Phosphorus-use efficiency of *Rytidosperma* species: understanding the morphological and physiological mechanisms that confer efficiency

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Summary

Phosphorus (P) is an essential element for plant growth. However, rock phosphate, which is used to manufacture P fertilisers, is a non-renewable resource. Australian soils have low levels of plant-available P and plants native to these environments are reputed to be well adapted to low-P soils. This thesis examined growth rates, P-utilisation efficiency and root morphological adaptations of *Rytidosperma* species to P fertilisers. *Rytidosperma* comprises an ideal genus to examine the response of Australian grasses to P fertilisers as it forms the dominant component of many grasslands. Preliminary data suggested there are a range of growth rates and P-utilisation efficiencies amongst *Rytidosperma* species in response to P fertiliser.

The growth response of nine *Rytidosperma* species relative to fast-growing *Bromus hordeaceus* and *Lolium perenne* was examined in Chapter 2. Growth response was measured as: maximum shoot yield, shoot yield in low-P soil, critical external P requirement (amount of P required for 90% of maximum growth) and agronomic P-use efficiency (shoot yield per unit P applied). There was a range of growth responses amongst the *Rytidosperma* species including slow and fast growth. Three *Rytidosperma* species, *R. duttonianum*, *R. racemosum* and *R. richardsonii* grew as fast as *B. hordeaceus* and *L. perenne* at high and low P supply. Species that were fast-growing at high P supply also had fast growth at low P supply, except for *B. hordeaceus*, which grew fast at high P supply, but had the lowest shoot yield at low P supply. Of the fast-growing *Rytidosperma* species, *R. duttonianum* and *R. racemosum* had a high critical external P requirement and lower agronomic P-use efficiency, whilst *R. richardsonii* had a low critical external P requirement and high agronomic P-use efficiency.

Shoot morphology traits and P-utilisation efficiency (PUE), was assessed using alternative measures of shoot P concentration or its reciprocal in Chapter 3. No measure of PUE was correlated with critical external P requirements of the species. However, one measure of PUE, shoot dry mass per unit shoot P when assessed at a common shoot P content, was positively correlated with maximum shoot yield. *Rytidosperma duttonianum*, *R. richardsonii*, *B. hordeaceus* and *L. perenne* mostly had similarly high PUE. However, the shoot morphology traits of these fast-growing *Rytidosperma* species differed from *B. hordeacues* and *L. perenne*. The *Rytidosperma* species had smaller leaf areas, lower specific leaf area, higher P content per unit leaf area and higher leaf dry
matter contents. These traits are associated with nutrient conservation and slow-growing species. However, *R. duttonianum* and *R. richardsonii* retained these traits without penalty to their shoot yields.

Root morphological traits associated with P uptake and critical external P requirement were examined in Chapters 4 and 5. Generally, species that had a larger root mass, longer root length and larger root hair cylinder volume (RHCV) took up more P and grew more quickly at all levels of applied P. Several root morphological traits were examined and it was found that no single trait was responsible for universally increasing root size and P uptake. Rather, different species utilised different traits. Hence, all root morphological traits should be considered in the context of root mass, root length and RHCV. There was no distinct grouping of fast- and slow-growing species, or critical external P requirements amongst *Rytidosperma* species with respect to specific root morphological traits. Therefore, root organic anions of two fast-growing and two slow-growing *Rytidosperma* species were examined. There was no clear difference in total carbon and carbon associated with organic anions exuded amongst the *Rytidosperma* species. Larger amounts of organic anions were detected at low P supply than at high P supply, indicating that low-P stress may stimulate organic anion exudation for some *Rytidosperma* species. Citrate and malate were detected in the highest amounts for the *Rytidosperma* species with high critical external P requirements. As *R. richardsonii*, a species with low critical external P requirement, is also reputed to exude citrate, the role of organic anions on the critical external P requirement of *Rytidosperma* species remains uncertain.

Of the *Rytidosperma* species, *R. richardsonii* cv Taranna was singled out on multiple occasions. It was comparable to *L. perenne* in shoot yield and PUE, but had a lower critical external P requirement and greater ability to accumulate shoot mass per unit leaf area. These characteristics make *R. richardsonii* of strong interest for use in low-P, productive pastures.
Statement of Candidate Contribution

The research presented in this thesis is an original contribution to the field of plant biology. For Chapters 2, 3 and 4 the hypotheses, experiments presented and discussions are my own original ideas and writing. Important contributions were made by my supervisors, Richard Simpson, Alan Richardson, Megan Ryan and Hans Lambers, in developing the research topic, critiquing hypotheses and preparation of this thesis. Assistance was also provided in experimental work including the collection of data and statistical analysis. These contributions are acknowledged as co-authors or in the acknowledgements of each chapter.

This thesis was completed during the course of enrolment in a PhD course at The University of Western Australia, in conjunction with CSIRO Agriculture, and has not been used previously for a degree or diploma at any institution.

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Publications arising from this thesis

The following manuscripts have either been published or have been submitted to scientific journals.


Chapter 4: Waddell HA, Simpson RJ, Ryan MH, Lambers H, Garden DL, Richardson AE. Root morphology and its contribution to a large root system for phosphorus uptake by *Rytidosperma* species (wallaby grass). Prepared for submission to Plant and Soil Conference proceedings (refereed)


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Chapter 1. General introduction and review of the literature
Introduction

Rock phosphate, which is used for the manufacture of phosphorus (P) fertilisers, is a non-renewable resource of which high-quality reserves are being depleted (Fixen and Johnston 2012; Scholz and Wellmer 2013). In order to maintain agricultural productivity, improvements of the P-acquisition and P-utilisation efficiency of crop and pasture species is required. Morphological and physiological adaptations, which enable plants to forage and mine the soil for P (i.e. P-acquisition efficiency) and utilise P for greater biomass gains (i.e. P-utilisation efficiency) are important. The native Australian pasture grasses of the *Rytidosperma* genus are valued for their occurrence in low-fertile soils where other pasture species are not able to persist (Scott and Whalley 1982; Scott and Whalley 1984; Boschma and Scott 2000; Garden et al. 2003). It is thought that there are a range of growth potentials and P-use efficiencies amongst *Rytidosperma* species (Bolger and Garden 1999). This thesis aims to examine the P-use efficiency of several *Rytidosperma* species and the shoot and root morphological and physiological adaptations which may confer P efficiency.

Soil phosphorus in agriculture

Phosphorus is an essential element for plant growth and development. However, it is often one of the more limiting nutrients in natural and agricultural systems (Vance et al. 2003). Many Australian soils have intrinsically low plant-available P concentrations (Handreck 1997) resulting in the need for addition of P fertilisers to maintain and increase the primary productivity of agricultural systems. Most P fertilisers are derived from rock phosphate. Whilst there is ongoing debate as to when ‘peak-P’ will occur, i.e. where global demand equals supply, following which demand will exceed supply, it is acknowledged that high-grade rock phosphate reserves are a finite resource and are currently being depleted resulting in increasing fertiliser costs (van Kauwenbergh 2010; Fixen and Johnston 2012; Scholz and Wellmer 2013).

Plants take up P from the soil solution in the orthophosphate forms $\text{H}_2\text{PO}_4^-$ and $\text{HPO}_4^{2-}$, both of which also readily form less-available complexes with iron and aluminium oxides and hydroxides in acidic soils and with calcium in alkaline soils (Vance et al. 2003). When P forms these complexes, plants are unable to readily take up the P until it has been desorbed (Holford 1997). Whilst continuing reactions between the P and the
soil particles release orthophosphate for plant uptake, this does not occur at a fast enough rate to maintain high agricultural productivity (Holford 1997; Simpson et al. 2011). Australian farms have low net farm-gate P-balance efficiency, the ratio of P outputs to P inputs, of approximately 1:2 for cropping and 1:5 for pasture systems (McLaughlin et al. 1992; Weaver and Wong 2011). A major cause of these P-balance inefficiencies is high P-sorbing soils which accumulate P as sparingly available phosphate.

Strategies for increasing the P-balance efficiencies of agricultural systems and reduce P input requirements generally use a multi-disciplinary approach. Cordell et al. (2009) advocates the use of manure, crop residues and animal and human excreta as an alternative form of P fertilisers to ensure that P is recycled back to agriculture. Others have focussed on the form and placement of P fertilisers to improve fertiliser P-uptake efficiencies (Holloway et al. 2001; McLaughlin et al. 2011). There are two key plant-based strategies, P-utilisation efficiency and P-uptake efficiency, both of which should potentially be addressed. Phosphorus-utilisation efficiency is the ability of the plant to use P, once taken up, to produce more dry matter. Improvements in P-utilisation efficiency allow plants to produce more per unit of P absorbed and plant material is removed from a field, this may also reduce the export of P from the farm system (Venkelaas et al. 2012). In grazing systems, the extra yield per unit plant P is potentially an advantage; however, it is important than P concentrations in herbage do not fall below the thresholds needed for grazing animal nutrition (Winks 1990, Simpson et al. 2011). Phosphorus-uptake efficiency refers to the ability of plants to acquire P from the soil. Improved P-uptake efficiency enables plants to achieve higher growth rates at low concentrations of plant-available P in soil, typically due to better nutrient foraging or mining by their roots (Richardson et al. 2011; Lynch 2007). This can improve yield in low P soils, but in itself is only a sustainable strategy when P export (through higher P-uptake) is replaced by P fertiliser applications (i.e. P input). Phosphorus fertiliser application to infertile soil is often accompanied by P sorption that reduces the effectiveness of the fertiliser application and leads to accumulation of P in the soil (McLaughlin et al. 2011). The rate of P sorption reactions is dependent on P concentration in the soil solution, the intrinsic chemistry of the soil and temperature (Barrow 1999). Plants with higher P-uptake efficiency are consequently expected to slow the rates of P sorption in such soils because they achieve maximum growth rates at
lower available P concentrations. In turn, this should reduce the amounts of P fertiliser inputs needed for high yields (Simpson et al. 2014).

