banksias*, there were no differences in Ci with increasing P or Ca concentrations.

*Chlorophyll fluorescence and dark-respiration rates*

The soil-indifferent *Hakea* showed higher Fv/Fm values with increasing P supply (*P*<0.001; Fig. 3), except in the no Ca treatment. There was a significant effect of Ca treatment (*P*<0.001; Fig.
3) on Fv/Fm of these plants, with increasing Ca supply reducing Fv/Fm at the low-P treatment, but not in plants growing under high-P conditions. In soil-indifferent *Banksia*, in contrast, the Fv/Fm did not change with increasing P or Ca supply, except in the 0.1 mM Ca treatment, in which Fv/Fm increased with high P supply. Similarly, calcifuge *Banksias* showed no differences in Fv/Fm with increasing P or Ca supplies (Fig. 3). In calcifuge *Hakeas*, Fv/Fm decreased with increasing P supply, except in the no Ca treatment. There was also a significant effect of the Ca treatment on Fv/Fm of these plants (*P*<0.001; Fig. 3), with increasing [Ca] reducing Fv/Fm of plants at the high-P treatment, a trend not found in low-P conditions.

There were virtually no differences in leaf dark respiration rates with increasing P and/or Ca supply (Fig. S1) for both soil-indifferent and calcifuge species. However, *Banksias* showed consistently slower rates than *Hakeas*.

![Graph showing Fv/Fm values](image)

**Fig. 3** Mean (± SE, *n= 10-14*) values of variable over maximal fluorescence (Fv/Fm) for soil-indifferent and calcifuge species (*Hakea* and *Banksia*) across different treatments of phosphorus (P) and calcium (Ca) supply. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.

**Photosynthesis and leaf calcium relationship**

In soil-indifferent *Hakea*, there was a negative correlation between light-saturated area-based photosynthetic rates and leaf [Ca], regardless of P supply (*P*=0.008, *R*²=0.15, β=-0.42 for plants under low-P conditions and *P*<0.001, *R*²=0.49, β=-0.71 for plants under high-P conditions; Fig. 4). This trend was also observed in calcifuge *Hakeas* (*P*=0.017, *R*²=0.13, β=--
0.39 for plants under low-P conditions and $P<0.001$, $R^2=0.50$, $\beta=-0.72$ for plants under high-P conditions; Fig. 4). However, soil-indifferent *Hakea* under high-P conditions showed consistently faster photosynthetic rates than those under low-P conditions, except when leaf [Ca] reached very high levels (≈30 mg g$^{-1}$). In contrast, calcifuge *Hakeas* at the high-P treatment either showed identical photosynthetic rates, under relatively low leaf [Ca], or slower rates than plants growing in low-P conditions, particularly when the leaf [Ca] exceeded 10 mg g$^{-1}$ (Fig. 4).

In soil-indifferent and calcifuge *Banksias*, there was no significant correlation between area-based photosynthetic rates and leaf [Ca], regardless of the P treatment ($P=0.100$ and $P=0.555$ for soil-indifferent *Banksias* in low- and high-P treatments, respectively; $P=0.063$ for calcifuge *Banksias* in both low- and high-P treatments; Fig. 4). However, in soil-indifferent *Banksia*, the photosynthetic rates were clearly faster in high-P conditions, a trend not observed in calcifuge *Banksias* (Fig. 4).

![Graph showing relationship between light-saturated rate of CO$_2$ assimilation ($A_{sat}$) and leaf calcium (Ca) concentration for soil-indifferent and calcifuge species (*Hakea* and *Banksia*) across different treatments of phosphorus (P) supply (0.1 µM, 10 µM). The significant relationships are expressed with corresponding $P$-values, $R^2$ and standardized $\beta$ values.]

**Fig. 4** Relationship between light-saturated rate of CO$_2$ assimilation ($A_{sat}$) and leaf calcium (Ca) concentration for soil-indifferent and calcifuge species (*Hakea* and *Banksia*) across different treatments of phosphorus (P) supply (0.1 µM, 10 µM). The significant relationships are expressed with corresponding $P$-values, $R^2$ and standardized $\beta$ values.
Leaf zinc and leaf phosphorus relationship

In soil-indifferent and calcifuge *Hakeas*, there was no significant correlation between leaf [Zn] and leaf [P], regardless of P supply ($P=0.112$ and $P=0.327$ for soil-indifferent *Hakea* in low- and high-P treatments, respectively; and $P=0.653$ and $P=0.233$ for calcifuge *Hakeas* in low- and high-P treatments, respectively; Fig. 5). In soil-indifferent and calcifuge *Banksias*, there was also no significant correlation between leaf [Zn] and leaf [P] for plants growing in low-P conditions ($P=0.094$ and $P=0.318$ for soil-indifferent and calcifuge species, respectively). Under high-P conditions, in contrast, there was a positive correlation between total leaf [Zn] and leaf [P] for both soil-indifferent and calcifuge *Banksias* ($P<0.001$, $R^2=0.44$, $\beta=0.67$ for soil indifferent *Hakeas*, and $P<0.001$, $R^2=0.36$, $\beta=0.61$ for calcifuge *Banksias*; Fig. 5).

**Fig. 5** Relationship between total leaf zinc (Zn) and phosphorus (P) concentrations for soil-indifferent and calcifuge species (*Hakea* and *Banksia*) across different treatments of P supply (0.1 μM, 10 μM). The significant relationships are expressed with corresponding $P$-values, $R^2$ and standardized $\beta$ values. Box-plots with medians, 25th, and 75th percentiles were included in the margins to outline the data distribution. Whiskers extend to 1.5 times the interquartile range. Data presented beyond whiskers represent outliers.
Leaf toxicity and/or deficiency symptoms

In *Hakea*, there was a distinct difference between soil-indifferent and calcifuge species in terms of their response to increasing P and Ca supply. The soil-indifferent *H. prostrata* showed slight leaf chlorosis with increased Ca supply under low-P conditions (0.1 μM P), a symptom aggravated at high-P conditions (10 μM P; Fig. 6). In treatments where no Ca was given to the plants, increased P supply alone did not visibly affect leaf health. The calcifuge species *H. flabellifolia* and *H. incrassata* also showed slight leaf chlorosis with increased Ca supply under low-P conditions (0.1 μM P, Fig. 6). However, under high-P conditions (10 μM P; Fig. 6), these species showed major chlorosis and extensive necrosis with increasing Ca supply.

In *Banksia*, the soil-indifferent and calcifuge species showed relatively similar responses to increasing P and/or Ca supply. The soil-indifferent *B. prionotes* showed slight leaf chlorosis and limited necrosis with increased Ca supply under both low- and high-P conditions (Fig. 6). The calcifuge species *B. menziesii* and *B. attenuata* also showed minor chlorosis with increased Ca supply under low- and high-P conditions (Fig. 6), but none of these plants showed necrosis, with the exception of *B. menziesii* under high-P/high-Ca conditions. In *Banksia*, increasing P supply within the same Ca treatments caused minor leaf chlorosis.
Fig. 6 Leaf toxicity and/or deficiency symptoms from two Proteaceae genera (*Hakea* and *Banksia*) growing under different phosphorus (P) and calcium (Ca) treatment combinations. The soil-indifferent species (*H. prostrata* and *B. prionotes*) naturally occur on both calcareous and acidic soils, while the calcifuge species (*H. flabellifolia*, *H. incassata*, *B. menziesii* and *B. attenuata*) occur exclusively on acidic soils. Scale bars are 1 cm.

**Discussion**

The studied Proteaceae species were affected in distinctly different ways by contrasting P and Ca supplies, with their responses dependent on genus and distribution type. Therefore, we will discuss each group separately.
Soil-indifferent Hakea

The soil-indifferent *Hakea* showed increased growth with increasing P supply, indicating that they experienced P limitation at 0.1 μM P. The photosynthetic rates and stomatal conductance of these plants also increased with additional P. Interestingly, the effects of P supply on photosynthesis and stomatal conductance were not entirely coupled: the positive effect of P on stomatal conductance was not observed at 0.6 and 6 mM Ca, whilst the rates of photosynthesis still increased with higher P supply at 0.6 mM Ca, suggesting that P and Ca, when interacting, affected these parameters differently. Limiting P supplies affect leaf expansion (Fredeen *et al.*, 1989) and the total number of leaves (Lynch *et al.*, 1991), reducing the sink demand for photosynthates (Dissanayaka *et al.*, 2018). They also affect leaf P concentration and decrease leaf P pools (Veneklaas *et al.*, 2012). This decrease in leaf P uptake is expected to negatively affect the plant’s photosynthetic activity, because the Calvin-Benson cycle requires P-rich metabolites (Dietz & Foyer, 1986; Ellsworth *et al.*, 2015; Foyer & Spencer, 1986; Giersch & Robinson, 1987). Orthophosphate is required for the export of triose-P from the chloroplast, as well as for ATP synthesis (Giersch & Robinson, 1987; Mächler & Nösberger, 1984). Phosphate limitation also restricts carbon assimilation and electron transport rates due to decreases in 3-phosphoglycerate (PGA) reduction and ribulose bisphosphate (RuBP) regeneration rates (Ellsworth *et al.*, 2015; Mächler & Nösberger, 1984; Giersch & Robinson, 1987; Brooks *et al.*, 1988). Increased plant growth, along with the alleviation of these constraints with additional P likely explains the positive effect of higher P supply on the photosynthetic rates of these plants. The relationship between leaf [P] and photosynthesis may also be key for understanding the effects of high Ca supply on the CO2-assimilation rates of these species.

Calcium is an essential plant nutrient that regulates several aspects of the photosynthetic process, particularly by modulating the activity of phosphatases and kinases in the Calvin cycle (Brand & Becker, 1984; Hochmal *et al.*, 2015). However, most studies that investigated the function of Ca in photosynthesis focused exclusively on its signalling role (Hochmal *et al.*, 2015; McAinsh & Pittman, 2009; Hirschi, 2004) and not on how it may interfere with other nutrients. Calcium and P, for example, are rarely co-localised in high concentrations (Conn & Gilliham, 2010; Hayes *et al.*, 2018; Guilherme Pereira *et al.*, 2018;
Chapter 2), presumably to avoid the deleterious precipitation of Ca-phosphates. Proteaceae (Hayes et al., 2018; Chapter 2) and other species from P-impoverished habitats (Guilherme Pereira et al., 2018) preferentially allocate P to mesophyll cells, and this might have implications for the way these nutrients interact in leaves. If the Ca concentration in the mesophyll becomes too high, it could interfere with the physiological availability of P, thus possibly leading to a chemical inhibition of photosynthesis. This might explain the decrease in Fv/Fm under low-P conditions and high Ca supplies, indicating a reduction in the efficiency of photosystem II (PSII; Krause & Weis, 1991; Maxwell & Johnson, 2000). This was not significantly reflected in photosynthetic rates, but $A_{sat}$ and $g_s$ did tend to decrease with extremely high Ca supplies.

The photosynthetic rates of plants growing in high-P conditions steadily decreased with increasing Ca supply. This might be partially explained by the negative interaction between P and Ca, causing immobilisation of both nutrients. However, immobilisation does not explain the slower photosynthetic rates at 0.1 to 0.6 mM Ca. Under “physiological” levels, the cytosol [Ca] is tightly controlled, rarely exceeding the nanomolar range (McLaughlin & Wimmer, 1999; White & Broadley, 2003). This is actively maintained to allow signalling events to occur through oscillations in cytosolic [Ca] (Hirschi, 2004; McAinsh & Pittman, 2009). In contrast, the Ca concentration in the chloroplasts may reach very high levels (15 mM; Kreimer et al., 1988), but virtually all of it is bound to thylakoids, so there is little free Ca to interact with P. How then could variation in the Ca supply affect the carbon assimilation rates? It has been suggested that increasing Ca supplies can significantly affect the uptake of other elements, either increasing their absorption, as happens with P (Bell et al., 1989; Robson et al., 1970), or decreasing it, which seems to be the case with micronutrients such as Zn (Chaudhry & Loneragan, 1972), iron (Fe; Taper & Leach, 1957) and manganese (Mn; Robson & Loneragan, 1970). Even though there was no significant increase in total leaf [P] with increasing Ca supplies, the [P] in particular cell-types did increase (P. E. Hayes et al., unpublished; Chapter 5). Increasing the Ca supply and consequently the leaf [Ca] may induce a displacement in the leaf P towards mesophyll cells (P. E. Hayes et al., unpublished; Chapter 5), and therefore affect photosynthesis. High cellular [P] might interfere with leaf Zn physiological availability (Boawn & Leggett, 1964; Cakmak & Marschner, 1987; Loneragan et al., 1979; Loneragan & Webb, 1993), leading to P-enhanced Zn requirement (Bhatti &
Loneragan, 1970; Cakmak & Marschner, 1987). If the plants are unable to up-regulate their Zn uptake, or if the Zn supply is inadequate, the availability of this micronutrient may limit photosynthesis (Wang & Jin, 2005; Ohki, 1976; Randall & Bouma, 1973).

In summary, soil-indifferent *Hakea* showed P-limitation at 0.1 μM P. Their growth increased at high P supply, but this positive effect gradually declined with increasing Ca supply. We surmise that this happened for two reasons: first, under 0 to 0.6 mM Ca, the increasing Ca supply likely induced a shift in leaf P towards mesophyll cells, even though there was no difference in the total leaf [P] (P. E. Hayes *et al.*, unpublished; Chapter 5). This increase in mesophyll [P] led to P-enhanced Zn requirement and micronutrient limitation, reducing photosynthetic rates. This explains why the steady decrease in assimilation rates was only observed at high P supply, when leaf P was high enough to interact and reduce the Zn availability. Second, extreme Ca conditions may have increased the leaf [Ca] to such a degree that it led to the formation of Ca-phosphates. In low-P conditions, this interference between Ca and P likely explains the reduction in Fv/Fm under extremely high Ca supplies.

*Calcifuge Hakeas*

The calcifuge *Hakeas* showed similar (0 and 0.1 mM Ca) or decreased growth (0.6 and 6 mM Ca) with additional P supply, and although this does not rule out P limitation at 0.1 μM P, it suggests that calcifuge *Hakeas* are much more sensitive to high P supplies than soil-indifferent ones. Most physiological parameters were affected in a similar way: stomatal conductance was either the same (0 and 0.1 mM Ca) or less (0.6 and 6 mM Ca) under high-P conditions, whilst the photosynthetic rates decreased with additional P in all treatments in which Ca was available. The effects of [P] on photosynthesis and stomatal conductance were not entirely coupled, indicating that these nutrients affected these parameters differently.