Plant adaptations to low phosphorus availability

P-uptake efficiency

Plant P-uptake efficiency is influenced by both “root foraging” and “soil mining”. Root foraging includes root architectural and morphological adaptations to increase P acquisition from the topsoil, the layer of the soil where most P is concentrated (McLaughlin et al. 1990). Plants can increase root foraging potential through an increase in biomass allocated to roots relative to shoots, a decrease in root angle to maximise root growth in the topsoil, and a decrease in lateral root density, which may be followed by an increase in density when nutrient-rich soil patches are encountered (Lynch 2007). There are also strategies plants may use to reduce the metabolic construction cost of roots involved in soil exploration. For example, an increase in specific root length can be achieved through a decrease in root diameter or through a decrease in root tissue density due to the formation of large air spaces, aerenchyma, in the root cortex (Lynch and Ho 2005; Wahl and Ryser 2000).

Root hairs and mycorrhizal fungi also play an important role in P acquisition through “root foraging” for many species. For example, Gahoonia et al. (1997) found a strong correlation between an increase in root exploration as measured by root hair length and surface area, and the depletion of P within the rhizosphere of a low-P soil for barley (*Hordeum vulgare* L. cvs. Hamu, Angora, Alexis, Canut) and wheat (*Triticum aestivum* L. cvs. Kraka, Kosak, Foreman). Further studies using rye (*Secale cereale* L. cv. Petkus II) demonstrated the uptake of P by root hairs and subsequent translocation of P to the shoots (Gahoonia and Nielsen 1998). Arbuscular mycorrhizal fungi (AMF) increase the potential surface area for P uptake and enable greater exploration of the soil than possible by root hairs alone as they extend into soil past the root zone where root hairs are present and enter into soil particles root hairs cannot get to (Smith and Reid 2008). A large volume of literature shows the presence of AFM to greatly improve plant P uptake and growth for a large range of plant species (e.g. Jakobsen et al. 2005; Koide and Mosse 2004). However, the applicability of these, mostly glasshouse-derived results, to field conditions is often unclear. For instance, within annual crops and pastures in southern Australia, there is no clear evidence that AFM can make an
agronomically-relevant contribution to crop P nutrition or yield, particularly when managed for optimal production at high levels of available soil P (Ryan and Kirkegaard 2012).

Specialised root structures such as cluster roots enable some plants to modify unavailable forms of P to organic forms that are available for plant uptake. Cluster roots are dense clusters of lateral roots (rootlets) covered in root hairs that form in specific regions of the root systems, typically in soil horizons that have high total P, but low plant-available P, concentrations (Lambers et al. 2006). In a process referred to as ‘P-mining’, cluster roots modify the soil chemistry through the release of exudates, particularly organic anions (carboxylates), in the small region immediately surrounding the cluster root (Lambers 2006, Ryan et al. 2001). Organic anions increase P availability in the rhizosphere by altering the surface characteristics of soil particles due to ligand exchange and ligand-promoted dissolution reactions; through the chelation of cations (particularly Al$^{3+}$, Fe$^{3+}$ and Ca$^{2+}$) by triboxylates (e.g. citrate$^{3-}$) and dicarboxylates (e.g. malate$^{2-}$) (Jones 1998; Ryan et al. 2001); and through the concomitant release of protons which, in alkaline soils, increase the acidity of the rhizosphere and thus increase the solubility of sparingly-available inorganic P compounds and/or affect the kinetics of orthophosphate adsorption-desorption reactions (Dinkelaker et al. 1989; Hoffland et al. 1989; Neumann and Römheld 1999). White lupin (*Lupinus albus* L.) is an example of a species that forms cluster roots and exudes carboxylates under low P stress. Some pot experiments have examined the potential use of white lupins in crop rotations with other species that lack similar adaptation. Inclusion of lupins allowed the other species access to P that would otherwise have been unavailable (Nuruzzaman et al. 2005). However, despite the potential promise of cluster-root forming species, there has so far been a lack of widespread incorporation of these species into agricultural systems. Challenges include the poor persistence of carboxylates in the soil, competition between intercropped species, a lack of understanding of how to best utilise this P-mining strategy to improve the P efficiency of agricultural systems and the economic reality that lupins do not command a reliably high price on grain legume markets (Lambers et al. 2006, Richardson et al. 2011).

Many non-cluster root forming species, including agriculturally important species such as canola (*Brassica napus* L.) and chickpea (*Cicer arietinum* L.), also exude carboxylates. However, the concentration of these carboxylates is species dependent and
generally less than that of cluster-root forming lupin species (Pearse et al. 2006; Ryan et al. 2012). The role of these carboxylates in plant P uptake is not fully understood. One hypothesis is that root exudates may also indirectly increase orthophosphate availability through interactions with the soil biota (Richardson et al. 2011). This occurs through the actions of beneficial associations whereby microorganisms break down soil detritus and mineralise nutrients, or harmful associations whereby microorganisms immobilise and/or consume orthophosphate thus reducing plant-available P. To date, the precise role of many types of root exudates remains unclear and further, studies have focussed on a small number of exudates types at a time (Richardson et al. 2011).

Internal P-utilisation efficiency

Improvements in plant internal P-utilisation efficiency have also been examined as a means to improve growth and biomass accumulation through improved use of P within plant tissue (Rose et al. 2011; Veneklaas et al. 2012). High photosynthetic P-use efficiency can be achieved in leaves that are relatively thin and have a low leaf mass per unit area (LMA) such as barley (Hordeum vulgare L.), or in thick leaves with a high LMA such as some Banksia species (Lambers et al. 2011), which have sunken stomata (Lambers et al. 2012). However, the end-use of a plant species must be taken into account when breeding species of greater internal P-efficiency. The very low shoot P concentration and high LMA, associated with slow growth and heavy investment in cell wall material, as in Banksia species, is undesirable for fast-growing crop and pasture plants, and for livestock health (Richardson et al. 2011).

Potential of Rytidosperma species

Historically the potential of native Australian species for use in agriculture has been considered to be poor due to perceived slow growth, low feed value and poor responses to applied fertilisers. The complete replacement of native species with introduced species has been advocated as ‘the only means’ to improve stocking density and livestock production (Donald 1970; Moore 1970). These views may have persisted today with more recent anecdotal and modelling studies claiming that profitability can best be achieved if more than 85% of pastures within a property are sown with exotic pasture species (Oram and Lodge 2003), and that the fertilisation of pastures that contain native species leads to the dominance of exotic weeds of low feed value (Vere et al. 2002). Despite these authors advocating the re-sowing of native pastures with
exotic pasture species, many native grassland species have persisted. In the cool-
temperate regions in eastern and southern Australia, native grasses form the dominant 
component of grasslands (Garden et al. 2000a; Pearson et al. 1997).

*Rytidosperma* is a genus of native Australian perennial grasses commonly known as 
wallaby grass which naturally occur in grasslands that have not previously been sown or 
re-sown with introduced species for several years (Garden et al. 2001). Particularly 
within the temperate areas of south-eastern Australia, *Rytidosperma* species are valued 
as a component within pastures as they are known to occur in acidic and infertile soils 
where other beneficial pasture species are not able to persist (Waters et al. 2009). Some 
*Rytidosperma* species have been identified as being as nutritious and palatable for 
livestock grazing as other highly-regarded exotic pasture species (Archer and Robinson 
1988). There are few commercially-available cultivars of *Rytidosperma* species, for 
example the Native Seeds website which is key source of *Rytidosperma* seeds (Chivers 
2014) lists just seven cultivars, *R. bipartitum* cv. Bunderra, *R. caespitosum* cv. Evans, 
*R. richardsonii* cv. Taranna and *R. tenuius* cv. Wirra. However, these cultivars are not 
widely sown by farmers due to the higher costs of seeds relative to those of other exotic 
seeds (Chivers 2014; Oram and Lodge 2003). Rather, many farmers rely on existing 
mature plants and seeds, combined with specific management practices to maintain 
naturally-occurring *Rytidosperma* species within their pastures (Garden et al. 2000b).

*Rytidosperma* species may provide insights into how P efficiency can be improved in 
agricultural systems. *Rytidosperma richardsonii* has been studied more than most other 
*Rytidosperma* species because seed of the cultivar Taranna was one of the first to be 
made commercially available (Oram and Lodge 2003). *Rytidosperma richardsonii* cv. 
Taranna has a low critical internal P concentration (1.16 mg P g DW⁻¹) of the whole 
shoot at 90% of maximum growth, in comparison with nine other pasture species 
including subterranean clover (*Trifolium subterraneum* L.) (1.46 mg P g DW⁻¹) (Hill et 
al. 2005). Further, *R. richardsonii* has a lower critical external P requirement (8.2 mg P 
kg⁻¹ soil), which is the amount of applied P required for 90% of maximum growth, in 
comparison with nine other species including subterranean clover (24.3 mg P kg⁻¹ soil) 
(Hill et al. 2005). This suggests that *R. richardsonii* has the two characteristics which 
Föhse et al. (1988) suggests are in the ideal P efficient plant, that is, high P uptake 
efficiency and a low internal P requirement.
Root morphological adaptations in *R. richardsonii* which may contribute to a higher rate of P acquisition relative to that of other species such as subterranean clover and barley grass (*Hordeum leporinum* Link) have been identified. These include an increase in root mass fraction (RMF), mycorrhizal colonisation, root hair length on lateral roots and SRL with decreasing P supply (Hill et al. 2006; Hill et al. 2010). The increase in SRL may have been driven by a decrease in root diameter and root mass density, both of which enable greater root foraging per root mass investment (Eissenstat 1991). Despite the plasticity demonstrated by *R. richardsonii*, it has a lower RMF, lower level of mycorrhizal colonisation, shorter root hairs and lower SRL compared to the other species examined in that study, including annual ryegrass (*Lolium rigidum* L.), and subterranean clover (Hill et al. 2006; Hill et al. 2010). Further, *R. richardsonii* is also reputed to exude citrate (2230 nmol g⁻¹ FW h⁻¹), a type of organic anion, when under low-P stress at a rate similar to that reported for white lupin (up to 2380 nmol g⁻¹ FW h⁻¹) (Barrett and Gifford 1999; Ryan et al. 2001).

Other *Rytidosperma* spp. may also be P efficient. Of particular interest is the reported P-efficiency of *R. erianthum*, *R. richardsonii*, *R. fulvum* and *R. pilosum*, which had a lower critical external P requirement for growth (average 18 kg P ha⁻¹) in comparison with *R. duttonianum*, *R. racemosum*, *R. auriculatum*, and *R. carphoides* (average 40 kg P ha⁻¹) (Bolger and Garden 1999). These species were also categorised according to high yielding, low critical P response (*R. fulvum*); low yielding, low critical P response (*R. pilosum*, *R. richardsonii*, and *R. erianthum*); high yielding, high critical P response (*R. racemosum*, and *R. duttonianum*); and low yielding, low critical P response (*R. carphoides* and *R. auriculatum*) (Bolger and Garden 1999). However, there is a lack of statistical analysis underpinning these groupings.

Despite the apparent potential of Australian native species such as *Rytidosperma* species to improve the P efficiency of agricultural systems, challenges exist. In particular, P toxicity at elevated levels of P supply have been reported for some Australian species, notably those in the Proteaceae family (Parks et al. 2000; Shane et al. 2004). However, although P toxicity has also been reported in some Australian herbaceous species, it is not widespread (Pang et al. 2010). A negative correlation between the occurrence and biomass produced by *Rytidosperma* species and increased P supply has also been recorded (Dorrough et al. 2011; Garden et al. 2003). It was further suggested that several *Rytidosperma* species may be “P sensitive” (Dorrough et al. 2011), although it is
not clear whether the authors specifically were referring to P toxicity. These field-based studies are confounded by many variables such as rainfall which are not always accounted for. Further, due to difficulties in identifying *Rytidosperma* species using vegetative traits alone (Humphreys et al. 2010; Linder et al. 2010), several species are often considered as one entity in field-based studies and differences in growth response by individual species to soil nutrient levels may have been overlooked.

An important consideration in harnessing the potential of native plants adapted to low-P soils is the pasture production potential of the native species relative to common exotic species. Studies of native Australian herbaceous legumes indicate some species such as *Cullen australasicum* (Schltdl.) J.W. Grime and *Ptilotus polystachyus* (Gaudich.) F. Muell., can yield as well as exotic species such as lucerne (*Medicago sativa* L.) and chicory (*Chicorium intybus* L.), especially at low levels of P supply (Ryan et al. 2009; Suriyagoda et al. 2010). However, many native Australian species may not consistently produce greater shoot mass than exotic species at the high P supply which farms may operate at (Pang et al. 2009). *Rytidosperma richardsonii* has been reported as having a similar relative growth rate as subterranean clover and annual ryegrass (Hill et al. 2005); however, no data exist for other *Rytidosperma* species.

In order to better understand whether *Rytidosperma* species have the potential to improve the P efficiency of agricultural systems it is necessary to confirm the growth responses of several species to P supply independent of other confounding factors. Further, confirmation of the different critical external P requirements of *Rytidosperma* species as reported by Bolger and Garden (1999) may provide insights into how P-efficient and P-inefficient species differ in shoot and root adaptations to low P supply. This may contribute to our understanding of how P efficiency of other agricultural species can be improved.

**Thesis aims, objectives and structure**

This thesis aimed to examine the growth responses of a number of *Rytidosperma* species to P supply, characterise root and shoot adaptations to low P supply, and relate these to the management of *Rytidosperma* grasslands. Experimental work comprised a series of glasshouse and growth cabinet experiments; the decision to undertake experiments under these conditions was based on the need to minimise confounding
effects of inter-species competition, different soil types and rainfall (Garden et al. 2001; Garden et al. 2000b; O'Dwyer and Attiwill 1999; Waters et al. 2009) on growth responses of *Rytidosperma* species.

The key objectives of this thesis were to:

- Determine the aboveground growth responses of nine *Rytidosperma* species, and two comparison species, perennial ryegrass (*Lolium prenne* L.) and soft brome (*Bromus hordeaceus* L.), to six levels of P supply ranging from no added P to 46 mg P kg⁻¹ soil) (Chapter 2)
- Examine the implications for the management of *Rytidosperma* grasslands with P fertilisation of differing growth responses of nine *Rytidosperma* species to six levels of P supply (Chapter 2)
- Verify the external P-use efficiency of nine *Rytidosperma* species (Chapter 2)
- Investigate P-uptake and internal P-use efficiency of nine *Rytidosperma* species (Chapter 3)
- Investigate shoot morphological traits of three fast-growing *Rytidosperma* species and examine how these traits differ from those of other fast-growing exotic pasture grass species, *L. perenne* and *B. hordeaceus* (Chapter 3)
- Investigate multiple root morphological adaptations known to affect root P foraging and mining of soil by nine *Rytidosperma* species when grown with six levels of P supply and compare adaptations between fast- and slow-growing species (Chapter 4)
- Investigate differences in amounts and composition of root exudates between two fast-growing and two slow-growing *Rytidosperma* species and compare these results with root exudates of four other agriculturally-important species (white lupin, sub clover, perennial ryegrass and soft brome) (Chapter 5)
- Draw the main findings together and discuss in Chapter 6, including recommendations for future work.

This thesis is presented as a series of three scientific papers, with one additional unpublished Results chapter, and is consistent with the Postgraduate and Research Scholarship Regulation 1.3.1.33(1) of the University of Western Australia, Australia. Three manuscripts have been submitted or prepared for publication and these form Results Chapters 2, 3 and 4. There are six main chapters in this thesis; General
Introduction; four experimental Results Chapters, of which three have been submitted or prepared for publication; and a General Discussion. The General Introduction covers the broad background and justification of the thesis objectives. A detailed and comprehensive literature review is presented at the beginning of each Results Chapter. Whilst each Results Chapter has been presented in the format of scientific papers, they are linked together as detailed in the Introduction and Discussion within each Results Chapter. The presentation of this thesis as a series of scientific papers has unavoidably resulted in some repetition, especially within the methodology. However, overlap has been kept to a minimum. Further, different formatting styles appear in each chapter, a reflection of the requirements of each scientific paper. All other chapters follow the style of Plant and Soil (Chapter 4). Finally, the General Discussion further links the scientific papers together, draws together the main findings of all Results Chapters and explores the significance of these findings.
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Chapter 2. Differential growth response of *Rytidosperma* species (wallaby grass) to phosphorus application and implications for grassland management
Differential growth response of *Rytidosperma* species (wallaby grass) to phosphorus application and its implications for grassland management

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Abstract

*Rytidosperma* species (formerly *Austrodanthonia*) are native grasses common in temperate grasslands of southern Australia. Nine *Rytidosperma* species, *Lebanon perenne* and *Brasilius horridacrum* were grown as microswards in pots in a glasshouse, and their growth response to six levels of applied P was measured. Shoot yield differed up to twofold between the highest- and lowest-yielding *Rytidosperma* species. Some *Rytidosperma* species were slow growing with minimal ability to respond to increased soil P availability. However, three species, *Rytidosperma authesioides*, *Rytidosperma tomentosum* and *Rytidosperma richardsonii*, had a similar shoot yield to *L. perenne*. Species that grew well at high P also grew well at low P, except *B. horridacrum*, which was the lowest-yielding species at low P, but had among the highest yields at high P. No species showed evidence of P toxicity. The species exhibited a range in critical external P requirement (i.e., amount of P applied for 90% maximum yield). Among the fast-growing *Rytidosperma* species, *R. richardsonii* was notable because it had a low critical external P (16.3 mg P pot⁻¹) and high agronomic P-use efficiency (94.1 g DW g⁻¹ P applied). In contrast, *R. authesioides* had a higher critical external P requirement (22.4 mg P pot⁻¹) and lower agronomic P-use efficiency (85 g DW g⁻¹ P applied). It was concluded that it is important to know which *Rytidosperma* species are present in a grassland to understand how they may respond to P fertilisation. The results help to explain the diverse opinions expressed about the productivity of pastures containing *Rytidosperma* species.

Keywords: agronomic efficiency, *Austrodanthonia*, critical phosphorus requirement, glasshouse experiment, pasture, perennial grass

Introduction

Pastures that comprise a mixture of perennial species and annual grasses and legumes are valued for their relative stability and productivity in the face of environmental and management stresses such as variation in rainfall, soil nutrient levels, weeds and grazing pressure (Sanford et al., 2003). However, the value of native Australian perennial grasses for use in pastures has often been considered to be relatively poor, due to perceived slow growth, low feed value and lack of response to fertilizers. Based on anecdotal evidence and modelling data, it has been suggested that the input of fertilizers to pastures that contain native species leads to the dominance of introduced annual grasses and broadleaf weeds of low feed value (Vere et al., 2002). The replacement of native species with introduced species that are responsive to phosphorus (P) fertilizers has been advocated as the only means of improving stocking density and livestock production from grazed pastures (Donald, 1970; Moore, 1970). These views have persisted and have contributed to farmers nominating native perennial grasses, such as *Rytidosperma* species and *Melaleuca supicea* (Labill.) R.Bru., as having similarly low value to introduced weeds, despite recent evidence of a useful role for these grasses within a pasture (Garden et al., 2009a).

*Rytidosperma* species, previously known as *Austroalpina* (Council of Heads of Australasian Herbaria, 1990),...
are native perennial grasses found in grasslands and pastures of the cool-temperate regions of Australia (Garden et al., 2001, 2003). There are seventy-three Rytidosperma species across New Guinea, Australia, New Zealand and South America (Linder et al., 2010), with approximately forty species present in Australia (Flora of Australia, 2009). Rytidosperma species have a number of beneficial attributes; for example, several species have been observed on soils of low pH (Bowling et al., 1996; Waters et al., 2009) (Table 1) although there are no data on long-term persistence. Populations of naturally occurring Rytidosperma plicatum, Rytidosperma richardsonii and Rytidosperma racemosum have been observed around the perimeter of sheep camps where grazing and nutrient levels were high (Scott and Whalley, 1984) (Table 1). Based on observations of the occurrence of several species at sites where grazing is known to occur, it has been hypothesized that some Rytidosperma species may be tolerant of grazing stress (Scott and Whalley, 1984; Garden et al., 2001; Waters et al., 2009; Dorrough et al., 2011). Danthonia longifolia (syn. Rytidosperma bipartitum, Rytidosperma plicatum) is reported to produce year-round highly nutritious and palatable feed, similar to that of the introduced species Festuca arundinacea Schreb. (tall fescue) and Phalaris aquatica L. (phalaris canary grass) (Archer and Robinson, 1988). In field studies, R. richardsonii cv. Taranu persisted better under drought and defoliation stress than did Lolium perenne L. (perennial ryegrass) and Dactylis glomerata L. (cocksfoot) (Boschma and Scott, 2000). A similar result for other Rytidosperma species has been found by Garden et al. (2003), and experimental results have confirmed drought tolerance for three other Rytidosperma species: R. caespitum, R. duttonianum and R. racemosum (Bolger et al., 2005). However, the close taxonomic affinity between many species combined with the fact that it is not possible to identify species using vegetative traits alone often results in the grouping of Rytidosperma species in grassland studies. Consequently, the association between species and their habitat can be difficult to define. Where information on a particular species is available, typically only some habitat characteristics have been recorded (Table 1).