Some Proteaceae are extremely sensitive to high P availability (Ozanne & Specht, 1981; Parks *et al.*, 2000; Lambers *et al.*, 2002; Shane *et al.*, 2004a), because they are unable to strongly down-regulate their P-uptake (Shane *et al.*, 2004a, b, 2008). Not surprisingly, calcifuge *Hakeas*, which typically do not occur in P-rich habitats, showed consistently higher total leaf [P] than soil-indifferent species under the same P supply, suggesting that P toxicity might help explain these species’ distribution. However, P toxicity cannot be entirely
explained by the high P availability. In fact, when Ca was withheld from these plants, increasing P supply had no negative effect on plant growth, photosynthesis or stomatal conductance. Similarly, increasing Ca supply had no effect on growth or physiology under low-P conditions. This suggests a strong interaction between Ca and P, resulting in the observed P toxicity.

The phenomenon of Ca-enhanced P toxicity has been described before (Grundon, 1972; Nichols & Beardsell, 1981), but the underlying mechanism remains uncertain. It has been suggested that increasing Ca supply might reduce plant growth (Grundon, 1972) or stimulate P uptake (Robson et al., 1970; Nichols & Beardsell, 1981). In both cases, it would be the resulting increase in total leaf [P] that intensifies the P-toxicity symptoms. However, these explanations are inconsistent with what we observed for the calcifuge Hakeas. Whilst plant growth was affected by increasing Ca supply at high P conditions, there was no difference in total leaf [P] (P.E. Hayes et al., unpublished; Chapter 3), which means that Ca-enhanced P toxicity symptoms were not ultimately caused by Ca-related increases in leaf [P].

It has recently been suggested that Ca-enhanced P toxicity might be related to leaf nutrient-allocation patterns at the cellular level (P.E. Hayes et al., unpublished; Chapter 3), particularly due to the negative interaction between these elements when co-localised at high concentrations. If there is a displacement in the leaf P towards photosynthetically-active cells with increasing Ca supply (P. E. Hayes et al., unpublished; Chapter 5), P-toxicity symptoms could develop without changes in total leaf [P]. Therefore, species with high leaf [P] should be more affected, and this is precisely the case for calcifuge Hakeas, which exhibited major symptoms of P toxicity (leaf chlorosis and necrosis) with increasing Ca. Leaf chlorosis, which has been described as a typical symptom of P toxicity and micronutrient deficiency (Robson & Pitman, 1983; Lambers et al., 2002; Shane et al., 2004a), likely occurs due to P interfering with Zn availability in the mesophyll. This is aggravated at high P supply, yet the extent of leaf necrosis under these conditions suggests something in addition to Zn deficiency. We surmise that, because of their high leaf [P], calcifuge Hakeas are more susceptible to the deleterious precipitation of Ca-phosphates. This may lead to cellular damage, which, in turn, explains the extensive necrosis with the increasing Ca supply at high-P conditions. This is consistent with the steady reduction in Fv/Fm values, thus lower PSII efficiency (Krause & Weis, 1991;
Maxwell & Johnson, 2000), and with the C\textsubscript{i} increase, which points towards reduced fixation of CO\textsubscript{2}.

In summary, calcifuge *Hakea* showed no positive responses to increasing [P], and their growth, photosynthesis and stomatal conductance were either the same or lower at high P supply. This response strongly depended on the Ca supply, suggesting a strong interaction between these elements. Calcium-enhanced P toxicity likely occurred due to the Ca-induced increase in mesophyll [P], leading to a P-enhanced Zn requirement that explains the leaf chlorosis. In addition, under high-P conditions, high [P] likely led to the precipitation of Ca-phosphates, causing cellular damage and necrosis, as was widespread in these species.

**Soil-indifferent Banksia**

The soil-indifferent *Banksia* showed increased growth with increasing P supply, indicating P limitation at 0.1 \mu M P. The photosynthetic rates also increased with the additional P, and, with the exception of plants growing under high Ca supply, this was tightly coupled with an increase in stomatal conductance. Interestingly, the high Ca supply had little to no effect on either growth or physiology. The overall positive responses on plant growth with additional P disappeared at 6 mM Ca, a pattern also seen in stomatal conductance. In contrast, the photosynthetic rates were unaffected by Ca supply. Whilst this might suggest that Ca-enhanced P toxicity is not a major issue for soil-indifferent *Banksia*, there is a significant aspect that needs to be further considered.

*Banksias* show a general separation of P and Ca in different leaf cell types, with P being preferentially allocated to the palisade mesophyll, whilst Ca is usually stored in spongy-mesophyll and/or hypodermal layers (Hayes *et al.*, 2018; Chapter 2). This allocation pattern is linked with high photosynthetic P-use efficiency (PPUE; Hayes *et al.*, 2018; Chapter 2) under P-limiting conditions, but it also poses some difficulties when leaf [P] is too high. Because most P is allocated to metabolically-active cells, increased leaf [P] might lead to more severe P-toxicity symptoms, particularly when high Ca supplies can increase palisade mesophyll [P] (P. E. Hayes *et al.*, unpublished; Chapter 5). Under these circumstances, why were soil-indifferent *Banksia* not affected by increasing Ca supply? The answer may lie in their greater capacity to take up Zn. Species with high Zn-uptake capacity would be able to compensate for
the P-enhanced Zn requirement. This may explain the high leaf [Zn] and the significant correlation between [P] and [Zn] under high-P conditions. This is also consistent with the overall lack of P-toxicity and micronutrient-deficiency symptoms, as well as the steady Ci and Fv/Fm values.

In summary, soil-indifferent Banksia showed increased growth, photosynthesis and stomatal conductance at 10 μM P. These positive responses were largely independent of the Ca supply: the only exception was at 6 mM Ca, when growth and conductance were not affected by the additional P. This might indicate that Ca-enhanced P toxicity is not a major issue for these species, and we surmise that this is due to their greater capacity to take up Zn. Because of their high leaf [Zn], these species tolerate higher leaf [P], even when Ca enhances P toxicity.

*Calcifuge Banksias*

Calcifuge Banksias grown under 0 and 0.1 mM Ca showed increased growth with increasing P supply. In contrast, those grown at 0.6 and 6 mM Ca were unaffected by the P concentration. The photosynthetic rates followed the same trend, but, interestingly, the stomatal conductance rate was unaffected by P supply, regardless of Ca treatment. This suggests that 0.1 μM P supply limited these plants' growth, but, unlike soil-indifferent Banksia, the response to high P supply strongly depended on the Ca supply.

The calcifuge Banksias showed lower leaf [Zn] than the soil-indifferent ones, likely due to a low Zn-uptake capacity and/or a lower leaf Zn allocation to leaves (P.E. Hayes *et al.*, unpublished; Chapter 3). Therefore, calcifuge Banksias were more vulnerable to Zn deficiency. The relatively low leaf [P] in these species might compensate for this, but at extremely high P supply, the leaf [P] will invariably reach high enough levels to interfere with Zn availability. Under these conditions, the Zn-uptake capacity and leaf Zn allocation will ultimately determine these plants' physiological responses to high [P]. Increasing Ca supply may also have a significant effect on this interaction, particularly by the shift of P towards photosynthetically-active cells in which Zn is required for carbonic anhydrase (Ohki, 1976; Randall & Bouma, 1973).
In summary, calcifuge Banksias showed increased growth and photosynthesis with increasing P supply, but, unlike the soil-indifferent species, this response strongly depended on Ca concentrations. We surmise that this apparently higher sensitivity in calcifuge Banksia species to Ca-enhanced P toxicity is a consequence of their lower leaf [Zn].

Concluding remarks

We observed Ca-enhanced P toxicity in all Proteaceae, but with considerable differences between calcifuge and soil-indifferent species in their physiological responses to increasing P and/or Ca supplies. Calcifuges were far more sensitive to P toxicity than soil-indifferent ones. In Hakea, this difference was attributed to the calcifuge species' limited ability to down-regulate their P-uptake which led to higher leaf [P]. If the leaf [P] becomes too high, it might interfere with Zn availability, leading to micronutrient deficiency and leaf chlorosis. In extreme cases, the interaction between P and Ca might also lead to precipitation of Ca-phosphates, causing cellular damage and leaf necrosis. In Banksias, on the other hand, there were no differences in total leaf [P] between calcifuge and soil-indifferent species. The leaf [Zn], however, differed significantly between these groups. Calcifuge species showed lower leaf [Zn] than soil-indifferent ones, likely due to lower Zn-uptake capacities and/or lower Zn allocation to leaves. Consequently, these species were unable to compensate for the P-enhanced Zn requirement, particularly when high Ca supplies enhanced P toxicity. This is why calcifuge Banksias showed reduced growth and photosynthesis with increasing P supply at high Ca supply, whilst this was not the case for soil-indifferent species. This might also explain differences in the severity of P-toxicity and Zn-deficiency symptoms between calcifuge and soil-indifferent Banksia.

This is the first study to evaluate, in depth, the physiology of Ca-enhanced P toxicity in a range of Proteaceae. We propose that the differences between calcifuge and soil-indifferent species in the way they respond to increasing P and Ca supplies is associated with their ability to regulate nutrient uptake, particularly that of P and Zn. These results not only improve our basic understanding of how these species function at different soil conditions, but also suggest that these species' distribution is strongly determined by Ca-enhanced P toxicity. This
knowledge will help in restoration efforts involving Proteaceae, as well as advance our fundamental understanding of plant mineral nutrition.

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References


Chapter 4 | Supporting Information

Fig. S1 Mean values of leaf dark respiration for soil-indifferent and calcifuge *Hakea* and *Banksia* species across different treatments of phosphorus and calcium supply.

Fig. S2 Mean values of plant growth and dry matter content for individual soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia, H. incrassata, B. menziesii and B. attenuata*) species across different treatments of phosphorus and calcium supply.

Fig. S3 Mean values of CO₂ assimilation rates, stomatal conductance and intercellular CO₂ concentration for individual soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia, H. incrassata, B. menziesii and B. attenuata*) species across different treatments of phosphorus and calcium supply.

Fig. S4 Mean values of variable over maximal fluorescence for individual soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia, H. incrassata, B. menziesii and B. attenuata*) species across different treatments of phosphorus and calcium supply.

Fig. S5 Relationship between CO₂ assimilation rates and leaf calcium concentration for individual soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia, H. incrassata, B. menziesii and B. attenuata*) species across different treatments of phosphorus supply.

Fig. S6 Relationship between leaf zinc and total leaf phosphorus concentrations for individual soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia, H. incrassata, B. menziesii and B. attenuata*) species across different treatments of phosphorus supply.

Table S1 Nutrient concentration used in the different hydroponics nutrient solutions.

Table S2 Generalised least square models for the plant growth of different groups analysed in the study.

Table S3 Generalised least square models for the dry matter content of different groups analysed in the study.

Table S4 Generalised least square models for the photosynthetic rates of different groups analysed in the study.

Table S5 Generalised least square models for the stomatal conductance of different groups analysed in the study.

Table S6 Generalised least square models for the internal CO₂ concentration of different groups analysed in the study.

Table S7 Generalised least square models for the variable over maximal fluorescence of different groups analysed in the study.

Table S8 Generalised least square models for the leaf dark respiration of different groups analysed in the study.
**Fig. S1** Mean (± SE, n= 10-14) values of leaf dark respiration (R_{dark}) for soil-indifferent and calcifuge *Hakea* and *Banksia* species across different treatments of phosphorus (P) and calcium (Ca) supply. All leaf dark respiration measurements were carried out at 400 µmol CO₂ mol⁻¹ air. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.
Fig. S2 Mean (± SE, n= 4-20) values of plant growth (as mass increase; (a)) and dry matter content (b) for soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia*, *H. incrassata*, *B. menziesii* and *B. attenuata*) species across different treatments of phosphorus (P) and calcium (Ca) supply. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.
Fig. S3 Mean (± SE, n= 4-12) values of light-saturated rate of CO₂ assimilation (A_{sat}, (a)), stomatal conductance (g_{s}, (b)) and intercellular CO₂ concentration (C_{i}, (c)) for soil-indifferent (Hakea prostrata and Banksia prionotes) and calcifuge (H. flabelifolia, H. incassata, B. menziesii and B. attenuata) species across different treatments of phosphorus (P) and calcium (Ca) supply. All photosynthetic measurements were carried out at 2500 μmol photons m⁻² s⁻¹ / 400 μmol CO₂ mol⁻¹ air. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.
Fig. S4 Mean (± SE, n= 4-12) values of variable over maximal fluorescence (Fv/Fm) for soil-indifferent (Hakea prostrata and Banksia prionotes) and calcifuge (H. flabelifolia, H. incrassata, B. menziesii and B. attenuata) species across different treatments of phosphorus (P) and calcium (Ca) supply. Different letters indicate significant differences among treatments within each panel, based on Tukey’s HSD test.
Fig. S5 Relationship between light-saturated rate of CO₂ assimilation ($A_{sat}$) and leaf calcium concentration (Ca) for soil-indifferent ($Hakea prostrata$ and $Bankia prionotes$) and calcifuge ($H. flabellifolia$, $H. incrassata$, $B. menziesii$ and $B. attenuata$) species across different treatments of phosphorus (P) supply (0.1 μM; 10 μM). The significant relationships are expressed with corresponding P-values, $R^2$ and standardized β values.
Fig. S6 Relationship between total leaf zinc (Zn) and leaf phosphorus (P) concentrations for soil-indifferent (*Hakea prostrata* and *Banksia prionotes*) and calcifuge (*H. flabellifolia*, *H. incrassata*, *B. menziesii* and *B. attenuata*) species across different treatments of P supply (0.1 µM; 10 µM). The significant relationships are expressed with corresponding *P*-values, $R^2$ and standardized $\beta$ values.
Table S1 Nutrient concentration used in the different hydroponics nutrient solutions. ‘Growth’ (25% growth and 100% growth) solutions were used during both growth and acclimation periods, whilst ‘basal’ solutions (25% basal, 33% basal and 50% growth) were used during the treatment periods. Nutrient solution recipes were based on Shane et al. (2004).

<table>
<thead>
<tr>
<th>Nutrient Solution</th>
<th>Treatments (μM)</th>
<th>Nutrient Concentration (μM)</th>
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</thead>
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<tr>
<td></td>
<td>PO₄²⁻</td>
<td>Ca²⁺</td>
</tr>
<tr>
<td>25% growth</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>100% growth</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>25% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
</tr>
<tr>
<td>33% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
</tr>
<tr>
<td>50% basal</td>
<td>0.1, 10</td>
<td>0, 100, 600, 6000</td>
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Table S2 Generalised least square (GLS) models for the plant growth (Fig. 1a) of different groups analysed in the study. Model identification and rationale, number of parameters (K), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc (∆AICc).