An increase in basal cover by Rytidosperma species has been recorded when soil nutrient levels, especially soil P, were increased (Garden et al., 2003). However, this finding contrasts with that of other reports, where a negative correlation between the occurrence and the biomass produced by Rytidosperma species has been found after increases in soil P availability (Garden et al., 2003; Dorrough et al., 2011). Dorrough et al. (2011) concluded that Rytidosperma species may be P-sensitive. However, it was unclear whether the authors meant the grasses could not tolerate high soil P levels or were adversely affected by increased interspecific competition in high-P soil. Field-based observations such as these may be confounded by other variables such as past management practices (Michalk et al., 2003; Sanford et al., 2003), soil type (Garden et al., 2001), rainfall (Garden et al., 2000b), competition with other species (Simpson and Langford, 1996; O’Dwyer and Attiwill, 1999) and grazing (Garden et al., 2003). For example, Garden et al. (2003) reported a decline in the representation by Rytidosperma species in a fertilized grazing experiment and noted that decreases in representation coincided with years in which above-average rainfall favoured annual species over perennials. The objective of the glasshouse studies reported here was to quantify the response of nine Rytidosperma species to P application. The Rytidosperma species were compared with two high-yielding introduced grasses. It was hypothesized that there would be differences in the applied P requirements for growth of different species within the Rytidosperma genus.

**Materials and methods**

**Plant material**

Nine species of Rytidosperma were examined: R. auriculatum (J.M. Black) Connor and Edgar, R. carphoides (F. Muell. ex Benth.) Connor and Edgar, R. duttonianum (Cashmore) Connor and Edgar, R. erianthum (Lindl.) Connor and Edgar, R. fulvum (Vickery) A.M. Humphreys and H.P. Linder, R. pilatum (R.B.) Connor and Edgar, R. racemosum (R.Br.) Connor and Edgar, R. richardsonii (Cashmore) Connor and Edgar cv. Taranu and R. setaceum (R.Br.) Connor and Edgar. For most species, seed was collected from naturally occurring plants from a single site at Yeaville near Hall, NSW (35°1′ S, 153°5′ E), as part of a field experiment (Garden et al., 2000b). Species were differentiated using seed morphological characteristics (Flora of Australia, 2009; Council of Heads of Australasian Herbaria, 2011). Seed was then increased by growing species outdoors in well-watered pots, which were spatially isolated to minimize possible cross-fertilization between species (Brock and Brown, 1961). However, R. fulvum was collected from Wyreeda near Dalgety, NSW (36°28′ S, 148°51′ E), and R. richardsonii was obtained from a commercial source (NSW Department of Primary Industries). These two Rytidosperma species were included as there was some information regarding their growth responses to applied nutrients (Bolger and Garden, 1999; Garden et al., 2001; Hill et al., 2005; Waters et al., 2009). Bromus inermis Leyss. (syn. Bromus mollisiformis), collected from the Ginninderra Experiment Station, Canberra, ACT (35°10′ S, 149°11′ E), was used as a control species.
<table>
<thead>
<tr>
<th>Species</th>
<th>Soil nutrient characteristics of topsoil (0-10 cm) of naturalized populations (Dowling et al., 1996)</th>
<th>Site characteristics of naturally occurring species (Scott and Whalley, 1984)</th>
<th>Critical external P requirement and shoot yield (Bolger and Garden, 1999)</th>
<th>Probability of occurrence relative to other species in response to soil P and grazing (Dorrough et al., 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. curtulatum</td>
<td>pH (CaCl₂) range: &lt;4.2 to &gt;4.8; modal value: &lt;4.2</td>
<td>Low-yielding, high critical external P</td>
<td>Negative correlation between occurrence and increasing soil P; tolerant of &quot;moderate&quot; (3 dry sheep equivalent ha⁻¹) levels of livestock grazing</td>
<td></td>
</tr>
<tr>
<td>R. acrophylax</td>
<td>pH (CaCl₂) range: &lt;4.2 to &gt;4.8; modal value: &lt;4.2</td>
<td>Low-yielding, high critical external P</td>
<td>Negative correlation between occurrence and increasing soil P; grazing-tolerant</td>
<td></td>
</tr>
<tr>
<td>R. duttonianum</td>
<td>pH (CaCl₂) range: 4.2–4.8, modal value: &lt;4.2</td>
<td>High-yielding, high critical external P</td>
<td>Negative correlation between occurrence and increasing soil P; grazing-tolerant</td>
<td></td>
</tr>
<tr>
<td>R. austromontanum</td>
<td>pH (CaCl₂) range: &lt;4.2 to &gt;4.8; modal value: &lt;4.2</td>
<td>Low-yielding, low critical external P</td>
<td>Moderately tolerant of a range of soil P concentrations and grazing intensities</td>
<td></td>
</tr>
<tr>
<td>R. fulvum (syn. Danthonia Siekki var. fulv)</td>
<td>pH (CaCl₂) range: 4.3–8.64</td>
<td>High-yielding, low critical external P</td>
<td>Low level of persistence across a range of soil P concentrations and grazing intensities</td>
<td></td>
</tr>
<tr>
<td>R. filiforme</td>
<td>pH (CaCl₂) range: &lt;4.2 to &gt;4.8; modal value: &lt;4.2</td>
<td>Low-yielding, low critical external P</td>
<td>Negative correlation between occurrence and increasing soil P; tolerant of &quot;moderate&quot; (3 dry sheep equivalent ha⁻¹) levels of livestock grazing</td>
<td></td>
</tr>
</tbody>
</table>

Sheep camps in fields under continuous stocking that had received 625 kg P ha⁻¹. Unclipped area sporadically grazed never fertilized.
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Soil nutrient characteristics of topsoil (0-10 cm) of naturalized populations (Dowling et al., 1996; Waters et al., 2009)</th>
<th>Site characteristics of naturally occurring species (Scott and Whalley, 1984)</th>
<th>Critical external P requirement and shoot yield (Bolger and Garden, 1999)</th>
<th>Probability of occurrence relative to other species in response to soil P and grazing (Dorrough et al., 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. racemosum</em></td>
<td>pH (CaCl₂) range: &lt;4.2 to &gt;4.8; modal value: &gt;4.8</td>
<td>Sheep camps in fields under continuous stocking that had received 625 kg superphosphate ha⁻¹</td>
<td>High-yielding, high critical external P</td>
<td>Moderately tolerant of a range of soil P concentrations and grazing intensities</td>
</tr>
<tr>
<td><em>R. richardsonii</em></td>
<td>Pasture under heavy grazing and never fertilized</td>
<td>Undeared area sporadically grazed never fertilized</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. santonum</em></td>
<td>Total P range: 4–31 mg P kg⁻¹ soil; pH (CaCl₂) range: 4.59–8.64</td>
<td></td>
<td>Low-yielding, low critical external P</td>
<td></td>
</tr>
</tbody>
</table>

Note: *Total* P is stated in Waters et al. (2009). However, these numbers indicate the values are more likely to be extractable P (method of extraction unknown). The values do suggest the sites are mostly low P (infertile) sites.
149°02'E, elevation 597 m), and *L. perenne* cv. Victorian (CleanSeeds Pty Ltd.) were used as comparison species because they were expected to exhibit contrasting responses to soil P fertility (Hill et al., 2005).

**Plant growth conditions and experimental design**

Plants were grown in a Yellow Chromosol soil (Isbell, 2002) collected from an unfertilized pasture at Ginninderra Experiment Station. Surface soil of 20–150 mm depth was sieved (5 mm) and steam-sterilized for 45 min at 70°C. Pots (90 mm diameter x 200 mm depth) were packed with 1.3 kg of dry soil. Initial soil properties are given in Table 2. Seeds were sown at a rate of 25 mg viable seed per pot. Pots were wrapped in reflective aluminium sleeves, and the height of the sleeve was increased with plant height to mimic the light conditions experienced within a grass stand and to ensure that equivalent incident radiation was experienced by all plants.

Plants were grown in a glasshouse in Canberra, Australia, from June to August 2011, the southern hemisphere winter (Experiment 1), or October to November 2012, the southern hemisphere spring (Experiment 2). Air temperature was partially regulated to permit temperatures to range between 23°C (day) and 12°C (night). Pots were arranged in a randomised block design with each block rearranged twice per week to account for any temperature or light variations within the glasshouse (Poorter et al., 2012). Pots were watered daily with demineralized water to 80% of field capacity.

**Table 2**: Initial properties of the soil used in the experiment.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH††</td>
<td>4.3</td>
</tr>
<tr>
<td>Phosphorus††</td>
<td>9 mg kg⁻¹</td>
</tr>
<tr>
<td>Phosphorus buffer index‡‡</td>
<td>0.40</td>
</tr>
<tr>
<td>Sulphate sulphur§</td>
<td>2.2 mg kg⁻¹</td>
</tr>
<tr>
<td>Calcium¶</td>
<td>0.95 cmol kg⁻¹</td>
</tr>
<tr>
<td>Potassium¶</td>
<td>0.46 cmol kg⁻¹</td>
</tr>
<tr>
<td>Magnesium¶</td>
<td>0.16 cmol kg⁻¹</td>
</tr>
<tr>
<td>Sodum¶</td>
<td>&lt;0.02 cmol kg⁻¹</td>
</tr>
<tr>
<td>Aluminium (KCl)***</td>
<td>0.6 cmol kg⁻¹</td>
</tr>
<tr>
<td>Cation exchange capacity††</td>
<td>2.39 cmol kg⁻¹</td>
</tr>
</tbody>
</table>

All soil analysis methods are described by Raymont and Lyons (2011): †pH in 0.1M CaCl₂ extract (method 481); ††Colwell bicarbonate extractable P (method 98); †‡Colwell phosphorus buffer index (method 95); §isolable S in KCl-10 (method 1001); §§exchangeable bases in 1 M ammonium acetate at pH 7.0 (method 1532); ***1 M KCl-extractable aluminium (exchange acidity) (method 15G1); ††cation exchange capacity is the sum of the exchangeable bases excluding aluminium.