<table>
<thead>
<tr>
<th>Group</th>
<th>Model ID</th>
<th>Model</th>
<th>K</th>
<th>Log L</th>
<th>AICc</th>
<th>∆AICc</th>
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</thead>
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<td>Hakea (soil-indifferent)</td>
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Table S3 Generalised least square (GLS) models for the dry matter content (DMC; Fig. 1b) of different groups analysed in the study. Model identification and rationale, number of parameters (K), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc (Δ AICc).

<table>
<thead>
<tr>
<th>Group</th>
<th>Model ID</th>
<th>Model</th>
<th>K</th>
<th>Log L</th>
<th>AICc</th>
<th>Δ AICc</th>
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### Table S4 Generalised least square (GLS) models for the light-saturated photosynthetic rates ($A_{sat}$; Fig. 2a) of different groups analysed in the study. Model identification and rationale, number of parameters ($K$), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc ($\Delta$ AICc).

<table>
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<th>Group</th>
<th>Model ID</th>
<th>Model</th>
<th>$K$</th>
<th>Log L</th>
<th>AICc</th>
<th>$\Delta$ AICc</th>
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<tr>
<td><strong>Hakea</strong></td>
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<td>597.31</td>
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<tr>
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<td>614.67</td>
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<td>654.51</td>
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Table S5 Generalised least square (GLS) models for the stomatal conductance ($g_t$; Fig. 2b) of different groups analysed in the study. Model identification and rationale, number of parameters ($K$), log likelihood ($\log L$), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc ($\Delta$ AICc).

<table>
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<th>$\log L$</th>
<th>AICc</th>
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<tr>
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<td></td>
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<td>116.64</td>
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<td>mm1</td>
<td>$g_t \sim P$ treatment * Ca treatment</td>
<td>9</td>
<td>-7.73</td>
<td>35.54</td>
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<tr>
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<td>mm2</td>
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<td></td>
<td>mm3</td>
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Table S6 Generalised least square (GLS) models for the internal CO₂ concentration in leaves (Cᵢ; Fig. 2c) of different groups analysed in the study. Model identification and rationale, number of parameters (K), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc (Δ AICc).

<table>
<thead>
<tr>
<th>Data</th>
<th>Model ID</th>
<th>Model</th>
<th>K</th>
<th>Log L</th>
<th>AICc</th>
<th>Δ AICc</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>mm3</td>
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<td>mm1</td>
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<td>-417.15</td>
<td>854.47</td>
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Table S7 Generalised least square (GLS) models for the variable over maximal fluorescence (Fv/Fm; Fig. 3) of the different groups analysed in the study. Model identification and rationale, number of parameters (K), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc (Δ AICc).

<table>
<thead>
<tr>
<th>Data</th>
<th>Model ID</th>
<th>Model</th>
<th>K</th>
<th>Log L</th>
<th>AICc</th>
<th>Δ AICc</th>
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<tr>
<td></td>
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<td>Fv/Fm ~ P treatment * Ca treatment</td>
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<td>mm4</td>
<td>Fv/Fm ~ P treatment</td>
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<td>mm1</td>
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<td>-239.51</td>
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Table S8 Generalised least square (GLS) models for the leaf dark respiration ($R_{\text{dark}}$, Fig. S1) of different groups analysed in the study. Model identification and rationale, number of parameters ($K$), log likelihood (log L), Akaike’s Information Criterion (AICc) and difference between selected and null model’s AICc ($\Delta$ AICc).

<table>
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<tr>
<th>Data</th>
<th>Model ID</th>
<th>Model</th>
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<th>Log L</th>
<th>AICc</th>
<th>$\Delta$ AICc</th>
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<td></td>
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<td>$R_{\text{dark}} \sim \text{P treatment + Ca treatment}$</td>
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<td>-37.31</td>
<td>80.89</td>
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<td>mm3</td>
<td>$R_{\text{dark}} \sim \text{Ca treatment}$</td>
<td>2</td>
<td>-39.32</td>
<td>82.77</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>mm4</td>
<td>$R_{\text{dark}} \sim \text{P treatment}$</td>
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<td>-38.73</td>
<td>88.12</td>
<td>5.35</td>
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<tr>
<td></td>
<td>null</td>
<td>$R_{\text{dark}} \sim \text{1}$</td>
<td>2</td>
<td>-39.32</td>
<td>82.77</td>
<td>-</td>
</tr>
</tbody>
</table>

| Hakea (soil-indifferent) | mm1      | $R_{\text{dark}} \sim \text{P treatment + Ca treatment}$ | 6   | -60.92 | 134.87 | 2.15          |
| Hakea (calcifuge)       | mm2      | $R_{\text{dark}} \sim \text{P treatment}$               | 5   | -61.07 | 132.88 | 0.16          |
|                        | mm3      | $R_{\text{dark}} \sim \text{Ca treatment}$              | 2   | -64.29 | 132.72 | -             |
|                        | mm4      | $R_{\text{dark}} \sim \text{P treatment}$               | 3   | -64.20 | 134.69 | 1.97          |
|                        | null     | $R_{\text{dark}} \sim \text{1}$                           | 2   | -64.29 | 132.72 | -             |

| Banksia (soil-indifferent) | mm1      | $R_{\text{dark}} \sim \text{P treatment + Ca treatment}$ | 6   | -26.90 | 66.79  | -6.25         |
| Banksia (calcifuge)       | mm2      | $R_{\text{dark}} \sim \text{P treatment}$               | 5   | -28.93 | 64.14  | -8.9          |
|                        | mm3      | $R_{\text{dark}} \sim \text{Ca treatment}$              | 3   | -32.43 | 75.56  | 2.52          |
|                        | mm4      | $R_{\text{dark}} \sim \text{P treatment}$               | 2   | -34.45 | 73.04  | -             |
|                        | null     | $R_{\text{dark}} \sim \text{1}$                           | 2   | -34.45 | 73.04  | -             |

| Banksia (calcifuge)       | mm1      | $R_{\text{dark}} \sim \text{P treatment + Ca treatment}$ | 6   | -50.21 | 113.36 | 5.94          |
| Banksia (calcifuge)       | mm2      | $R_{\text{dark}} \sim \text{P treatment}$               | 5   | -50.68 | 112.02 | 4.6           |
|                        | mm3      | $R_{\text{dark}} \sim \text{P treatment}$               | 3   | -51.18 | 108.63 | 1.21          |
|                        | mm4      | $R_{\text{dark}} \sim \text{P treatment}$               | 2   | -51.65 | 107.42 | -             |
Chapter 5

Calcium modulates cell-specific allocation of phosphorus in leaves of Proteaceae

Abstract

Over 650 Proteaceae species occur in south-western Australia, contributing to the region’s exceptionally high biodiversity. Most Proteaceae occur exclusively on severely nutrient-impoverished, acidic soils (calcifuge), whilst few also occur on young, calcareous soils (soil-indifferent), higher in available calcium (Ca) and phosphorus (P). The calcifuge habit of most Proteaceae is explained by Ca-enhanced P toxicity and is likely linked to the leaf cell-specific allocation of Ca and P. Cellular Ca and P must be separated to avoid the deleterious precipitation of Ca-phosphate. We used quantitative X-ray microanalysis to determine leaf cell-specific nutrient concentrations of two calcifuge and two soil-indifferent Proteaceae grown in hydroponics at a range of Ca and P concentrations. Calcium enhanced the relative distribution of P to palisade mesophyll (PM), resulting in a greater PM [P] in calcifuges, despite no change in whole leaf [P]. Calcifuges showed a greater PM [P] compared with soil-indifferent ones, corresponding with their increased sensitivity to Ca-enhanced P toxicity. This study advances our mechanistic understanding of Ca-enhanced P toxicity, supporting our proposed model, and demonstrating its role in limiting Proteaceae distribution. This research furthers our fundamental understanding of plant mineral nutrition and highlights the importance of considering nutrient interactions at the cellular level.
Introduction

Different nutrients within leaves tend to be allocated to specific cell-types (Karley et al., 2000b; Conn and Gilliham, 2010). This process of cell-specific nutrient allocation must be tightly regulated, as particular elements can negatively interact with each other. For example, calcium (Ca) and phosphorus (P) must be allocated to separate cell types in order to avoid the deleterious precipitation of Ca-phosphates, which reduces the availability of both nutrients and severely impacts cellular processes (McLaughlin and Wimmer, 1999; White and Broadley, 2003; Conn and Gilliham, 2010).

The separate cell types in which Ca and P are allocated varies among different groups of plants (Leigh and Tomos, 1993; Karley et al., 2000b; Conn and Gilliham, 2010; Hayes et al., 2018; Chapter 2). Most eudicots studied tend to allocate P to epidermis and bundle sheath cells, and Ca to mesophyll cells (Outlaw Jr. et al., 1984; Treeby et al., 1987; Conn and Gilliham, 2010). In contrast, eudicots from severely P-impoverished habitats (including Proteaceae; Shane et al., 2004a; Hawkins et al., 2008; Hayes et al., 2018; Guilherme Pereira et al. 2018; Chapter 2) and monocots (Boursier and Läuchli, 1989; Dietz et al., 1992; Leigh and Storey, 1993; Williams et al., 1993; Karley et al., 2000a) tend to allocate P to mesophyll cells and Ca to non-mesophyll cells. Nevertheless, the cell-specific allocation of Ca and P has been studied under a relatively narrow range of conditions. Consequently, we still know very little of how much Ca and P allocation can change within individual species and the impact this may have on their physiology.

The ability of eudicots from severely P-impoverished habitats to preferentially allocate P to mesophyll cells and Ca to other cell types represents an important adaptation to increase P-use efficiency (Hayes et al., 2018; Guilherme Pereira et al. 2018; Chapter 2). By preferentially allocating P to where it is needed in the greatest amount - photosynthetic cells - these species are able to use P more efficiently (Stitt et al., 2010; Tsuji et al., 2017). However, this allocation pattern is also linked to P toxicity in P-sensitive Proteaceae from south-western Australia (Shane et al., 2004a). The reduced ability of these species to down-regulate P uptake, coupled with their preferential allocation of P to mesophyll cells increases mesophyll [P], leading to P-toxicity symptoms, even under relatively low P supply. Furthermore, Ca increases the severity of these P-toxicity symptoms, likely related to their
cell-specific allocation of Ca and P (Grundon, 1972; Nichols and Beardsell, 1981; Hayes et al. unpublished; Guilherme Pereira et al. unpublished; Chapters 3, 4).

Proteaceae are mainly found across the southern hemisphere and most abundantly in severely P-impoverished regions, such as south-western Australia (Weston, 2007), where over 650 species occur. They represent an iconic component of the vegetation, contributing to the region’s exceptionally high biodiversity (Cowling and Lamont, 1998; George, 1998; Myers et al., 2000; Hopper and Gioia, 2004). Very few Proteaceae occur on young (<7,000 yr) calcareous soils in south-western Australia (Hayes et al., 2014; Zemunik et al., 2015). Instead, most occur on older, nutrient-impoverished acidic soils and are calcifuge (Hayes et al., 2014; Zemunik et al., 2015). The few Proteaceae able to grow on young calcareous soils also occur on older acidic soils and are soil-indifferent. A well-documented example of this distribution is along the Jurien Bay dune chronosequence in south-western Australia (Hayes et al., 2014; Zemunik et al., 2015). The calcareous soils of this chronosequence exhibit relatively high total and plant available [P] compared with acidic soils, although this soil [P] is unlikely high enough to exclude P-sensitive Proteaceae (Shane and Lambers, 2005; Turner and Laliberté, 2015; Turner et al., 2018). These calcareous soils are also very high in [Ca] and have a low available [micronutrient] (Turner and Laliberté, 2015).

Previous research has indicated that Ca-enhanced P toxicity, alongside low soil micronutrient availability can explain the exclusion of most Proteaceae from these calcareous soils (Hayes et al. unpublished; Guilherme Pereira et al. unpublished; Chapters 3, 4). Increasing Ca supply enhances sensitivity to P and the severity of P-toxicity symptoms in P-sensitive Proteaceae (Grundon, 1972; Nichols and Beardsell, 1981; Hayes et al. unpublished; Guilherme Pereira et al. unpublished; Chapters 3, 4). Calcium-enhanced P toxicity is thought to result from interactions between cell-specific Ca and P. Under high-P conditions, increasing Ca supply is thought to increase PM [P], leading to P-toxicity. An increase in PM [P] with increasing Ca supply accounts for Ca-enhanced P-toxicity symptoms and the lack of an increase in whole leaf [P] with increasing Ca supply (Hayes et al. unpublished; Chapter 3). However, the mechanism by which this operates remains largely unknown. The main visual symptoms of P toxicity are leaf chlorosis and necrosis (Grundon, 1972; Groves and Keraitis, 1976; Nichols and Beardsell, 1981; Webb and Loneragan, 1990; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008). Leaf chlorosis likely results from higher PM [P] reducing
physiological availability of Zn and other micronutrients, whilst leaf necrosis may result from precipitation of Ca-phosphate, where separation is not maintained. The overall aim of this research was to explore evidence to support this proposed mechanism for Ca-enhanced P toxicity.

We used quantitative X-ray elemental microanalysis to determine leaf cell-specific nutrient concentrations of four Proteaceae grown in hydroponics at a range of Ca and P concentrations. This included two calcifuge and two soil-indifferent *Banksia* and *Hakea* species. Our aims were to: 1) study the effects of Ca and P supply on the cell-specific allocation of these nutrients across different Proteaceae species; and 2) compare responses in cell-specific nutrient allocation between calcifuge and soil-indifferent species and their link to Ca-enhanced P toxicity and species distribution.

First, we hypothesised that increasing Ca supply would alter the cell-specific allocation of P, that it would enhance the relative distribution of [P] to PM, and that this would be most evident under high P supply. Second, we hypothesised that calcifuge Proteaceae would show a greater response to increased Ca supply than soil-indifferent ones, resulting in a greater PM [P] in calcifuges, particularly under high-P conditions. Finally, a comparison of calcifuge and soil-indifferent Proteaceae would allow us to understand the strategies by which soil-indifferent species tolerate increased Ca and P supplies, providing a mechanistic understanding of Ca-enhanced P toxicity and its role in the calcifuge habit of most Proteaceae.