Phosphorus treatments were established by applying P as K₂HPO₄ at six rates, i.e., 4.5, 10.5, 21, 42 and 60 mg P pot⁻¹ with five replicates. The P treatment was applied once at the beginning of the experiment as an aqueous solution sufficient to wet the top 50 mm of the soil to mimic the situation in the field, where most P is in the topsoil layer. All other nutrients were applied at regular intervals as an aqueous solution which consisted of 2 mmol MgSO₄·7·5 mmol CaSO₄, 20 mmol KNO₃, 2·5 mmol (NH₄)₂S₂O₃, and the following micronutrients: 25 μmol H₂BO₃, 46 μmol MnCl₂, 15 μmol ZnSO₄, 1·6 μmol CuSO₄, 0·7 μmol (NH₄)₂MoO₄·4·0 μmol CoCl₂ and 0·05 mmol FeNaEDTA. Each pot had received a total of 382 mL of the P-free nutrient solution by the end of the experiment.

**Harvest and measurements**

For Experiment 1, half of the plants for each P treatment were harvested at 26 d after germination, with the remainder harvested at 47 d after germination. At 26 d after germination, plants were harvested by removing them from the pots, washing them clean of soil and separating into root and shoot portions. At 47 d after germination, the youngest fully expanded blade (YEB) of ten tillers was separated from the remaining shoot portions. All plant material was dried at 70°C for 48 h before being weighed.

Experiment 2 was conducted to confirm the asymptotic yield relationship for the five highest-producing species: *B. hordeaceus*, *L. perenne*, *R. davotiana*, *R. racemosa* and *R. richardsonii*. Plants were grown for 41 d and harvested as per Experiment 1. Particular care was taken to ensure the most productive species did not overtop the reflective sleeves and potentially capture more light than lower-producing species.

**Statistical analyses**

Mitscherlich nonlinear curves of the form \( y = \frac{a - b \exp(-c x)}{x} \) for parameters \( a \), \( b \) and \( c \), response \( y \) (yield) and \( x \) (P application rate) were fitted to each species using the statistical language (R Core Team, 2013). This function was used because it often captures the curvature and the asymptotic nature of yield in response to nutrient application (Mengel et al., 2001). The growth of the species in response to soil P supply was then assessed using four criteria: maximum shoot yield, shoot yield at low P, critical external P requirement and agronomic P-use efficiency (AE).

Maximum shoot yield and critical external P requirement (the amount of P applied to achieve 90% of maximum shoot growth) were defined by the asymptote of the Mitscherlich curve, and shoot yield
at low P by the origin of the curve (y-intercept). Agronomic P-use efficiency was defined in two ways: (i) AE\textsubscript{F} = (maximum shoot yield – shoot yield at low P)/critical external P requirement of the species that required the largest amount of P to achieve near-maximum yield. This reflected a common definition of AE (e.g. Balladar et al. 2001) for use in pasture studies, where AE is defined relative to a specified amount of applied fertilizer P irrespective of the specific P requirements of each species; (ii) AE\textsubscript{E} = (maximum shoot yield – shoot yield at low P)/critical external P requirement of the species being assessed. This quantifies the efficiency with which fertilizer P is used for herbage production when applied at a rate appropriate for the assessed species and reflects the intrinsic attributes of the species that support acquisition of P from soil and its utilization for shoot growth.

Mitscherlich nonlinear growth curves fitted to shoot yield data were found to fit the data well for all species. A small number of observations were identified as having large residuals, but no observations were omitted. Estimates and confidence intervals associated with the growth response and P-use criteria were determined by least squares and assumed that the model was approximately linear around the estimate. Estimates and confidence intervals for the shoot yield at low P supply were obtained by reparameterizing the model so that the y-intercept was a key parameter. Differences between critical external P requirement values, maximum yields (i.e. the asymptotes) and the low-P yields (i.e. the intercepts) were tested by considering the estimates and approximate standard errors for each measure simultaneously, and testing the significant pairwise differences. Significance was determined by calculating a standardized difference that weighted the two contributing standard errors. Values greater than two standard errors were considered significantly different at the P = 0.05 level. No adjustment was made for multiple comparisons. For both measures of AE, standard errors were calculated using the delta method. Relations between the growth response and P-use criteria were explored by linear regression using Genstat (13th edition; VSN International, Harpenden, UK).

Results

Growth response to P applications

Experiment 1

The first harvest of Experiment 1 at 26 d post-germination was conducted to determine whether differences in light capture and growth during the period before full canopy closure would confound the analysis of each species’ growth response to P supply. However, most shoot growth (89%) occurred between 26 and 47 d from germination ($R^2 = 0.99$; Figure 1), and it was concluded that shoot yields at the second harvest at 47 d post-germination adequately reflected the growth response of all species to P supply. No further analysis was made using the shoot yields at 26 d.

All plants remained vegetative, and shoot yield of all species reached an asymptote within the P application rates tested (Figure 2a). Growth of all species was increased by application of P to the soil. However, maximum shoot yield varied 2.5-fold between the lowest yielding (R. erianthum, 1.45 g) and the highest

![Figure 1: Difference in shoot yield between 26 and 47 d after germination plotted against shoot yield at 47 d after germination for Brachytrichon hordeaceus (●), L. perenne (•), Rytidosperma curvatum (Δ), Rytidosperma cuneifolius (Ο), R. duttonianum (●), R. ohiensis (Ω), R. fukum (•), R. erianthum (●), R. richardsonii (Δ) and R. selaeceum (Ο). Data are means of 3 replicates within each treatment. Solid line indicates significant correlation.](image-url)
Figure 2. Shoot yield of Bromus hordeaceus (♀), Lolium perenne (■), Rydiosperma auriculatum (▲), Rydiosperma carphoides (○), Rydiosperma cuttonanum (●), Rydiosperma erianthum (□), Rydiosperma fukum (▲), Rydiosperma pleuran (○), Rydiosperma rachensum (△), Rydiosperma ritchsonii (△) and Rydiosperma setocoem (△) at six levels of applied P in Experiment 1 (a) and Experiment 2 (b). Data points are the mean for each species ± standard error (s.e.). The least significant difference (LSD) at P = 0.05 is given, and curves are fitted with a Mitscherlich growth response function.

yielding (L. perenne, 3.61 g) species (Figure 2a, Table 3). The highest yielding Rydiosperma species, R. cuttonanum (2.78 g) and R. richardsonii (2.37 g), produced about double the shoot biomass of the lowest yielding Rydiosperma species, R. auriculatum (1.54 g), R. carphoides (1.58 g) and R. erianthum (1.45 g). Shoot yield in soil to which no P had been applied varied threefold between the lowest (B. hordeaceus, 0.37 g) and the highest (L. perenne, 1.11 g) yielding species (Figure 2a, Table 3).

There was no significant relationship between the maximum potential yield of a species and the yield achieved in the low-P soil (Figure 3a). However, B. hordeaceus was an outlier as it had a high potential maximum yield and low shoot yield in the low-P soil. When B. hordeaceus was removed from the regression, a correlation between maximum potential yield of a species and its growth in the low-P soil was evident for the other species (P < 0.001; R² = 0.77). At intermediate levels of applied P (4.5 and 10.5 mg P pot⁻¹), there was a positive correlation (P < 0.001; R² = 0.68 and 0.92 at 4.5 and 10.5 mg P pot⁻¹, respectively) between maximum shoot yield and yield achieved at lower soil fertility (Figure 3b,c). However, B. hordeaceus remained an outlier.

Experiment 2

In Experiment 2, shoot yield at low rates of P application varied 3.5-fold between the lowest-yielding (B. hordeaceus, 0.22 g) and highest-yielding (R. racemosum, 0.76 g) species (Figure 2b, Table 3). However, R. racemosum seed heads emerged during this experiment and its shoot yield response was very different to that observed in Experiment 1. The other species remained vegetative, and their maximum shoot yields
Table 3 Measures of Puse efficiency including the maximum shoot yield as determined by Mitchelmore asymptote; shoot yield at low applied P as determined by the origin of the asymptote curve; critical external P requirement at 95% of maximum yield (mg P pot⁻¹); agronomic P-use efficiency (AEₚ) defined as the shoot yield due to applied P divided by the critical external P requirement of the species with the highest P requirement for growth; agronomic P-use efficiency (AEₚ) defined as the shoot yield at a level of applied P divided by the critical P requirement of the species assessed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Maximum shoot yield (g)</th>
<th>Shoot yield at low P (g)</th>
<th>Critical external P (mg P pot⁻¹)</th>
<th>Agronomic P-use efficiency (AEₚ) (g DW g⁻¹ P)</th>
<th>Agronomic P-use efficiency (AEₚ) (g DW g⁻¹ P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>3.61 ± 0.10 a</td>
<td>1.11 ± 0.12 a</td>
<td>15.0 ± 1.2 ab</td>
<td>81.9 ± 6.1 ab</td>
<td>126.9 ± 11.7 a</td>
</tr>
<tr>
<td>Bromus hordeaceus</td>
<td>3.31 ± 0.07 b</td>
<td>0.37 ± 0.08 c</td>
<td>22.4 ± 1.6 ef</td>
<td>72.0 ± 3.0 b</td>
<td>85.0 ± 7.2 bc</td>
</tr>
<tr>
<td>Rytidosperma dactylosum</td>
<td>2.78 ± 0.06 c</td>
<td>0.88 ± 0.07 ab</td>
<td>22.4 ± 1.6 ef</td>
<td>72.0 ± 3.0 b</td>
<td>85.0 ± 7.2 bc</td>
</tr>
<tr>
<td>Rytidosperma richardii</td>
<td>2.57 ± 0.06 d</td>
<td>0.84 ± 0.08 abc</td>
<td>16.3 ± 1.7 cd</td>
<td>58.1 ± 4.7 c</td>
<td>94.1 ± 11.2 ab</td>
</tr>
<tr>
<td>Rytidosperma ramosum</td>
<td>2.13 ± 0.07 c</td>
<td>0.61 ± 0.07 cd</td>
<td>24.7 ± 2.6 ef</td>
<td>55.3 ± 5.4 c</td>
<td>59.2 ± 7.0 d</td>
</tr>
<tr>
<td>Rytidosperma fulvum</td>
<td>1.90 ± 0.05 f</td>
<td>0.76 ± 0.06 bc</td>
<td>16.8 ± 1.8 cd</td>
<td>43.1 ± 3.6 d</td>
<td>67.5 ± 8.4 cd</td>
</tr>
<tr>
<td>Rytidosperma pinnatum</td>
<td>1.80 ± 0.04 fg</td>
<td>0.71 ± 0.06 bc</td>
<td>13.9 ± 1.5 bc</td>
<td>41.3 ± 3.4 d</td>
<td>78.3 ± 9.6 bcd</td>
</tr>
<tr>
<td>Rytidosperma pilosum</td>
<td>1.75 ± 0.05 g</td>
<td>0.72 ± 0.06 bc</td>
<td>11.0 ± 1.3 ab</td>
<td>39.0 ± 3.3 d</td>
<td>93.5 ± 12.6 ac</td>
</tr>
<tr>
<td>Rytidosperma caespitodes</td>
<td>1.58 ± 0.03 h</td>
<td>0.53 ± 0.05 de</td>
<td>17.1 ± 1.5 cd</td>
<td>39.8 ± 3.9 d</td>
<td>61.6 ± 6.2 d</td>
</tr>
<tr>
<td>Rytidosperma aristulatum</td>
<td>1.54 ± 0.04 hi</td>
<td>0.48 ± 0.05 e</td>
<td>14.6 ± 1.5 bc</td>
<td>40.6 ± 3.2 d</td>
<td>72.6 ± 8.4 bcd</td>
</tr>
<tr>
<td>Rytidosperma ericetum</td>
<td>1.45 ± 0.04 i</td>
<td>0.40 ± 0.07 de</td>
<td>7.9 ± 1.4 a</td>
<td>35.7 ± 3.7 d</td>
<td>119.3 ± 24.2 ab</td>
</tr>
</tbody>
</table>

Within Experiment 1 and Experiment 2 in each column, treatments marked with different letters indicate significantly different means (P < 0.05).

Phosphorus requirements for growth

The critical external P requirements for species differed 3-3-fold between the lowest (R. ericetum, 7.9 mg P pot⁻¹) and the highest (B. hordeaceus, 26.4 mg P pot⁻¹) species (Table 3). The relative rankings for critical external P requirement among the species used in Experiments 1 and 2 were consistent, except for R. racemosa, for which seed heads had emerged during Experiment 2. The critical external P requirement of R. racemosa was lower in Experiment 2 relative to that observed in Experiment 1. There was a positive correlation (P < 0.005; R² = 0.47) between critical external P requirement and maximum shoot yield (Figure 4a). The lower-yielding Rytidosperma species generally had lower critical external P requirements. Rytidosperma richardii was notable as a fast-growing species with a consistently low critical external P requirement (Table 3). However, across all species, variation in maximum yield only explained 47% (linear regression) of the variation in critical external P requirement.

Agronomic efficiency estimated relative to a single maximum rate of P application to the soil (AEₚ, Table 3) varied threefold between the highest (B. hordeaceus) and lowest (R. ericetum) species, and there was a positive linear (P < 0.001; R² = 0.92) correlation with the maximum potential yield of each species. When determined using the shoot yield and critical external P requirement of each species, agronomic efficiency (AEₚ) varied twofold between the highest (L. perenne, 121.4 g DW g⁻¹ P) and the lowest (R. racemosa, 59.2 g DW g⁻¹ P) species. There was no correlation between AEₚ and maximum shoot yield (Figure 4b).

Discussion

Growth response to rate of P application

All of the grasses examined in this experiment increased their shoot yield in response to increased P supply. However, the maximum growth rates of the Rytidosperma species in high P soil differed markedly.
Figure 3  Shoot yield at 0 (a), 4.5 (b) and 10.5 mg applied P per pot (c) graphed relative to maximum shoot yield as determined by the asymptote of the fitted Mitscherlich function for: Bromus hordeaceus (●), Lolium perenne (■), Rytidosperma auriculatum (▲), Rytidosperma carpoides (◇), Rytidosperma duttonianum (○), Rytidosperma erianthum (■), Rytidosperma julianum (◇), Rytidosperma plicatum (+), Rytidosperma racemosum (∆), Rytidosperma richardsonii (▲) and Rytidosperma setosum (○). Data points are means ± s.e. solid lines are linear correlation of all species with 95% confidence intervals and the broken line (a) is the linear correlation for all species with B. hordeaceus omitted.

(Figure 2), Several Rytidosperma species (e.g. R. auriculatum, R. carpoides and R. erianthum) grew relatively slowly and did not respond further to higher rates of P application. The asymptotic growth pattern of all species suggests no P toxicity at the higher rates of P supply. Phosphorus toxicity is relatively common among Australian plant species from nutrient-impoverished environments when they are fertilized with P (Handreck, 1997; Shane et al., 2004), and Dorrrough et al. (2004) have previously described Rytidosperma species as having a “negative response to P”. The introduced grasses, L. perenne and B. hordeaceus, were highly responsive to P applications, and three Rytidosperma species (R. duttonianum, R. racemosum and R. richardsonii) also yielded well in fertile soil. The actual shoot yields of R. duttonianum and R. richardsonii did not differ markedly from those of the introduced grasses in the high-P soil in Experiment 2. The relative response of R. racemosum could not be confirmed, because it did not remain vegetative under the warmer, longer day-length conditions that prevailed in October–November, the southern hemisphere spring.
For most species, there was a significant positive correlation between the yield at high P supply and yield at low P supply (Figure 3). This reflects the common observation of short-term experiments that an intrinsic capacity for faster shoot and root growth confers a greater ability to capture limiting nutrients as a result of more effective root foraging (Mahmoud and Grime, 1976; Campbell et al., 1991; Lambers and Poorter, 1992; Ryser and Lambers, 1995). However, *B. hordeaceus* was an outlier in this respect, and despite having among the highest growth rates when grown in the fertile soil, it was the least-productive species at low P supply. Hill et al. (2005) also observed low shoot biomass of *B. hordeaceus, Holcus lanatus* L. (Yorkshire fog) and *Trifolium subterraneum* L. (subterranean clover) at no applied P, relative to maximum shoot biomass at high P. It is hypothesized that *B. hordeaceus* may have been unable to maintain root foraging scale and/or precision, defined as the ability of a species to proliferate roots in nutrient-rich patches (Lambers and Poorter, 1992; Kembel et al., 2008), at the lowest levels of P nutrition. *Bromus hordeaceus* has previously been found to have a high total root length at high P relative to that at low P (Hill et al., 2010).

**Efficiency of phosphorus use**

The critical external P requirement of a plant (defined here as the amount of P applied to achieve 90% of maximum shoot growth) provides a useful guideline for fertilizer use in grassland management (Simpson et al., 2014) and can indicate evolutionary adaptation to a given habitat (Table 1). Although there was a continuum from low to high growth rates, a number of the *Rytopsperma* species examined in this study fit the definition of slow-growing species (Grime and Hunt, 1975; Lambers and Poorter, 1992) given their low maximum shoot yields (Table 3). Slow growth is
a common adaptation to low-nutrient environments (Grime and Hunt, 1975; Claydon, 1980; Lambers and Poorter, 1992). The observed slow growth of the present species aligns with the widely held view that many native Australian grasses and forbs are well adapted to infertile soils (Moore, 1970; Pang et al., 2010; Suriyagoda et al., 2010). It should not be assumed, however, that the widespread occurrence of low soil fertility in natural Australian grasslands precludes the occurrence of highly productive native species, or species that have high-P requirements. In the present study, three Rytodopserma species were potentially as productive as the sown (L. perenne) and weedy (B. hordeaceae) grasses with which they were compared. These fast-growing species had a range of critical external P requirements with evidence for both high (R. obtusifolium, B. hordeaceae) and low (R. richardsonii, L. perenne) critical external P requirement. This is consistent with data previously reported for B. hordeaceae, R. richardsonii and Lolium rigidum (Hill et al., 2005), which is an annual species closely related to L. perenne (Balloufier et al., 1998). Rytodopserma richardsonii cv. Taranza was particularly notable among the Rytodopserma species because it had a similar critical P requirement to that of two of the slower-growing species, R. ariocaulum and R. carphodes, and was more productive than either of these species in the fertilized and unfertilized soil. This cultivar was selected from a single plant of a natural population from the New England Tablelands of northern New South Wales and was chosen primarily for high seed yield (Lodge, 1993). However, it was noted as being as productive as other undomesticated accessions of the same species (Lodge and Schipp, 1993).

There was no correlation between the critical agronomic efficiency (AE) and critical external P requirement, or between AE and maximum shoot biomass. Hence, the AE of these species does not indicate why differences in critical external P requirements can be found within the more productive Rytodopserma species. Although the critical external P requirement of a species is a measure of its fertilizer requirement, the amount of fertilizer that is applied to a grassland may be determined by the other species present in the pasture. In many temperate Australian grasslands, where nitrogen inputs are derived from pasture legumes, it is the P requirement of the legume that determines how much P fertilizer needs to be applied to achieve the production goals of a farm (Simpson et al., 2014). Legumes, including white clover (Trifolium repens L.) and subterranean clover (T. subterraneum), have higher P requirements than the grasses with which they are typically grown (Ortman et al., 1969; Davis, 1991; Hill et al., 2005). Under these circumstances, the alternative calculation of agronomic P-use efficiency (AEp) is important, because it demonstrates that high potential yields dictate which species will make best use of the fertilizer that is being applied (Baligar et al., 2001). Although B. hordeaceae can be classed as a P-responsive and productive grass, its failure to thrive in low-P soil is not ideal given that many farmers choose to operate with low-to-moderate soil P fertility, or find themselves in circumstances where changing management objectives results in reduced P fertilizer use.