**Materials and Methods**

**Species selection**

We selected four Proteaceae species, from two genera, *Banksia* and *Hakea*, which occur along the Jurien Bay dune chronosequence in south-western Australia, approximately 200 km north of Perth (Laliberté *et al.*, 2012; Hayes *et al.*, 2014; Zemunik *et al.*, 2015). We selected two calcifuge (*Hakea incrassata* R.Br., and *Banksia menziesii* R.Br.) and two soil-indifferent species (*Hakea prostrata* R.Br., and *Banksia prionotes* Lindl.). Species distributions were identified through published information (Hayes *et al.*, 2014; Zemunik *et al.*, 2015).
Glasshouse hydroponic experiment

Plants were grown as part of a larger experiment investigating Ca-enhanced P toxicity in Proteaceae (Hayes et al. unpublished; Chapter 3 for full details). Plants were grown from seed and transferred to hydroponics. Hakeas were grown between February and September 2014, and Banksias between June 2014 and March 2015. Seeds were sterilised and germinated on moist filter paper (15°C, 12 h : 12 h, light : dark), before being transferred to trays containing 3 l of continuously-aerated nutrient solution. The strength of this nutrient solution (pH 5.8) was increased as plants became larger, from ‘25% growth’ to ‘100% growth’ (Table S1 for concentrations). The solution was replenished once to three times per week. After ten (Banksia) or six (Hakea) weeks, seedlings were transferred to 4.5 l pots and placed in a glasshouse.

Each 4.5 l pot contained two plants and 4 l of continuously-aerated nutrient solution, maintained at 18°C in a root-cooling tank. During acclimation in the glasshouse, the strength of the nutrient solution was increased from ‘25% growth’ to ‘50% growth’ for Banksias and kept at ‘25% growth’ for Hakeas. During growth and acclimation, P and Ca were supplied at 0.1 μM and 10 μM, respectively.

After six (Banksia) or eight (Hakea) weeks of acclimation in the glasshouse, 64 (Banksia) or 80 (Hakea) plants of uniform size were selected for each species and exposed to four treatments. During the treatment period, plants were supplied with ‘50% basal’ (Banksia) or ‘25% to 33% basal’ (Hakea) nutrient solution (Table S1 for concentrations) and were supplemented with different P (0.1, 10 μM; supplied as KH₂PO₄) and Ca (0, 600 μM; supplied as CaCl₂) concentrations. All nutrient solutions were replenished three times per week. Despite no additional Ca being added under the 0 μM Ca treatment, there would be a very low background level of Ca in the deionised water. Plants were grown under treatment in a temperature-controlled glasshouse; at a mean temperature, of 21°C (Banksia) and 17°C (Hakea). After 11-weeks of treatment, three representative plants were selected for each species/treatment combination and sampled for cell-specific element analysis.
Field collection

Samples for cell-specific element analysis were collected in November 2014, from three sites of contrasting soil type (two acidic, one calcareous) along the Jurien Bay chronosequence (Table S2 for full soil details). Collections from acidic sites were part of a larger study (Hayes et al. 2018; Chapter 2 for full details). Calcareous sites and their comparison with acidic sites has not been previously published.

Two sites were selected on the severely P-impoverished acidic soils of the oldest Bassendean stage (~2 million years), and one site on the much younger, relatively P-richer, calcareous soils of the old Quindalup stage (~6,500 years; Hayes et al., 2014; Turner and Laliberté, 2015; Turner et al., 2018). All samples were collected within <100 m, at each site. Calcifuge and soil-indifferent species were collected from acidic sites, whilst soil-indifferent species were collected from both acidic and calcareous sites. At each site, three healthy mature individuals were selected and sampled for cell-specific element analysis.

Three representative soil samples were taken at each site, from 0-20 cm depth, in June 2015. Samples were sieved (<2 mm) to remove any large organic material, before being air-dried and homogenised prior to chemical analyses. Samples were analysed at the Smithsonian Tropical Research Institute (Panamá, República de Panamá). Soil pH was determined in a 1:2 soil-to-solution ratio in water using a glass electrode. Total soil [P] was measured by ignition and acid digestion. Readily-exchangeable phosphate was determined by extraction with anion-exchange membranes and molybdate colourimetry. Total nitrogen (N) was measured by dry combustion followed by colourimetric analysis. Exchangeable Ca and other cations were determined by BaCl₂ (0.1 M) extraction and inductively coupled plasma optical emission spectrometry.

Leaf sampling for cell-specific element analysis

Only mature, undamaged, fully-expanded and sun-exposed leaves were sampled for each individual plant, grown either in hydroponics or collected in the field. Three samples were collected from each individual plant, from one to two leaves. Upon removal of a leaf, small sections (~2x3 mm) were cut from either side of the mid-rib, mid-way along the leaf, avoiding
large secondary veins. These sections were then mounted onto aluminium pins using optimal cutting temperature compound and plunged into liquid N, thereby immediately immobilising and preserving cellular ions. Samples were then stored in liquid N until preparation in a cryomicrotome and analysis using cryo-SEM. With this, all samples were collected fresh, were immediately plunge frozen in liquid N, and always kept under cryo-conditions, such that all elements were preserved in situ.

**Cell-specific element analysis by X-ray microanalytical mapping**

The method of cell-specific element analysis followed that of Hayes et al. (2018; Chapter 2 for full details). Transverse regions of frozen hydrated leaf samples were prepared by cryoplaning a flat surface with a glass knife, followed by a diamond knife, at -120°C in a cryomicrotome (Leica EM FC6 cryochamber integrated with Leica Ultracut EM UC6 microtome). Leaves were progressively microtomed flat, initially on a glass knife at 1 μm, 750 nm, and 500 nm steps, followed by a diamond knife at 250 nm and 100 nm steps. The pin was then mounted on a custom-made substage, transferred under a N gas environment to a Leica MED020 cryopreparation system, and sputter coated with 25-50 nm chromium, without sublimation. After coating, samples were transferred under vacuum to a Zeiss field emission SEM fitted with a Leica VCT100 cryotransfer and stage, and an Oxford X-Max80 SDD X-ray detector interfaced to Oxford Instruments AZtecEnergy software. This preparation method and this fully integrated Oxford analytical system are highly suitable for elemental analysis and quantitation of biological samples (Huang *et al.*, 1994; Marshall and Xu, 1998; Marshall and Clode, 2009; McCully *et al.*, 2010; Marshall *et al.*, 2012; Jin *et al.*, 2017; Marshall, 2017; Hayes *et al.*, 2018).

Samples were analysed at -150°C, 15 kV, and a 2 nA beam current (measured using a Faraday cup), in high-current mode. Prior to each map acquisition, the instrument was calibrated, and the beam current measured using a pure copper standard. Elemental maps were acquired at a resolution of 512 pixels, for > 3000 frames with a dwell time of 10 μs per pixel. Drift correction and pulse-pile up correction were activated. Using the Oxford Instruments AZtecEnergy software, quantitative numerical data were subsequently extracted from regions of interest drawn on the element maps, with individual spectra from each pixel.
within the region of interest summed and processed to yield element concentration data. Summed spectra from regions of interest were quantified using the AZtec XPP model for matrix corrections using standard files included in the software package. Percentage hydrogen (H) and N were fixed at 11.11% and 3.3%, respectively, with oxygen (O) determined by difference. Regions of interest represented various areas and individual cells of interest. Different cells were readily identified and classified, based on their leaf anatomy, cell appearance, and element levels. Only cells that were clearly identifiable and had a flat surface were analysed. Since the central vacuole occupies most of the volume in a plant cell, the reported measurements typically reflect vacuolar concentrations.

The following six cell types were confidently identified and analysed: epidermis (EP), hypodermis (HY), palisade mesophyll (PM), spongy mesophyll (SM), internal parenchyma (IP), and sclerenchyma (SC; excluding the hydrated sclerenchyma lumen). In total, 3,075 cells were analysed in the glasshouse experiment, with 8 - 107 cells for each cell type within each species/treatment combination (Table S3). For field-collected material, a total of 1,206 cells were analysed, with 18 - 78 cells for each cell type within each species/soil type combination (Table S4). The analytical resolution was ~2 μm (sufficient for the analysis of individual plant cells; Marshall, 1982) and the detection limit was a few mmol (Roomans and Dragomir, 2007).

**Statistics**

For glasshouse-grown plants, differences in [P] and [Ca] across all cell types and treatments, as well as across treatments within PM cells, were tested using general linear mixed-effect models, with individual plants as the random effect (Pinheiro and Bates, 2000). For field-collected plants, differences in [P] and [Ca] across cell types, at the species level were tested using general linear mixed-effect models, with individual plants as the random effect (Pinheiro and Bates, 2000). The residuals of each model were visually inspected for heteroscedasticity. In the presence of heteroscedasticity, appropriate variance structures were specified if they significantly improved the model, based on Akaike and Bayesian Information Criterion (AIC and BIC, respectively) values (Pinheiro and Bates, 2000). Differences among cell types and treatments were defined using Tukey *post hoc* tests. All statistical analyses were performed using the R software platform (R Core Team, 2017), with
the ‘nlme’ (Pinheiro et al., 2016), effects (Fox, 2003), and ‘multcomp’ (Hothorn et al., 2008) packages.

Results

Hakeas

The cell-specific allocation of P depended on Ca and P supply in both Hakeas (cell type x Ca treatment x P treatment interaction: $P < 0.01$; Fig. 1A). The calcifuge H. incrassata showed preferential allocation of P to IP, under low Ca / high P supply, with at least a ~7.6-fold greater [P] compared with other cell types (EP, PM, and SC; Figs 1A, 2). Under the same high-P condition, increasing Ca supply caused a ~10-fold increase in PM [P], from 9 to 100 μmol g$^{-1}$ (Figs 1A, 2). Also, the [P] increased in EP and SC, but not in IP. Thus, PM [P] increased from ~13% of IP [P] under low Ca supply, to ~147% under high Ca supply, a significant change in P allocation. In summary, increasing Ca supply under high-P conditions significantly changed the cell-specific allocation of P in H. incrassata, strongly increasing the relative distribution of [P] to PM and increasing the PM [P] to an exceptionally-high level.

The soil-indifferent H. prostrata showed a preferential allocation of P to both IP and PM under low Ca / high P supply (Figs 1A, 2). Under the same high-P condition, increasing Ca supply strongly decreased the [P] of all cell types, except PM (EP: 16 to 4 μmol g$^{-1}$, IP: 53 to 7 μmol g$^{-1}$, and SC: 12 to 4 μmol g$^{-1}$; Figs 1A, 2). This change in the allocation of P resulted in PM [P] reaching at least ~910% that of other cell types, without a significant increase in PM [P]. Therefore, under high-P conditions, increasing Ca supply induced a major change in the cell-specific allocation of P in H. prostrata, with a greater proportion of P allocated to PM; however, this was not associated with an increase in PM [P].
Fig. 1. Leaf cell-specific phosphorus ([P]) and calcium ([Ca]) concentrations of two *Hakea* species across different P and Ca treatments. Comparison of a typical calcifuge (CF) and soil-indifferent (SI) *Hakea* species. Only plants grown under high P (10 μM) / high Ca (600 μM) showed symptoms of P toxicity. Concentrations are per unit fresh weight, from fully-hydrated cells. Bars are means and error bars represent 95% confidence intervals, from linear mixed-effect models. Different letters indicate significant differences among cell types within each Ca treatment and panel (*post hoc* Tukey test, *P* <0.05). *P*-values (*, <0.05; **, <0.01; ***, <0.001) represent significant differences between Ca treatments (*post hoc* Tukey test). 0.1 μM P and 10 μM P, indicate the two P treatments. EP, epidermis; PM, palisade mesophyll; IP, internal parenchyma; SC, sclerenchyma.

Both *Hakeas* tended to show lower [P] under low P supply (*H. incrassata*: 2–6 μmol g\(^{-1}\), *H. prostrata*: 2–4 μmol g\(^{-1}\)), compared with those under high P supply (*H. incrassata*: 2–100...
μmol g⁻¹, *H. prostrata*: 4-61 μmol g⁻¹). Cellular [P] did not differ between Ca supplies, except for a small change in SC of *H. prostrata* (Fig. 1A). Therefore, under low-P conditions both *Hakeas* showed reduced cell [P], no major changes with Ca supply, and no clear preferential allocation of P.

The cell-specific allocation of Ca was also dependent on Ca and P supply in both *Hakeas* (cell type x Ca treatment x P treatment interaction: P < 0.001; Fig. 1B). *Hakea incrassata* showed preferential allocation of Ca to EP under low Ca supply, irrespective of P supply (Fig. 1B). Under high Ca supply, IP [Ca] increased substantially in *H. incrassata* (Fig. 1B, 2). For example, under high-P conditions EP [Ca] increased ~10.8-fold (96 to 1,040 μmol g⁻¹) and IP increased ~17.4-fold (7 to 122 μmol g⁻¹). Other cell types also showed small increases in [Ca] (P < 0.01; Fig. 1B). Similar changes in Ca allocation were evident under low- and high-P conditions. In summary, increasing Ca supply changed the cell-specific allocation of Ca in *H. incrassata*. The most notable was the acute allocation of Ca to IP under high Ca supply, a cell type that accumulated no Ca under low Ca supply.

*Hakea prostrata* showed very low [Ca] across all cell types under low Ca supply, irrespective of P supply (Fig. 1B). However, under high Ca supply, *H. prostrata* showed a preferential allocation of Ca to EP and IP, with a ~25-fold increase in both under high P supply (Fig. 1B, 2). The [Ca] increased in almost all cell types, except in PM under high P supply (Fig. 1B, 2). In summary, increasing Ca supply significantly changed the cell-specific allocation of Ca in *H. prostrata*, with a strong preferential allocation of Ca to EP and IP under high Ca supply, but no preferential allocation under low Ca supply. However, unlike *H. incrassata*, *H. prostrata* showed no change in PM [Ca] under high-P conditions.

Under high Ca / high P supply, *H. incrassata* co-allocated Ca and P within IP cells. This resulted in the apparent formation of Ca-phosphate crystals within the IP (Fig. 2). Other Ca-based crystals, presumably oxalates, formed in the EP of *H. incrassata* (Fig. 2). All crystal regions were avoided when selecting cellular regions for analysis, so as not to misrepresent cellular [Ca] or [P]. In contrast, *H. prostrata* did not co-allocate Ca and P within IP. Instead, *H. prostrata* showed a decrease in IP [P], concomitant with an increase in [Ca]. In summary, the co-allocation of Ca and P in IP of *H. incrassata* at high Ca / high P supply resulted in the precipitation of Ca-phosphates, not present under other treatments. In addition, the
exceptionally high [Ca] in EP of *H. incrassata* under high Ca / high P supply was associated with the formation of Ca-based crystals.

![Image of anatomical schematics](image)

**Fig. 2.** Qualitative element maps and corresponding anatomical schematics, showing phosphorus (P), calcium (Ca), and oxygen (O) distributions in transverse leaf sections of two *Hakea* species, grown under two Ca treatments (0 and 600 μM) and high P supply (10 μM). Comparison of a typical calcifuge (CF) and soil-indifferent (SI) *Hakea* species. All leaves were isobilateral, with images capturing at least half of the transverse section. Phosphorus and Ca maps are corrected for peak overlaps and background subtraction, providing a visualisation of the distribution, with quantified concentrations in Figs 1 and 3 (Supplementary Table S3). Only plants grown under high P (10 μM) / high Ca (600 μM) showed symptoms of P toxicity. Arrows indicate Ca-phosphate crystals; arrowheads indicate Ca-based crystals; asterisk indicates accumulation of P in sclerenchyma lumen (blue) and xylem parenchyma (white) surrounding a vein. Scale bar: 50 μm.


**Banksias**

The cell-specific allocation of P depended on Ca and P supply in *B. menziesii* (*P* < 0.001), but not *B. prionotes* (cell type x Ca treatment x P treatment interaction: *P* = 0.39; Fig. 3A). The calcifuge *B. menziesii* showed a preferential allocation of P to SM under low Ca / high P supply, with at least a ~2.5-fold greater [P] than in other cell types (EP, HY.1, HY.2, PM, and SC; Fig. 3A, 4). Under the same high-P condition, increasing Ca supply changed P allocation to PM, and away from SM, with a ~2-fold increase in PM [P] (9 to 18 μmol g⁻¹), coupled with a ~50% decrease in SM (26 to 12 μmol g⁻¹; Fig. 3A, 4). The [P] also increased in HY.1, decreased in HY.2 and was unchanged in EP and SC. Therefore, increasing Ca supply under high-P conditions changed the cell-specific allocation of P in *B. menziesii*, enhancing preferential allocation of P to PM, increasing the PM [P], with a concomitant decrease in SM [P].

The soil-indifferent *B. prionotes* showed a preferential allocation of P to PM, SM, and SC (9, 10, and 10 μmol g⁻¹, respectively) under low Ca / high P supply (Fig. 3A, 4). Under the same high-P condition, increasing Ca supply caused no change in [P] of most cell types, except PM, which showed a minor increase from 9 to 14 μmol g⁻¹ (*P* = 0.02; Fig. 3A, 4), which was non-significant (*P* = 0.07) when PM [P] were analysed separately (Fig. 5). Although the increase in PM [P] was small, it changed the relative distribution of [P], increasing PM [P] from ~90% to ~162% of that in SM and SC. Therefore, an increased Ca supply under high-P conditions caused a small increase in PM [P] of *B. prionotes*, enhancing the relative distribution of [P] to PM.

Both *Banksias* tended to show lower [P] under low P supply (*B. menziesii*: 1-4 μmol g⁻¹, *B. prionotes*: 2-5 μmol g⁻¹), compared with those under high P supply (*B. menziesii*: 2-26 μmol g⁻¹, *B. prionotes*: 2-14 μmol g⁻¹). Cellular [P] did not differ between Ca supplies (Fig. 3A). There were some differences in cell-specific [P] among cell types, but there was no clear preferential allocation. Therefore, under low-P conditions, both *Banksias* showed a low cell [P], no change in P allocation with increasing Ca supply, and no preferential allocation of P, thus showing similar responses to *Hakeas* under low P supply.
Fig. 3. Leaf cell-specific phosphorus ([P]) and calcium ([Ca]) concentrations of two Banksia species across different P and Ca treatments. Comparison of a typical calcifuge (CF) and soil-indifferent (SI) Banksia species. Only plants grown under high P (10 μM) / high Ca (600 μM) showed symptoms of P toxicity. Concentrations are per unit fresh weight, from fully-hydrated cells. Bars are means and error bars represent 95% confidence intervals, from linear mixed-effect models. Different letters indicate significant differences among cell types within each Ca treatment and panel (post hoc Tukey test, P <0.05). P-values (*, <0.05; **, <0.01; ***, <0.001) represent significant differences between Ca treatments (post hoc Tukey test). 0.1 μM P and 10 μM P, indicate the two P treatments. EP, epidermis; HY.1, upper layer of hypodermis; HY.2, lower layer of hypodermis; PM, palisade mesophyll; SM, spongy mesophyll; SC, sclerenchyma.
The cell-specific allocation of Ca depended on Ca and P supply in both *Banksias* (cell type x Ca treatment x P treatment interaction: *P* <0.001; Fig. 3B). *Banksia menziesii* showed low [Ca] under low Ca supply (2-29 μmol g⁻¹), regardless of P supply (Fig. 3B). However, increasing Ca supply caused a major change in the allocation of Ca, depending on P conditions. For example, under high Ca / low P supply, Ca was preferentially allocated to SM (524 μmol g⁻¹), followed by HY.2 (154 μmol g⁻¹). Conversely, under the same high-Ca condition, but with high P supply, Ca was instead preferentially allocated to HY.2 (470 μmol g⁻¹), and secondarily to SM (273 μmol g⁻¹). Therefore, increasing Ca supply significantly changed the cell-specific allocation of Ca in *B. menziesii*, the pattern of which also depended on P supply.

*Banksia prionotes* preferentially allocated Ca to SM (92 μmol g⁻¹) under low Ca / low P supply (Fig. 3B). In contrast, under low-Ca conditions, but with high P supply, *B. prionotes* showed low [Ca] across all cell types (3-22 μmol g⁻¹). Increasing Ca supply caused an acute increase in SM [Ca], at least ~26-fold and ~4.4-fold greater than that in other cell types, under low- and high-P conditions, respectively (Fig. 3B, 4). In summary, increasing Ca supply caused a major change in Ca allocation, with Ca preferentially allocated to SM under high Ca-supply. In addition, this preferential allocation of Ca to SM was less under high-P conditions, with SM [Ca] decreasing with increasing P supply.

Both *Banksias* possessed Ca-based crystals throughout SM cells, primarily under high Ca supply (Fig. 4).
**Fig. 4.** Qualitative element maps and corresponding anatomical schematics, showing phosphorus (P), calcium (Ca), and oxygen (O) distributions in transverse leaf sections of two *Banksia* species, grown under two Ca treatments (0 and 600 μM) and high P supply (10 μM). Comparison of a typical calcifuge (CF) and soil-indifferent (SI) *Banksia* species. All leaves were dorsiventral with stomatal crypts on the abaxial surface. Images capture upper part of transverse sections, with adaxial surface at the top. Phosphorus and Ca maps are corrected for peak overlaps and background subtraction and provide a visualisation of the distribution, with quantified concentrations in Figs 1 and 3 (Supplementary Table S3). Only plants grown under high P (10 μM) / high Ca (600 μM) showed symptoms of P toxicity. Arrowheads indicate Ca-based crystals. Scale bar: 50 μm.

**Palisade mesophyll**

A direct comparison of PM [P] and [Ca] across treatments revealed differences between calcifuge and soil-indifferent species (Fig. 5A). Only calcifuges showed an increase in PM [P] with increasing Ca supply and only under high-P conditions (P <0.05; Fig. 5A). Soil-indifferent
species showed only an increasing trend in PM [P] with increasing Ca supply ($P > 0.05$; Fig. 5). No species showed a difference in PM [P] under low P supply.

In both Banksias, the PM [Ca] depended only on Ca treatment ($P < 0.001$; Fig. 5B). In contrast, cellular [Ca] in both Hakeas changed between Ca treatments, dependent on P treatment (Ca treatment x P treatment: $P < 0.001$). For example, H. prostrata showed no increase in PM [Ca] under high-P conditions, only increasing under low-P conditions (Fig. 5B). Also, the PM [Ca] in H. incassata increased more strongly with increasing Ca supply under high-P conditions than under low-P conditions.

![Graph A](image1)

**Fig. 5.** Phosphorus ([P]) and calcium ([Ca]) concentrations of palisade mesophyll cells across different P and Ca treatments. Comparison of typical calcifuge and soil-indifferent Hakea and Banksia species. Only plants grown under high P (10 μM) / high Ca (600 μM) showed symptoms of P toxicity. Concentrations are per unit fresh weight, from fully-hydrated cells. Bars are means and error bars represent 95% confidence intervals, from linear mixed-effect models. Different letters indicate significant differences among treatments within each panel (post hoc Tukey test, $P < 0.05$).

**Field-collected material**

All species collected from acidic soils showed a clear preferential allocation of P to PM cells (Fig. 6). Cellular [P] did not differ between plants collected from different soil types in H. prostrata ($P = 0.84$; Fig. 6A). However, in B. prionotes the effect of soil type on [P] depended
on cell type (soil type x cell type interaction: $P < 0.01$). For example, PM [P] in *B. prionotes* was 17 $\mu$mol g$^{-1}$ on acidic soils, versus 23 $\mu$mol g$^{-1}$ on calcareous soils, with no difference in other cell types.

Both *Hakeas* showed relatively low cellular [Ca] on acidic soils, with *H. incrassata* showing a preferential allocation of Ca to EP (Fig. 6B). Both *Banksias* preferentially allocated Ca to SM, with *B. menziesii* also allocating Ca to HY. The effect of soil type on [Ca] depended on cell type in both soil-indifferent species (soil type x cell type interaction: $P < 0.001$; Fig. 6B). For example, [Ca] increased across all cell types in *H. prostrata*. In contrast, *B. prionotes* showed a much lower SM [Ca] on acidic soils, 263 $\mu$mol g$^{-1}$ versus 537 $\mu$mol g$^{-1}$ on calcareous soils. In summary, *B. prionotes* allocated extra P to PM and Ca to SM on calcareous soils, whilst *H. prostrata* maintained a stable PM [P], but increased [Ca] across cell types.
Fig. 6. Leaf cell-specific phosphorus ([P]) and calcium ([Ca]) concentrations in natural populations of Proteaceae collected from field locations of contrasting soil type along the Jurien Bay chronosequence, south-western Australia. Comparison of typical (A) soil-indifferent (SI) and (B) calcifuge (CF) *Hakea* and *Banksia* species, indicating soil type(s) on which they were collected. (A) SI species are found on both acidic and calcareous soils, (B) CF species are only found on acidic soils. Calcareous soils are higher in total and available soil [P], and exchangeable [Ca], compared with acidic soils (Table S2). None of the field-collected plants showed signs of P toxicity. Concentrations are per unit fresh weight, from fully-hydrated cells. Bars are means and error bars represent 95% confidence intervals, from linear mixed-effect models. Different letters indicate significant differences among cell types within each soil type and panel (post hoc Tukey test, \( P < 0.05 \)). (A) \( P \)-values (*, \( < 0.05 \); **, \( < 0.01 \); ***, \( < 0.001 \)) represent significant differences between soil types (post hoc Tukey test) in SI species. EP, epidermis; HY.1, upper layer of hypodermis; HY.2, lower layer of hypodermis; PM, palisade
mesophyll; SM, spongy mesophyll; IP, internal parenchyma; SC, sclerenchyma. Data for plants
collected on acidic soils are from Hayes et al. (2018; Chapter 2).

Discussion

Calcium supply altered the cell-specific allocation of P

Increased Ca supply altered the cell-specific allocation of P in all species. Specifically, higher
Ca supply enhanced the relative distribution of [P] to PM, resulting in PM typically showing
the highest mean [P] (Fig. 7). Furthermore, the effect of Ca supply on P allocation was only
evident at high P supply. At low P supply, the cellular [P] was very low, showed no clear
preferential allocation, and was independent of Ca supply. Therefore, Ca supply modulated
the cell-specific allocation of P under high-P conditions, by enhancing the relative distribution
of [P] to PM.

We surmise that Ca supply altered the cell-specific allocation of P through the
following mechanism. Under low Ca supply, all cells had exceptionally low [Ca] (typically <30
μmol g⁻¹). This low [Ca] allowed for excess P under high P supply to accumulate in cells other
than PM (Fig. 7). For example, P typically accumulated in IP in Hakeas and in PM, SM and SC
in Banksias, under low Ca supply. However, under high Ca supply, Ca also accumulated in the
non-PM cells, EP and IP in Hakeas, and SM and HY.2 in Banksias. Associated with this increase
in non-PM [Ca] was a strong change in the relative distribution of [P], away from non-PM (high
[Ca]) and towards PM (low [Ca]). This change in relative distribution of [P] to PM was due to
either an increase in PM [P] or a decrease in non-PM [P]. This mechanism of increased Ca
supply enhancing the relative distribution of [P] to PM was evident across all species.
This study supports our model of Ca-enhanced P toxicity, demonstrating, for the first time, the mechanism by which an increase in Ca supply enhanced sensitivity to, and severity of, P-toxicity symptoms, with no change in whole leaf [P] (Guilherme Pereira et al. unpublished, Hayes et al. unpublished; Chapters 3, 4). Increasing Ca supply enhanced the preferential distribution of P to PM, tending to increase PM [P], with no effect on whole leaf [P]. This increase in PM [P] likely reduced physiological availability of micronutrients, particularly Zn, resulting in leaf chlorosis, a main symptom of P toxicity (Cakmak and
Marschner, 1987; Broadley et al., 2012). Furthermore, in species where the separation of Ca and P could not be adequately maintained and there was co-allocation of high [Ca] and [P], there was apparent precipitation of Ca-phosphate crystals, as observed in IP of *H. incrassata*. The precipitation of Ca-phosphates was associated with leaf necrotic regions, another symptom of P toxicity and one that was evident in *H. incrassata*.

In summary, all species showed a general change in P allocation with increased Ca supply. We surmise that this change in P allocation was strongly influenced by changes in cellular [Ca] and the requirement of plants to separate cellular Ca and P (McLaughlin and Wimmer, 1999; White and Broadley, 2003; Conn and Gillham, 2010). This is the first example of Ca supply influencing the cell-specific allocation of P, representing a significant advancement in our understanding of P regulation and highlighting the importance of considering interactions in cell-specific nutrient concentrations.

*Calcifuge species showed a greater response to increased Ca supply than soil-indifferent species*

Calcifuges showed an increase in PM [P] in response to increased Ca supply, whereas soil-indifferent species showed no increase in PM [P] (*H. prostrata*) or a small increase (*B. prionotes*; Fig. 5A). Furthermore, within each genus, calcifuges showed a higher PM [P], compared with soil-indifferent species (Fig. 5A), but only under high P supply.

The calcifuge *H. incrassata* showed the greatest increase in PM [P] in response to increased Ca supply (9 to 100 μmol g⁻¹). This was an extreme increase in cellular [P], considering PM [P] in field-collected plants was 12 μmol g⁻¹ and the highest reported in eudicots under standard growth conditions is ~42 μmol g⁻¹ (Conn and Gillham, 2010; Hayes et al., 2018). The extreme PM [P] in *H. incrassata* likely severely reduced physiological [Zn] and thus explain the severe chlorotic symptoms under high Ca / high P supply.