**Soil fertility management in Rytodopserma species grasslands**

Most of the Rytodopserma species examined in this study were collected from a single grassland grazed by sheep and maintained at a low level of soil P fertility (deficient for maximum potential pasture growth; extractable $P = 7.3\ mg\ kg^{-1}$ (Bray and Kurtz, 1945)). In this grassland, 59% of the herbage mass was represented by Rytodopserma species (Garden et al., 2000b). The seed sources for two species in the present study were exceptions to this; R. fulvum was only a minor component at the site where seed of most species was collected, and was obtained from another site. Rytodopserma richardsonii was not found in the local area, but was included because it was one of the few commercially available cultivars of Rytodopserma (Lodge, 1993; Lodge and Schipp, 1993) and has a low P requirement for growth (Hill et al., 2005). The relative abundance of each species within the grassland was not recorded. This is often the case in Rytodopserma grasslands because it is not possible to identify species using vegetative characteristics alone (Humphreys et al., 2010). The objective of using seed sourced largely from a single location was to explore the likely responses to fertilizer use by coexisting species of Rytodopserma.

Grasslands containing Rytodopserma species typically have several other species present including introduced legumes and forbs. Commonly they contain more than three Rytodopserma species (Waters et al., 2009), with up to ten species reported for one grassland on the Monaro Tableland of New South Wales, Australia (Dowells et al., 2012). These grasslands are natural pastures and are not sown because seed is generally not available or is very expensive. Given the large differences in shoot yields and P responses among Rytodopserma species in the present study, it is likely that problems in understanding the impact of soil P fertility on grassland botanical composition will arise unless the growth characteristics of the species present are known. For example, if low-yielding Rytodopserma, such as R. ariocaulum, R. carphodes or R. erianthum, form the dominant component of the
grassland at the time of initial fertilizer application, it would not be surprising that grasslands containing native species may be viewed as low-yielding and unresponsive to P fertilizer (Donald, 1970). The botanical composition of *Rytidosperma* grasslands is usually considered to be relatively stable when only low amounts of P fertilizers are applied (Garden et al., 2000b). The large range in growth responses to P supply also indicates that sustained use of P fertilizers is likely to change the botanical composition of a pasture towards more P-responsive *Rytidosperma* species. This could be expected to also potentially increase the productivity of the grassland. These results and inconsistencies in other reported responses of *Rytidosperma* grasslands to P fertilizer highlight the need to identify which *Rytidosperma* species are present in a grassland when examining the response to P fertilizer use. Waters et al. (2009) also stressed the importance of identifying which species are present for understanding the influence of soil acidity on *Rytidosperma* species distribution and grassland botanical composition. They hypothesized that failure to identify the *Rytidosperma* species in other botanical surveys was likely to be the reason why soil acidity had not been found to influence grassland composition, despite known differences between *Rytidosperma* species in acid-soil tolerance (Dowling et al., 1996; Iblain et al., 2006; Waters et al., 2009; Table 1). The difficulty in identifying *Rytidosperma* species in botanical surveys based on vegetative traits alone (Linder et al., 2010) will inevitably confound the development of sound grassland management protocols. More detailed methods of species identification based on molecular phylogeny using chloroplast and nuclear ribosomal DNA sequence markers combined with morphological and ecological data (Humphreys et al., 2010; Linder et al., 2010) may need to be used to resolve these issues.

**Conclusions**

All *Rytidosperma* species in this study increased shoot biomass with increasing P supply. No *Rytidosperma* species showed signs of P toxicity at any of the P levels applied. There was a wide range of potential growth rates among species. Three *Rytidosperma* species (*R. antirrhonum*, *R. australophilum* and *R. richardsonii*) had high shoot yields relative to other species at both low and high levels of applied P. *Rytidosperma richardsonii* was particularly notable as a fast-growing species with a consistently low critical external P requirement. This study highlights the importance of identifying *Rytidosperma* species present in grasslands when examining the productivity, yield responses to P fertilizer and impacts of P fertilizer on botanical composition.

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**References**


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Chapter 3. Phosphorus utilisation efficiency and leaf morphology traits of *Rytidosperma* species (wallaby grasses) that differ in their growth response to phosphorus fertilisation.
Abstract

Rytidosperma species are perennial grasses found in cool temperate grasslands of Australia. The species differ in their intrinsic growth rates, response to phosphorus (P) fertiliser application and critical external P requirements (P required for 90% maximum growth). This study examined whether internal P utilisation efficiency (PUE) by Rytidosperma species influenced these differences. The PUE of nine Rytidosperma species and two grasses of Mediterranean origin: Bromus hordeaceus L. and Lolium perenne L. was assessed using alternative measures of shoot P concentration or its reciprocal. Plants were grown in pots in a glasshouse with six levels of applied P for 47 days (Experiment 1) or 41 days (Experiment 2). No measure of PUE was correlated with the critical external P requirements of the species and most measures of PUE were not correlated with their potential growth rates. However, shoot dry matter per unit P when assessed at a common shoot P content was the exception ($P<0.001; R^2 = 0.86$).

All of the fast-growing species (B. hordeaceus, L. perenne, R. duttonianum (Cashmore) Connor & Edgar and R. richardsonii (Cashmore) Connor & Edgar) exhibited high PUE, but PUE varied substantially among the slower-growing species. The fast-growing Rytidosperma species differed in the contribution that area-based P concentration of leaves and specific leaf area (SLA) made to the achievement of high PUE, and they retained shoot morphology traits normally associated with slow-growing species such as smaller leaf area, smaller SLA and higher leaf dry matter content.
Preface

This chapter follows on from Chapter 2 in that it refers to the same experiments as those presented in Chapter 2 (i.e. Experiment 1 conducted over 47 days and Experiment 2 conducted over 41 days). Chapter 3 examines the P concentration, P-utilisation efficiency and shoot morphological traits in order to better understand the growth rates observed in Chapter 2.

Introduction

*Rytidosperma* species (formerly *Austrodanthonia*) are perennial grasses found in grasslands and pastures in cool-temperate regions of Australia (Garden *et al.* 2001; Garden *et al.* 2003). Traditionally native Australian grasses have been considered to be adapted to low phosphorus (P) soils. For example, *Rytidosperma* species form the dominant component of grasslands that have no or low levels of P fertilisers applied (Garden *et al.* 2003). Similar to other native species, *Rytidosperma* species have also been considered to be unresponsive or ‘sensitive’ to P fertilisers (Donald 1970; Dorrough *et al.* 2011). Indeed, Garden *et al.* (2003) recorded a decrease in the occurrence and yield of *Rytidosperma* species with increasing levels of P fertiliser. However, these field-based observations may have been confounded by variables such as grazing and competition with other species (Simpson and Langford 1996; Garden *et al.* 2000; Garden *et al.* 2003).

The growth of nine *Rytidosperma* species in response to applied P was examined without such confounding variables by Waddell *et al.* (2015). A range of growth responses to P fertilisers was observed amongst the *Rytidosperma* species. For example, *R. auriculatum*, *R. carphoides* and *R. erianthum* were slow-growing and poorly responsive to P fertiliser applications. In contrast, *R. duttonianum* and *R. richardsonii* were highly responsive to P fertiliser and grew as fast as *Bromus hordeaceus* and *Lolium perenne*, fast-growing species of Mediterranean origin, at both high and low P supply. The P uptake efficiency of the *Rytidosperma* species varied substantially. The slow-growing species generally had low critical external P requirements (the amount of P required for 90% of maximum growth). However, *R. richardsonii* also achieved a low critical external P requirement equivalent to that of some slow-growing species, despite its capacity for fast growth (Waddell *et al.* 2015).
Little is known about the internal efficiency with which *Rytidosperma* species utilise P for shoot growth (i.e. phosphorus utilisation efficiency; PUE), or whether PUE contributes to the differing responses of *Rytidosperma* species to P applications, or to their contrasting critical external P requirements.

There are various ways in which PUE is estimated for comparison of plant species. Typically, PUE measures are variants of either the concentration of P in the shoots, or its reciprocal: (i) critical internal P concentration, i.e. the shoot P concentration that corresponds with 90% of maximum shoot yield (White and Hammond 2008); (ii) the amount of shoot dry mass (DM) produced per unit shoot P, over a responsive P application range for the plant (termed, physiological P efficiency of shoots, PPE e.g. Baligar *et al.* 2001); and (iii) shoot DM per unit P (i.e. the reciprocal of shoot P concentration) when measured at a common shoot P content in the species being compared (PUEc, Rose *et al.* 2015).

The P concentration of shoots is often reported to differ among slow- and fast-growing species, with slow-growing species tending to have higher shoot P concentrations (Chapin 1980). Large differences in critical internal P concentrations are also known to occur among pasture species, but there are relatively few data for *Rytidosperma* species (Pinkerton *et al.* 1997; Hill *et al.* 2005).

Leaf P concentration is also used in conjunction with shoot morphology traits to understand how plants utilise P for growth. Ryser *et al.* (1997) found a strong negative correlation between leaf P productivity (plant growth rate per unit leaf P content) and the area-based P concentration of leaves (Parea, leaf P content per unit leaf area) of three grass species. Leaf P productivity was correlated with Parea irrespective of whether the species varied in their P concentration per unit DM, leaf thickness or leaf DM content (LDMC; DM per unit fresh mass). They surmised that the association between nutrient use efficiency and area-based leaf nutrient concentration may be caused by increased shading within leaves of species with higher nutrient content per unit leaf area. In a similar way, positive associations between specific leaf area (SLA) and potential growth rate have also been recorded across a wide range of species, with species from nutrient-poor habitats typically displaying slow potential growth rates and a low SLA (Poorter and Remkes 1990; Lambers and Poorter 1992; Poorter and van der Werf 1998). However, Shipley (2002, 2006) has argued that these relationships may not be universal.
because herbaceous plants can exhibit a large amount of plasticity between SLA and net assimilation rate (NAR) to maintain their growth rate at different levels of incident radiation.

In this study we assessed PUE of nine Rytidosperma species and compared them with two fast-growing species, B. hordeaceus and L. perenne. The shoot morphology and \( P_{\text{area}} \) of the three fastest-growing Rytidosperma species and B. hordeaceus and L. perenne were also examined to determine whether PUE had influenced growth responses to P supply or critical external P requirements. It was hypothesised that slow-growing species would exhibit low PUE relative to fast-growing species because of the reports that high shoot P concentrations are associated with slow-growing species. It was also hypothesised that high PUE would be associated with relatively low leaf P concentration per unit leaf area.