*Hakea incrassata* also showed a high [P] in most non-PM cells; IP showed the highest non-PM [P]. These non-PM cells also showed large increases in [Ca] under high Ca supply and thus caused Ca and P to be co-localised. This inability to separate Ca and P resulted in their co-allocation and formation of crystals in IP cells. Increased [P] across most cells of *H. incrassata* was not associated with an increase in whole leaf [P] (Hayes et al. unpublished;
Chapters 3). This increase in cell-specific [P], despite no change in leaf [P], may result from the displacement of P into cellular regions, driven by changes in [Ca]. This P may be displaced from non-quantified regions within the leaf, such as apoplastic regions, xylem parenchyma, and sclerenchyma lumen. Therefore, the inability of *H. incrassata* to separate Ca and P likely resulted in the precipitation of Ca-phosphates and consequently explains the severe symptoms of necrosis in this species. This is the first evidence of a direct interaction between cellular Ca and P, resulting in precipitation of Ca-phosphate crystals (Treeby *et al.*, 1987; McLaughlin and Wimmer, 1999; Karley *et al.*, 2000b; White and Broadley, 2003; Storey and Leigh, 2004; Conn and Gilliam, 2010). In summary, severe sensitivity to Ca-enhanced P toxicity in the calcifuge *H. incrassata* was likely due to an extreme increase in PM [P], coupled with an inability to separate P from Ca.

The soil-indifferent *H. prostrata* showed no increase in PM [P] (Fig. 5A). The PM [P] of *H. prostrata* remained below 62 μmol g\(^{-1}\), regardless of the Ca supply. This PM [P] was less than the 100 μmol g\(^{-1}\) in *H. incrassata*, grown under the same conditions and less than the 136 to 233 μmol g\(^{-1}\) observed for *H. prostrata* under highly P-toxic conditions (Shane *et al.*, 2004a). In addition, *H. prostrata* also showed no difference in PM [Ca]. Therefore, *H. prostrata* showed tighter control of PM [P] and [Ca] under increasing Ca supply, likely resulting in its reduced sensitivity to Ca-enhanced P toxicity and consequently reduced chlorotic symptoms.

*Hakea prostrata* showed a reduction in [P] of non-PM cells, with increasing Ca supply. By reducing [P] in non-PM cells, *H. prostrata* was better able to avoid co-allocation of Ca and P. This general reduction in cellular [P] was likely achieved by allocating P to other regions, such as xylem parenchyma or sclerenchyma lumen. Therefore, *H. prostrata* responded very differently to increased Ca supply than did *H. incrassata* and was able to better tolerate Ca-enhanced P toxicity because it maintained stable PM [P]/[Ca] and avoided the co-allocation of Ca and P in non-PM cells.

The calcifuge *B. menziesii* showed a ~2-fold increase in PM [P] with increasing Ca supply. This increase in PM [P] severely impacted glasshouse-grown plants (severe chlorosis), yet field-collected plants appeared healthy. The severe impact of this relatively small change in PM [P] may be partly explained by differences in cellular inorganic P concentration ([P]) between glasshouse-grown and field-collected plants. A high [Pi] would severely reduce physiological [Zn] and result in severe chlorosis (Oykman and Marschner, 1987; Zhao *et al.*, 2004b).
1998; Broadley et al., 2012). In this study we measured total cellular [P] (which includes P_i and organic P); however, leaves with higher [P] generally show a greater proportion of P_i and hence should show a greater cellular [P_i] (Veneklaas et al., 2012). Therefore, since glasshouse-grown plants showed leaf [P] ~6.6-fold greater than field-collected plants, we would expect glasshouse-grown plants to show a much greater proportion of [P_i] in the total cellular [P] (Hayes et al. unpublished; Chapters 3). Therefore, we surmise that although PM [P] are only marginally greater in glasshouse-grown plants than field-collected plants, the glasshouse-grown plants likely contain a greater [P_i], which would more severely reduce the physiological [Zn], thus explaining the chlorosis in B. menziesii under high Ca/ high P supply.

_Banksia menziesii_ altered its allocation of P under high Ca supply. The two Ca-accumulating cell types in _B. menziesii_ (SM and HY.2) showed a significant reduction in [P] with increasing Ca supply, coupled with an increase in the [P] of non-Ca accumulating cell types (PM and HY.1). This strategy effectively reduced the co-allocation of Ca and P in _B. menziesii_. In summary, _B. menziesii_ reduced co-allocation of Ca and P, by shifting the allocation of P away from Ca-accumulating cell types and towards non-Ca-accumulating cell types, PM and HY.1. However, this shift increased the PM [P] and this likely reduced physiological [Zn] and thus explains the severe chlorosis associated with P-toxicity in _B. menziesii_ (Fig. 5A).

The soil-indifferent _B. prionotes_ showed only a minor increase in PM [P] under high Ca supply. This PM [P] was below that of _B. menziesii_ and that of field-collected _B. prionotes_. The development of mild chlorosis in glasshouse-grown _B. prionotes_ under the most severe treatments, may indicate that glasshouse-grown plants had a greater cellular [P_i] than field-collected plants. This notion is supported by the ~10.8-fold greater whole leaf [P] of glasshouse-grown plants, expected to result in a greater [P_i] (Veneklaas et al., 2012).

_Banksia prionotes_ showed no change in the [P] of any non-PM cell type. In addition to minimising changes in P allocation, _B. prionotes_ also showed a greater whole leaf [Zn], compared with calcifuge _B. menziesii_ (Hayes et al. unpublished; Chapter 3). A greater leaf [Zn] in _B. prionotes_ further explains why this species is more tolerant to Ca-enhanced P toxicity, because an increased [Zn] would allow _B. prionotes_ to better tolerate P-enhanced Zn requirements. Therefore, _B. prionotes_ is more tolerant of Ca-enhanced P toxicity than _B. menziesii_, due to its ability to minimise changes in cellular [P], particularly PM [P] and because
it operates at a higher leaf [Zn], allowing it to tolerate higher [P], without critically reducing the physiological [Zn].

A mechanism for Ca-enhanced P toxicity

The main visual symptoms of P toxicity are leaf chlorosis and necrosis (Grundon, 1972; Groves and Keraitis, 1976; Nichols and Beardsell, 1981; Webb and Loneragan, 1990; Lambers et al., 2002; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008). Calcium increases the severity of P-toxicity symptoms, termed Ca-enhanced P toxicity (Grundon, 1972; Nichols and Beardsell, 1981; Guilherme Pereira et al. unpublished, Hayes et al. unpublished; Chapters 3, 4). Calcium-enhanced P toxicity likely results from the leaf cell-specific allocation of P and Ca, and the requirement of plants to allocate these nutrients separately (Treeby et al., 1987; McLaughlin and Wimmer, 1999; Karley et al., 2000b; White and Broadley, 2003; Storey and Leigh, 2004; Conn and Gillham, 2010). We propose that P-toxicity symptoms and their enhancement under high Ca supply is explained by two processes. First, an inability to strongly down-regulate P-uptake capacity, and, second, the cell-specific allocation of P and Ca.

Phosphorus-sensitive Proteaceae show a weak ability to down-regulate P uptake, resulting in excess uptake of P, leading to P toxicity even at relatively low P availability (Handreck, 1997; Shane et al., 2004b; Shane and Lambers, 2006; de Campos et al., 2013). This is considered the primary physiological mechanism causing P-toxicity and has been demonstrated for several P-sensitive species (Handreck, 1997; Shane et al., 2004b; Shane and Lambers, 2006; de Campos et al., 2013). Second, is the preferential allocation of P to photosynthetic mesophyll cells in species from severely P-impoverished habitats (Shane et al., 2004a; Hawkins et al., 2008; Lambers et al., 2015; Hayes et al., 2018; Guilherme Pereira et al. 2018; Chapter 2). Preferential allocation of P to photosynthetic mesophyll cells increases photosynthetic P-use efficiency (PPUE), but also leads to very high [P] in these cells at high P supply (Shane et al., 2004a; Hayes et al., 2018; Chapter 2). Therefore, preferential allocation of P to mesophyll cells disproportionally increases mesophyll [P], enhancing their sensitivity to P, even at relatively low P availability.
Increased Ca supply can significantly increase the preferential allocation P to PM cells, generally resulting in higher PM [P], enhancing the severity of P-toxicity symptoms. This mechanism explains why Ca enhances symptoms of P toxicity, despite no changes in whole leaf [P], because the allocation of P within the leaf is changed, and not the total amount. This tended to increase PM [P] with severe consequences for plant health.

A greater [P] can reduce leaf physiological [Zn] and when not separated from Ca can form Ca-phosphate, rendering both nutrients unavailable and disrupting cellular processes. Precipitation could also occur with other metals, such as aluminium (Al), iron (Fe) or Zn (Robson and Pitman, 1983; Lambers et al., 2002; Broadley et al., 2012). These mechanisms support the visual symptoms of P toxicity, with chlorosis, caused by reduced physiological [Zn], and necrosis, caused by the precipitation of Ca-phosphates, resulting in cell death.

Concluding remarks

Calcium enhanced the relative distribution of P to PM, generally resulting in a greater PM [P] and thus supporting our model of Ca-enhanced P toxicity. Calcifuge Proteaceae showed a greater response to increased Ca supply than soil-indifferent ones, corresponding with their increased sensitivity to Ca-enhanced P toxicity. Specifically, calcifuges showed a greater PM [P] compared with soil-indifferent species, of the same genus. Calcifuge Hakeas also showed a reduced ability to separate Ca and P; forming Ca-phosphate crystals under high Ca / high P supply. Therefore, we surmise that Ca-enhanced P toxicity is due to an inability to down-regulate P uptake, in conjunction with leaf cell-specific interactions between Ca and P. This results in higher leaf PM [P] under high Ca supply, thus interfering with the physiological [Zn] and/or precipitating with Ca.

The comparison of calcifuge and soil-indifferent species demonstrates that cell-specific [P] in soil-indifferent species was less impacted by higher Ca supply, with soil-indifferent species showing a tighter control of PM [P] with increasing Ca supply. We surmise that it is the higher soil [P] of young calcareous soils, in combination with higher soil [Ca] and low available [Zn] that excludes most Proteaceae from calcareous habitats and that soil-indifferent Proteaceae can overcome this restriction: by reducing changes in PM [P], by separating Ca and P, and through several traits that increase leaf [Zn]. These strategies reduce

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the impact of Ca on PM [P], reduce deleterious interactions between Ca and P, and compensate for P-enhanced Zn requirement.

This study advances our mechanistic understanding of Ca-enhanced P toxicity and highlights the importance of considering P, Ca, and micronutrients in the nutrition of P-sensitive species. This is critically important in the management of Proteaceae in restoration projects and in the horticultural industry (Bunn and Dixon, 1992; Enright and Lamont, 1992; Fuss et al., 1992; Stephenson, 2005; Cross and Lambers, 2017). This research has important implications for our understanding of P nutrition in higher plants, particularly with regards to plants that preferentially allocate P to mesophyll cells (Conn and Gilliam, 2010; Hayes et al., 2018; Guilherme Pereira et al. 2018; Chapter 2). This includes monocot crop species (Dietz et al., 1992; Leigh and Tomos, 1993; Williams et al., 1993; Fricke et al., 1994, 1996; Karley et al., 2000a) as well as eudicot species from severely P-impoverished habitats (Shane et al., 2004a; Hawkins et al., 2008; Hayes et al., 2018; Guilherme Pereira et al., 2018; Chapter 2). Species that preferentially allocate P to the mesophyll may be particularly susceptible to Ca-enhanced P toxicity and possibly other Ca-driven changes in cell-specific nutrient allocation. We speculate that similar relationships may be observed between P and metals such as Al, Fe or Mn which warrants further investigation (Robson and Pitman, 1983; Lambers et al., 2002; Broadley et al., 2012).

The exact mechanisms and pathways with which plants regulate the cell-specific preferential allocation of nutrients are not yet fully understood (Conn et al., 2011; Conn and Gilliam, 2010; Gilliam et al., 2011; White and Broadley, 2003). Future research should focus on the cell-specific regulatory pathways of nutrients, including the differential expression of membrane-bound transporters and channels, for example, Ca^{2+}-transporting P-type-ATPases and Ca^{2+}/H^{+} antiporters (Conn et al. 2011). Differences in the cell-specific expression of transporters and channels are likely to play an important role in regulating the cell-specific preferential allocation of nutrients, such as Ca, P, and Zn. Although this study does not delve into the realm of transporter/channel regulation, this would certainly be the next logical step.

Knowledge of the interactions among nutrients at the cell-specific level highlights the limitations in inferring physiological implications from whole-leaf nutrient concentrations. There can be physiologically significant changes in cellular concentrations, with no apparent change at the whole leaf level. Therefore, this research advances our fundamental
understanding of plant mineral nutrition and highlights the importance of considering nutrient interactions at the cell-specific level.

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Bunn E, Dixon KW. 1992. Micropropagation of Stirlingia latifolia (Proteaceae), an important cut flower from Western Australia. HortScience 27, 368–369.


Chapter 5 | Supporting Information

**Table S1** Concentrations of nutrient solutions used in hydroponic experiments.

**Table S2** Soil chemical properties from selected sites along the Jurien Bay dune chronosequence, south-western Australia.

**Table S3** Cellular phosphorus (P) and calcium (Ca) concentrations of plants grown under different P and Ca supplies.

**Table S4** Cellular phosphorus (P) and calcium (Ca) concentrations of Proteaceae species collected from contrasting soil types along the Jurien Bay dune chronosequence, south-western Australia.
### Table S1. Concentrations of nutrient solutions used in hydroponic experiments

<table>
<thead>
<tr>
<th>Nutrient solution</th>
<th>Treatments (µM)</th>
<th>Nutrient solution concentrations (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PO₄³⁻</td>
<td>Ca²⁺</td>
</tr>
<tr>
<td>25% growth</td>
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</tr>
<tr>
<td>100% growth</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>25% basal</td>
<td>0.1, 10</td>
<td>0, 600</td>
</tr>
<tr>
<td>33% basal</td>
<td>0.1, 10</td>
<td>0, 600</td>
</tr>
<tr>
<td>50% basal</td>
<td>0.1, 10</td>
<td>0, 600</td>
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</table>

‘Growth’ nutrient solutions were used prior to the start of treatments.

‘Basal’ nutrient solutions were used during treatment period.
Table S2. Soil chemical properties from selected sites along the Jurien Bay dune chronosequence, south-western Australia

<table>
<thead>
<tr>
<th>Soil type</th>
<th>Total P (mg kg⁻¹)</th>
<th>Resin P (mg kg⁻¹)</th>
<th>Exchangeable Ca (mg kg⁻¹)</th>
<th>pH (H₂O)</th>
<th>CEC (cmol. kg⁻¹)</th>
<th>Total N (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>10 ± 0.6</td>
<td>0.42 ± 0.17</td>
<td>142 ± 19</td>
<td>6.2 ± 0.1</td>
<td>0.98 ± 0.12</td>
<td>0.11 ± 0.04</td>
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<td>Calcareous</td>
<td>57 ± 20</td>
<td>0.91 ± 0.23</td>
<td>1136 ± 109</td>
<td>8.3 ± 0.1</td>
<td>6 ± 0.53</td>
<td>0.5 ± 0.04</td>
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</table>

Resin P, a measure of plant-available P. All values are mean ± standard error (n = 3–6).

CEC = cation exchange capacity. Acidic soil data is from Hayes et al. (2018).
**Table S3.** Cellular phosphorus (P) and calcium (Ca) concentrations of plants grown under different P and Ca supplies. Values are means (standard error). Different letters indicate significant differences within each species, across all cell types and treatments (Tukey post hoc test, P <0.05). CF, calcifuge; SI, soil-indifferent; EP, epidermis; HY.1, upper layer of hypodermis; HY.2, lower layer of hypodermis; PM, palisade mesophyll; SM, spongy mesophyll; SC, sclerenchyma; IP, internal parenchyma.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell type</th>
<th>Treatment (µM)</th>
<th>Cellular [P] (µmol g⁻¹)</th>
<th>Cellular [Ca] (µmol g⁻¹)</th>
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<td></td>
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<td>P</td>
<td>Ca</td>
<td>n</td>
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**Table S4.** Cellular phosphorus (P) and calcium (Ca) concentrations of Proteaceae species collected from contrasting soil types along the Jurien Bay dune chronosequence, south-western Australia. Values are means (standard error). Different letters indicate significant differences within each species and soil-type combination (Tukey *post hoc* test, $P < 0.05$). CF, calcifuge; SI, soil-indifferent; EP, epidermis; HY.1, upper layer of hypodermis; HY.2, lower layer of hypodermis; PM, palisade mesophyll; SM, spongy mesophyll; SC, sclerenchyma; IP, internal parenchyma. Data for plants collected on acidic soils is from Hayes et al. (2018).  

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<th>Cellular [Ca] (µmol g$^{-1}$)</th>
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1. Introduction

Proteaceae represent an ecologically important component of the Australian flora, contributing towards south-western Australia’s exceptionally high biodiversity (Cowling & Lamont, 1998; George, 1998; Myers et al., 2000; Hopper & Gioia, 2004). Many Australian Proteaceae species are highly P-sensitive and occur exclusively on old nutrient-impoverished acidic soils (calcifuge), whilst some species also occur on young calcareous soils (soil-indifferent), higher in available Ca and P (Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/). It is important to understand the ecophysiological reasons for this distribution, to ensure sustainable management of this iconic family and that of other P-sensitive species. This is of increasing importance, as mining, agriculture, land-clearing, climate change, and other anthropogenic factors continue to influence Proteaceae distribution in south-western Australia (Enright & Lamont, 1992; Miller et al., 2007). The primary objective of my PhD project was to discover the physiological basis for Ca-enhanced P toxicity in the plant family Proteaceae and its role in their distribution. However, it also explored aspects of cell-specific nutrient allocation of far broader relevance. In the following sections, I will highlight the major findings of this research, their broader implications and the areas in which I believe future research should focus.

2. Proteaceae species from P-impoverished habitats preferentially allocated P to photosynthetic cells

Plants tend to allocate different nutrients to different cell types within their leaves (Karley et al., 2000b; Conn & Gillham, 2010). Most eudicots were generally thought to preferentially allocate P to epidermis and bundle sheath cells, and Ca to mesophyll cells (Conn & Gillham, 2010). However, none of the Proteaceae species I studied showed this pattern of nutrient allocation. Instead, Proteaceae species from extremely P-impoverished habitats (south-
western Australia) preferentially allocated P to photosynthetic mesophyll cells, whilst those from P-richer habitats (Brazil and Chile) showed no preferential allocation of P (Chapter 3). Similarly, Guilherme Pereira et al. (2018) recently reported preferential allocation of P to photosynthetic cells in a range of eudicot species from P-impoverished habitats, other than Proteaceae. These results suggest that preferential allocation of P to photosynthetic cells evolved as an adaptation to an extremely P-impoverished habitat and that it is not a family-wide trait. In addition, the allocation of Ca varied among species and did not follow the model for most eudicots, with variation found even within a single genus.

I surmise that by preferentially allocating P, species adapted to severely P-impoverished habitats are able to reduce their whole leaf [P], while maintaining high [P] in photosynthetic cells and achieve rapid rates of photosynthesis (Stitt et al., 2010). Therefore, the ability of Proteaceae species from severely P-impoverished habitats to allocate P preferentially to photosynthetic cells offers a partial explanation for their extremely high PPUE and may represent a critically important adaptation to surviving in a severely P-limited habitat. This complements several other important traits contributing to a high P-use efficiency in these species, including the replacement of phospholipids with sulfolipids and galactolipids (Lambers et al., 2012), and functioning at very low levels of ribosomal RNA (Sulpice et al., 2014). At higher P availability, this greater mesophyll [P] may also contribute to the extreme P sensitivity of many Proteaceae species, due to interference with physiological [Zn] in mesophyll cells, resulting in a P-enhanced Zn requirement (Chapman & Vanselow, 1937; Boawn & Leggett, 1964; Cakmak & Marschner, 1987; Loneragan & Webb, 1993).

My thesis highlights the role of soil [P] in driving the evolution of ecologically relevant nutrient-allocation patterns and that these patterns cannot be generalised across families. Furthermore, preferential allocation of P to photosynthetic cells may provide a new and exciting strategy to improve P-use efficiency in crop species, thus reducing agricultural fertiliser demand. Future research may utilise plant breeding to target crop varieties with more efficient nutrient allocation patterns, thus improving P-use efficiency. In addition, cell-specific P allocation appeared to be linked with higher leaf-P resorption, with Proteaceae and monocots both showing relatively higher P resorption, and both preferentially allocating P to mesophyll (Vergutz et al., 2012; Hayes et al., 2014). This link requires further investigation to
confirm its validity and the underlying mechanisms involved. Such research should be combined with further investigation of the extent to which cell-specific element accumulation varies among a range of ecologically and phylogenetically diverse species, under a range of conditions. This knowledge will improve our understanding of the movement, accumulation and overall regulation of essential elements within plants.

3. A mechanistic understanding of Ca-enhanced P toxicity

The main visual symptoms of P toxicity are leaf chlorosis and necrosis (Grundon, 1972; Groves & Keraitis, 1976; Nichols & Beardsell, 1981; Webb & Loneragan, 1990; Lambers et al., 2002; Shane et al., 2004a,b; Parks et al., 2007; Hawkins et al., 2008). Calcium increases the severity of these symptoms, a phenomenon termed Ca-enhanced P toxicity (Grundon, 1972; Nichols & Beardsell, 1981). A key objective of my study was to develop a mechanistic understanding for Ca-enhanced P toxicity, as it plays an important role in the distribution of Proteaceae (Chapters 3, 4, 5). Calcium-enhanced P toxicity explains the calcifuge habit of most Proteaceae species and accounts for their exclusion from calcareous habitats (higher in available Ca and P; Chapters 3, 4, 5). This thesis not only presents the first clear evidence for Ca-enhanced P toxicity across multiple species, but also proposes the first model describing Ca-enhanced P toxicity; highlighting interactions among P, Ca and Zn (Fig. 1). I propose that P-toxicity symptoms and their enhancement under higher Ca supply are explained by two main processes. First, an inability to strongly down-regulate P-uptake capacity, and, second, a preferential allocation of P to palisade mesophyll cells, which is enhanced by a high Ca availability.

A reduced ability to down-regulate P-uptake capacity is considered the primary physiological cause for P toxicity in P-sensitive species (Shane et al., 2004b; Shane & Lambers, 2006; de Campos et al., 2013), as it reduces their capacity to regulate P uptake, therefore leading to excess leaf [P], even under relatively low P availability (Handreck, 1997). The effect of excess P uptake is further exacerbated within leaves due to the preferential allocation of P to palisade mesophyll (PM) cells, leading to excessively high PM [P] (Shane et al., 2004a; Hawkins et al., 2008; Hayes et al., 2018; Chapter 2). This is advantageous in increasing P-use efficiency under P-limited conditions but can be deleterious when excess P is available.
Calcium does not increase leaf [P], as was previously proposed, but instead changes the allocation of P within leaves (Chapter 5). Calcium increased the relative distribution of P to PM cells, generally leading to a greater PM [P] and enhancing the severity of P-toxicity symptoms (Chapters 3, 4, 5). A greater PM [P] can reduce physiological [Zn], resulting in chlorosis. These Proteaceae species operate at exceptionally low leaf [Zn], with low Zn allocation to leaves (Chapter 3) and are therefore sensitive to decreased physiological [Zn]. Low leaf [Zn] is further reduced under high P supply due to increased growth (effectively diluting [Zn]) and decreased root:shoot ratio (reducing the amount of roots for Zn uptake) (Cakmak & Marschner, 1987; Loneragan & Webb, 1993; Chapter 3). A similar relationship may also occur for other micronutrients, such as iron (Fe) and copper (Cu); however, Zn appears the most prominent in these Proteaceae species (Fig. 1).

A greater leaf [P], when not separated from Ca, can precipitate to form Ca-phosphate crystals, rendering both nutrients unavailable and disrupting cellular processes, resulting in necrosis (Fig. 1). This study provides the first evidence of a direct interaction between cellular Ca and P, resulting in the precipitation of Ca-phosphate crystals (Treeby et al., 1987; McLaughlin & Wimmer, 1999; Karley et al., 2000b; White & Broadley, 2003; Storey & Leigh, 2004; Conn & Gillham, 2010).

The model of Ca-enhanced P toxicity explains the visual symptoms of P toxicity (Fig.1). Chlorosis was likely caused by reduced physiological [Zn] and necrosis by the precipitation of Ca-phosphates, resulting in cell death. These symptoms significantly impact leaf functioning, generally reducing growth, photosynthesis and stomatal conductance (Chapter 4). The findings of this thesis represent an important advancement in our understanding of P toxicity and Ca-enhanced P toxicity, with important implications for management of Proteaceae and other P-sensitive species. This research highlights the importance of considering P, Ca and micronutrients in the management of P-sensitive species. This is of critical importance to the future management of Proteaceae in restoration projects and in the horticultural industry (Bunn & Dixon, 1992; Enright & Lamont, 1992; Fuss et al., 1992; Stephenson, 2005; Cross & Lambers, 2017).
My research has important implications for understanding P nutrition in higher plants, particularly plants that preferentially allocate P to mesophyll cells. This includes many Proteaceae (Hayes et al., 2018; Chapter 2), but also other eudicots from severely P-impoverished habitats (e.g., some Asteraceae, Fabaceae, Myrtaceae, Vochysiaceae; Guilherme Pereira et al., 2018) and monocot species (e.g., barley, sorghum, wheat; Conn & Gillham, 2010). The implications for monocot crop species are of particular interest, as cell-specific P allocation has only been assessed in a few monocot species (barley, sorghum, wheat; Boursier & Läuchli, 1989; Malone et al., 1991; Williams et al., 1993; Karley et al., 2000a). Other important monocot crops such as rice and maize would be of particular interest, especially considering the importance of micronutrients to these species (Nichols et al., 2012; Moore et al., 2014). Species preferentially allocating P to mesophyll cells may be particularly susceptible to Ca-enhanced P toxicity and possibly other Ca-driven changes in cell-
specific nutrient allocation. I speculate that P allocation in these species may also be influenced by the allocation of other metals, such as Al, Fe or Mn, warranting further investigation.

Knowledge of the important interactions among nutrients at the cell-specific level highlights the limitations of inferring physiological implications from whole-leaf nutrient concentrations. My thesis demonstrates that there can be physiologically significant changes in cellular concentrations, with no apparent change at the whole leaf level. Therefore, my research advances our understanding of plant mineral nutrition and highlights the importance of considering nutrient interactions at the cell-specific level. Whole leaf nutrient concentrations provide an excellent proxy for plant nutrient status but may not always reflect physiologically important cellular concentrations.

4. The role of Ca-enhanced P toxicity in the distribution of Proteaceae

Many of the 650 Proteaceae species in south-western Australia are highly P-sensitive, occurring exclusively on old nutrient-impoverished acidic soils (calcifuge), whilst very few species also occur on young calcareous soils (soil-indifferent; Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/). The calcifuge habit of species along the Jurien Bay chronosequence in south-western Australia is typical of most Proteaceae, with very few species found on young calcareous soils (Hayes et al., 2014; Zemunik et al., 2015; Western Australian Herbarium, https://florabase.dpaw.wa.gov.au/).

All Proteaceae showed signs of Ca-enhanced P toxicity; however, there were clear differences in sensitivity between calcifuge and soil-indifferent species (Chapters 3, 4, 5). Visual P-toxicity symptoms, nutrition, growth, physiology, and cell-specific nutrient allocation were more severely impacted in calcifuges than in soil-indifferent species (Chapters 3, 4, 5). Calcifuges showed more severe symptoms and at lower Ca and P supplies than soil-indifferent species. These differences would severely inhibit the ability of calcifuges to grow in calcareous soils, and hence, Ca-enhanced P toxicity likely contributes to the calcifuge habit of most Proteaceae.

I surmise that the higher soil [P] of young calcareous soils, in combination with higher soil [Ca] and low available [Zn] excludes most Proteaceae species from calcareous habitats.
and that soil-indifferent species can overcome this restriction: by reducing changes in PM [P], by separating Ca and P (Chapter 5), and through several traits that increase leaf [Zn] (Chapters 3, 4). These strategies reduce the impact of Ca on PM [P], reduce the deleterious interactions between Ca and P, and compensate for P-enhanced Zn requirement. All soil-indifferent species utilised the above-mentioned strategies to reduce Ca-enhanced P toxicity; however, there were some important differences among genera.

*Banksias* showed severe leaf chlorosis, suggesting they were severely impacted by reduced physiological [Zn], and less by a reduced ability to separate Ca and P. The ability of soil-indifferent *Banksias* to tolerate Ca-enhanced P toxicity appears mainly related to maintaining higher leaf [Zn], thus enabling them to meet P-enhanced Zn requirements. Conversely, calcifuge *Banksias* showed lower leaf [Zn] and greater increases in PM [P], resulting in severe chlorosis.

*Hakeas* showed severe leaf necrosis, suggesting they were severely impacted by a reduced ability to separate Ca and P and less by reduced physiological [Zn]. Soil-indifferent *Hakeas* showed a clear ability to separate Ca and P within leaves. In addition, soil-indifferent *Hakeas* also showed lower leaf [P], suggesting a somewhat greater capacity to down-regulate P-uptake. This, along with higher leaf [Zn] in *H. trifurcata* may explain why soil-indifferent *Hakeas* better tolerate Ca-enhanced P toxicity. The ability of soil-indifferent *Hakeas* to tolerate Ca-enhanced P toxicity appears mainly related to reducing the co-localisation of Ca and P within leaves and reducing whole leaf [P], thus reducing the deleterious effects of Ca-phosphate precipitation. Conversely, calcifuge *Hakeas* showed co-localisation of Ca and P, and precipitation of Ca-phosphate, along with greater leaf [P], resulting in severe necrosis and mild chlorosis.

5. **Conclusions**

Calcium-enhanced P toxicity can explain the exclusion of most Proteaceae species from calcareous habitats. Calcifuges have a greater sensitivity compared with soil-indifferent species. The proposed physiological mechanism for Ca-enhanced P toxicity is based on a reduced ability to down-regulate P-uptake capacity and the leaf cell-specific interactions of Ca and P. Despite no changes in total leaf [P] with increasing Ca supply, leaf mesophyll [P]
generally increased; this increase interfered with the physiological [Zn] and/or precipitated with Ca, resulting in leaf chlorosis and necrosis.

Future research to support these findings would include a glasshouse soil experiment, in which species would be grown in field-collected acidic and calcareous soils. A soil-based experiment such as this would also take into account many of the potentially important plant-soil interactions, such as those observed in wheat (Ova et al., 2015). The formation of cluster roots and measurements of their activity in both soil types would be of particular interest. Measurements of physiological [Zn], both at the whole leaf and cell-specific level would also help support the proposed model, as it would demonstrate the importance of physiological [Zn], supporting its roles in P-toxicity.

My thesis presents a novel strategy to improve P-use efficiency in crop species, through the cell-specific allocation of P to leaf photosynthetic cells. It also highlights the importance of considering cell-specific nutrient concentrations in plant physiology, by demonstrating its role in Ca-enhanced P toxicity. Most notable is the impact of other nutrients on the allocation of P, a phenomenon that likely occurs in relation to other essential and non-essential elements. Through a thorough exploration of responses in nutrition, biomass, physiology, growth, and leaf cell-specific nutrient allocation, my thesis presents the first model describing Ca-enhanced P toxicity. This will improve current management practices involving Proteaceae and other P-sensitive species, highlighting the importance of considering P, Ca and Zn (as well as other micronutrients) in management of this ecologically important family. This will aid in the preservation of south-western Australia’s exceptionally high biodiversity, improving the region’s environmental sustainability into the future. Finally, my thesis presents Ca-enhanced P toxicity as a critically important factor in Proteaceae distribution, accounting for the absence of most Proteaceae species from calcareous habitats.

References


Appendix

Additional publications during PhD


A climosequence of chronosequences in southwestern Australia

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Summary

To examine how climate affects soil development and nutrient availability over long timescales, we studied a series of four long-term chronosequences along a climate gradient in southwestern Australia. Annual rainfall ranged from 533 to 1185 mm (water balance from -900 to -52 mm) and each chronosequence included Holocene (<6.5 ka), Middle Pleistocene (120–500 ka) and Early Pleistocene (~2000 ka) dunes. Vegetation changed markedly along the chronosequence, from shrubland at the driest site to Eucalyptus forest at the wettest. Soil pH was similar in the youngest soil of each chronosequence, although the carbonate and P contents of the parent sand declined from dry to wet along the chronosequence, presumably linked to variations in offshore productivity. Despite this, soil development and associated nutrient status followed remarkably consistent patterns along the four chronosequences. Pedogenesis involved decalcification and secondary carbonate precipitation in Holocene soils and leaching of iron oxides from Middle Pleistocene soils, leading ultimately to bleached quartz sands in the oldest soils. Along all chronosequences soil pH and total P declined, whereas Ca and Na increased, which is consistent with the predicted change from C-N to P limitation of vegetation during ecosystem development. The expected unimodal pattern of leaf area index was most pronounced along wetter chronosequences, suggesting an effect of climate on the expression of retrogression. The four chronosequences do not appear to span a pedogenic climate threshold, defined as an abrupt change in soil properties across a relatively small change in climate, because exchangeable phosphate and base cations declined consistently during long-term pedogenesis. However, the proportions of total P in organic form was greater along wetter chronosequences. We conclude that soil and nutrient availability on the coastal sand plains of southwestern Australia change consistently during long-term pedogenesis, despite marked variation in modern vegetation and climate. The four chronosequences provide a rare soil-age x climate framework within which to study long-term ecosystem development.

Highlights

- We describe four long-term coastal dune chronosequences spanning a climate gradient in a global biodiversity hotspot.
- Pedogenesis involves depletion of phosphorus and cations linked to decalcification and subsequent podzolization.
- Climate has relatively little effect on patterns of nutrient availability during ecosystem development along the chronosequences.
- The age by climate framework enables study of effect of edaphic change on above- and below-ground communities.

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Peppermint trees shift their phosphorus-acquisition strategy along a strong gradient of plant-available phosphorus by increasing their transpiration at very low phosphorus availability

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Abstract Some plant species use different strategies to acquire phosphorus (P) dependent on environmental conditions, but studies investigating the relative significance of P-acquisition strategies with changing P availability are rare. We combined a natural P availability gradient and a glasshouse study with 10 levels of P supplies to investigate the roles of rhizosphere carbohydrates and transpiration-driven mass flow in P acquisition by Agonis flexuosa. Leaf P concentrations of A. flexuosa decreased and leaf manganese (Mn) concentrations increased with decreasing soil P concentration along a dune chronosequence. In the glasshouse, in response to decreasing P supply, shoot growth and root length decreased, leaf P and Mn concentrations decreased, rhizosphere carbohydrates decreased, transpiration rate and transpiration ratio increased and the percentage of root length colonized by arbuscular mycorrhizal fungi was unchanged. Although it was proved leaf Mn concentration was a good proxy for rhizosphere carbohydrate amounts in the glasshouse study, the enhanced plant P acquisition at low P supply was related to transpiration-induced mass flow rather than carbohydrates. We deduced that the higher leaf Mn concentrations in low soil P availability of the field were likely a result of increased mass flow. In summary, as soil P availability declined, A. flexuosa can shift its P-acquisition strategy away from a mycorrhizal mode towards one involving increased mass flow.

Keywords Agonis flexuosa - Arbuscular mycorrhizal fungal - Leaf manganese concentration - Mass flow - Phosphorus supply - Leaf phosphorus concentration - Transpiration

Introduction

Generally, soils in old landscapes tend to be severely P-impoverished due to long-term erosion, teaching and occlusion in geological time (Walker et al. 1976). In ecosystems in such landscapes, net primary productivity tends to be limited by low soil P availability (Jalbert et al. 2012). However, some of severely nutrient-impoverished regions are also biodiversity hotspots, and this includes south-western Australia (Myers et al. 2000; Hopper and Gioia 2004). The variety of plant P-acquisition strategies that plants have evolved in landscapes with very low soil P availability (Lambers et al. 2010; Zenonik et al. 2015).

Numerous plant responses and adaptations to enhance P acquisition in P-impoverished environments have been reported (Lambers et al. 2008, 2010). Three common types of adaptation are mycorrhizal symbioses, specialized root structures, and rhizosphere carbohydrates. By far the most common of these strategies are arbuscular mycorrhizal symbioses (Smith and Read 2008), which increase a plant's acquisition of soil P because they increase the volume of soil explored by the plant through growth of a hyphal network.
Greater root phosphatase activity in nitrogen-fixing rhizobial but not actinorhizal plants with declining phosphorus availability

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Summary

1. The abundance of nitrogen (N)-fixing plants in ecosystems where phosphorus (P) limits plant productivity poses a paradox because N fixation entails a high P cost. One explanation for this paradox is that the N-fixation strategy allows greater root phosphatase activity to enhance P acquisition from organic sources, but evidence to support this contention is limited.

2. We measured root phosphomonoesterase (PME) activity of 10 N-fixing species, including rhizobial legumes and actinorhizal Alnus species, and eight non-N-fixing species across a regressive soil chronosequence showing a clear shift from N to P limitation of plant growth and representing a strong natural gradient in P availability.

3. Legumes showed greater root PME activity than non-legumes, with the difference between these two groups increasing markedly as soil P availability declined. By contrast, root PME activity of actinorhizal species was always lower than that of co-occurring legumes and not different from non-N-fixing plants.

4. The difference in root PME activity between legumes and actinorhizal plants was not reflected in a greater or similar reliance on N fixation for P acquisition by actinorhizal species compared to co-occurring legumes.

5. Synthesis: Our results support the idea that N-fixing legumes show high root phosphatase activity, especially at low soil P availability, but suggest that this is a phylogenetically conserved trait rather than one directly linked to their N-fixation capacity.

Keywords: Fabaceae, nitrogen paradox, nutrient-acquisition strategies, organic phosphorus, phosphomonoesterase, plant-soil (below-ground) interactions, soil chronosequence

Introduction

Biological nitrogen (N) fixation from N-fixing symbiotic associations involving micro-organisms and vascular plants is the primary source of N input in many terrestrial ecosystems (Cleveland et al. 1999). Symbiotic N fixation enhances plant performance on N-poor soils (Vitousek et al. 2002; Mengel, Lichtenstein & Angèle-Pérez 2014), but should not be favoured on phosphorus (P)-impoverished soils (Houlton et al. 2008; Hedin et al. 2009) because symbiotic N fixation entails a high P cost (Spence & Raven 1985; Vitousek & Howarth 1991; Hartwig 1998; Sprung 1999; Raven 2012). However, plants possessing the capacity to form a symbiosis with N-fixing bacteria (hereafter referred to as ‘N-fixing’ plants or species) are abundant in many ecosystems with strongly weathered, P-impoverished soils such as lowland tropical rain forests, where P rather than N is likely to limit plant productivity (Cousens 1999; Hedin et al. 2009). This has been referred to as the ‘nitrogen paradox’ (Hedin et al. 2009).

One potential explanation for the nitrogen paradox is that the ability to symbiotically fix N could allow a greater investment in extracellular phosphatase enzymes, since enzymes are N-rich organic molecules (Houlton et al. 2008). Phosphatase enzymes catalyse the hydrolysis of organic P esters, releasing...
Leaf manganese accumulation and phosphorus-acquisition efficiency

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Plants that deploy a phosphorus (P)-mobilising strategy based on the release of carbohydrates tend to have high leaf manganese concentrations (Mn)\textsuperscript{[1]}. This occurs because the carbohydrates mobilise not only soil inorganic and organic P, but also a range of micronutrients, including Mn. Concentrations of most other micronutrients increase to a small extent, but Mn accumulates to significant levels, even when plants grow in soil with low concentrations of exchangeable Mn availability. Here, we propose that leaf Mn can be used to select for genotypes that are more efficient at acquiring P when soil P availability is low. Likewise, leaf Mn can be used to screen for belowground functional traits related to nutrient-acquisition strategies among species in low-P habitats.

**Phosphorus-acquisition strategies**

Here we explore the idea of using leaf Mn to indicate a carbohydrate-releasing P-acquisition strategy. The rationale behind this contention is that the availability of both P and Mn are increased when roots release carbohydrates into the rhizosphere [1] (Figure 1; see Glossary). The availability of some other micronutrients is also enhanced, but, at least in these data, leaf Mn does not lead to a signal as strong as that provided by Mn. The release of carbohydrates into the rhizosphere is important for P acquisition, because they mobilise not only inorganic P, but also organic P, which can be a major fraction of soil P, especially when P availability is low [2].

Addressing this topic is timely, because there is a growing interest among plant ecologists in belowground functional traits, to complement the suite of ‘easy-to-measure’ aboveground traits [3]. Furthermore, because of the gradual decline in phosphate rock that is used to produce P fertilisers [4], there is an increasing need for more P-efficient cropping systems [5]. Therefore, a simple tool to screen for P-acquisition efficiency in crop species would be welcomed by agronomists and plant breeders.

**Glossary**

- **Arbuscular mycorrhizal**: a type of mycorrhizal association that forms arbuscules on root hair tips and highly branched hyphae (endomycorrhiza) in the cortex of roots of the host.
- **Carbohydrate**: an organic anhydride, which is the organic acid minus the water. For example, glucose is the carbohydrate released from the degradation of the organic acid, gluconic acid.
- **Cluster**: a group of cells that conserve resources, usually high affinity, with a root (e.g., barnyard grass, sugarcane, sunflower).
- **Cluster roots**: a suite of leaves or leaf clusters that function as a single root (e.g., sugarcane), extending the root systems in the rhizosphere, thus facilitating access to exchangeable nutrients (e.g., P) in the soil.
- **Citronellal**: a small, highly volatile compound that, in woody species, has a strong, pleasant smell that is used in perfumery and in foods.
- **Heavy metals**: metals with a mass density exceeding 6.0 g ml\(^{-1}\).
- **Hydrocarbons**: plants that typically accumulate 100 times more of a specific heavy metal than the surrounding air that occurs in non-vegetation plants growing in the same environment. For most elements, including Pm, the threshold concentration is 0.006 g g\(^{-1}\) dry weight (DW), except for zinc (2.000 g g\(^{-1}\) DW), iron (1.5 g g\(^{-1}\) DW), and cadmium (0.050 g g\(^{-1}\) DW).
- **Organic phosphorus transporter (ORT)**: a transporter associated with the uptake of Mn from the rhizosphere into root cells. It is highly specific and transports other nutrients and nutrients.
- **Micronutrient**: organic phosphorus that a plant requires in relatively small quantities, such as copper, iron, Mn, molybdenum, and zinc.
- **Mycorrhiza**: a structure arising from a symbiotic association between a mycorrhizal fungus and the root of a higher plant. The mycorrhizal fungus (endomycorrhizal) forms a network of fine, thread-like structures that extend into the soil and transport nutrients to the host plant.
- **Natural resistance associated mycorrhiza protein (NRAMP)**: a differentiation transporter associated with the uptake of transition metals, such as copper, iron, Mn, and zinc.
- **Non-mycorrhizal plant family**: a plant family whose members tend to be mycorrhizal (e.g., acorns).

Manganese as a plant nutrient

The significance of Mn as an essential plant nutrient was firmly established in 1922 [6]. More recent work has revealed the role of Mn in redox processes, as an activator of a large range of enzymes, and as a cofactor of a small number of enzymes, including proteins required for light-induced water oxidation in photosystem II [7,8]. Crop plants that contain 50 μg Mn g\(^{-1}\) dry weight (DW) in their