**Materials and Methods**

Phosphorus utilisation efficiency

Experiment 1: Eleven species were examined: Bromus hordeaceus L. (synon. B. molliformis), Lolium perenne L. cv. Victorian, Rytidosperma auriculatum (J.M.Black) Connor & Edgar, R. carphoides (F.Muell. ex Benth.) Connor & Edgar, R. duttonianum (Cashmore) Connor & Edgar, R. erianthum (Lindl.) Connor & Edgar, R. fulvum (Vickery) A.M.Humphreys & H.P.Linder, R. pilosum (R.Br.) Connor & Edgar, R. racemosum (R.Br.) Connor & Edgar, R. richardsonii (Cashmore) Connor & Edgar cv. Taranna, and R. setaceum (R.Br.) Connor & Edgar. These species have a range of growth responses to P, and some differ significantly in P uptake efficiencies as defined by the critical external P requirement and agronomic P-use efficiency (Waddell et al. 2015).

Plants were grown as described by Waddell et al. (2015). Briefly, 25 mg of viable seeds were sown into each 90 mm × 200 mm cylindrical pot, which contained 1.3 kg of dry yellow chromosol soil (Isbell 2002) and had a bicarbonate extractable P concentration of 9 mg kg\(^{-1}\) (Colwell 1963). Pots were wrapped in reflective aluminium sleeves, and the height of each sleeve was increased with increasing plant height so that approximately 1 cm of leaf blade was above the reflective sleeve at all times. This
mimicked the light conditions experienced within a grass sward and ensured equivalent incident radiation was experienced by all plants. All plants were watered daily with deionised water to 80% of field capacity. Plants were grown in a glasshouse in Canberra, Australia, without supplementary light from June to August; the southern hemisphere winter. Air temperature was partially regulated to permit temperatures to range between a maximum of 23 °C (day) and minimum of 12 °C (night). Pots were arranged in a randomised block design with each block rearranged twice per week to minimise any temperature or light variations. Phosphorus treatments were established by adding P as KH₂PO₄ as an aqueous solution, sufficient to wet the top 50 mm of the soil, at six rates: nil, 4.5, 10.5, 21, 42 and 60 mg P per pot. All other nutrients were applied at regular intervals as an aqueous solution, which consisted of 2 mM MgSO₄, 7.5 mM CaSO₄, 20 mM KNO₃, 2.5 mM (NH₄)₂SO₄, and the following micronutrients; 23 µM H₃BO₃, 46 µM MnCl₂, 15 µM ZnSO₄, 1.6 µM CuSO₄, 0.7 µM (NH₄)₂MoO₄, 1.0 µM CoCl₂, and 0.05 mM FeNaEDTA. Each pot had received a total of 382 ml of the P-free solution by the end of the experiment.

All plants were harvested at 47 days after germination. Shoot portions were separated from roots at the crown, and the youngest fully-expanded blade (YEB) of 10 tillers was collected by excising at the ligule (i.e. the junction of the leaf blade and leaf sheath). Whole shoot and YEB portions were dried at 70°C for 48 hours before being weighed.

The dry shoot and leaf samples were milled and 20-50 mg of each sample was ashed at 550°C for five hours. The ash was dissolved in 2 M HCl at 1 ml HCl per 10 mg dry weight plant material before being analysed using a modified malachite green method in which disodium phosphate (Na₂HPO₄) was used for the P standards (Matomizu et al. 1983).

Maximum shoot yield was used as a measure of the potential growth rates of the species and was determined as the asymptote of the Mitscherlich non-linear growth curve fitted to the shoot DM and P application data for each species using the R statistical language (R Core Team 2013). Standard errors were calculated using the delta method in which independence of the fixed asymptote and fixed intercept of the Mitscherlich growth curves were assumed, as reported by Waddell et al. (2015). Relative shoot yield at each level of applied P was determined as shoot yield divided by the asymptote of the Mitscherlich function fitted to shoot DM. Low relative shoot yields indicate high levels
of P stress and values close to one indicate that soil P supply was sufficient or better than that required for maximum plant growth. Shoot P contents were determined as the product of shoot P concentration and shoot DM.

Critical internal P concentration (mg P g\(^{-1}\) DM) was determined as the shoot and YEB P concentrations that corresponded with 90% of maximum shoot DM (White and Hammond 2008). The Mitscherlich model was reparameterised to determine the critical internal P concentration by calculating the shoot P concentration at the critical external P requirement of each species (Waddell \textit{et al.} 2015). Significance was determined by calculating a standardised difference that weighted the two contributing standard errors. Values greater than two standard errors were considered significantly different at the \(P \leq 0.05\) level (Moore and McCabe 2003).

Physiological P efficiency of shoots (PPE; g DM g\(^{-1}\) P) was calculated following the definition of Baligar \textit{et al.} (2001).

\[
PPE = (\text{shoot DM}_{\text{critical}} - \text{shoot DM}_{0P}) / (\text{shoot P}_{\text{critical}} - \text{shoot P}_{0P})
\]

Where: \text{shoot DM}_{\text{critical}} and \text{shoot P}_{\text{critical}} were the yield and P content of shoots at 90% of maximum yield, and \text{shoot DM}_{0P} and \text{shoot P}_{0P} were the yield and P content of shoots when no P had been applied to the soil.

Shoot DM per unit shoot P content at a common shoot P content (PUE\(_c\); g DM g\(^{-1}\) P) was determined after considering the arguments for estimating PUE proposed by Rose \textit{et al.} (2011; 2015). Shoot DM per unit shoot P content was regressed exponentially against shoot P content and PUE\(_c\) for each species was estimated, by interpolation, at the common shoot P content of 4 mg P pot\(^{-1}\). Some comparisons were also made with PUE\(_c\) determined at shoot P contents of 2 and 7 mg P pot\(^{-1}\). These estimates inevitably meant that some species were compared at different levels of P stress (e.g. at a shoot P content of 4 mg P pot\(^{-1}\) the relative shoot yield ranged between 0.59 - 0.79). Shoot DM per unit shoot P was, therefore, also determined at a common relative shoot yield of 0.7 (PUE\(_s\)) to compare PUE among the species at an equivalent level of P stress. This was determined by linear regression of shoot DM per unit shoot P content against relative shoot yield over the range in which shoot P concentrations were changing in response to P stress (i.e. relative shoot yields >0.5).
Leaf morphology and P utilisation by fast-growing species

Experiment 2: This experiment was conducted to examine leaf morphology traits considered likely to influence shoot PUE and to reconfirm the shoot yield ranking of the five fastest-growing species: *B. hordeaceus*, *L. perenne*, *R. duttonianum*, *R. racemosum* and *R. richardsonii*. By this stage of the experiments it was also recognised that these species all exhibited relatively high PUE. The experimental design for Experiment 2 was the same as that for Experiment 1, except that plants were grown in the glasshouse from October to November (the southern hemisphere spring) and were harvested at 41 days after germination. Particular care was taken to ensure these species did not overtop the reflective sleeves and potentially capture light outside of the grass sward conditions.

From each pot, five YEB were excised at the ligule and placed in deionised water in the dark and stored at 4°C for 24 – 48 hours until leaf fresh mass and leaf area (LA) were measured (Garnier *et al.* 2001b). Leaf area was estimated by recording the length and width of each fully-hydrated leaf and applying a conversion factor derived from additional YEB of each species of known length and width that had been scanned at 400 dots per inch resolution for assessment of leaf area using WinRhizo software (Régent Instruments, Quebec, Canada). From each YEB, a 1 cm section was taken at one-third the distance towards the top of the leaf blade from the ligule (van Arendonk and Poorter 1994) and placed in 70% (v/v) ethanol until leaf thickness was measured. Mean leaf thickness (LT) was calculated as the ratio of area and width of transverse sections stained with toluidine blue and observed under a Zeiss Axio Imager light microscope (Carl Zeiss Inc, Germany) (Garnier and Laurent 1994). The remainder of the YEB were then dried at 70 °C for 48 hours and DM determined. The YEB specific leaf area (SLA) and its inverse, leaf mass per unit area (LMA), were calculated as the ratio of LA to leaf DM. The YEB LDMC was calculated as the ratio of leaf DM to leaf fresh mass. All shoot structure terms, abbreviations and units are detailed in Table 1.
Table 1 Abbreviations related to phosphorus utilisation efficiency and shoot growth analysis, and the units in which they are expressed

<table>
<thead>
<tr>
<th>Trait</th>
<th>Abbreviation</th>
<th>Units</th>
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<tr>
<td>Aboveground leaf mass fraction</td>
<td>ALMF</td>
<td>g g(^{-1})</td>
</tr>
<tr>
<td>Leaf area</td>
<td>LA</td>
<td>m(^2) leaf(^{-1})</td>
</tr>
<tr>
<td>Leaf dry matter content</td>
<td>LDMC</td>
<td>mg g(^{-1})</td>
</tr>
<tr>
<td>Leaf mass per area</td>
<td>LMA</td>
<td>g m(^{-2})</td>
</tr>
<tr>
<td>Leaf thickness</td>
<td>LT</td>
<td>µm</td>
</tr>
<tr>
<td>Area based leaf phosphorus content</td>
<td>P(_{area})</td>
<td>mg P m(^{-2})</td>
</tr>
<tr>
<td>Physiological phosphorus efficiency of shoots</td>
<td>PPE</td>
<td>g shoot DM g(^{-1}) shoot P</td>
</tr>
<tr>
<td>Phosphorus utilisation efficiency</td>
<td>PUE</td>
<td>-</td>
</tr>
<tr>
<td>Shoot mass per unit phosphorus when measured at a common shoot phosphorus content</td>
<td>PUE(_{c})</td>
<td>g DM g(^{-1}) P</td>
</tr>
<tr>
<td>Shoot mass per unit phosphorus when measured at a common relative shoot yield</td>
<td>PUE(_{s})</td>
<td>g DM g(^{-1}) P</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>SLA</td>
<td>m(^2) kg(^{-1})</td>
</tr>
<tr>
<td>Youngest fully expanded blade</td>
<td>YEB</td>
<td>-</td>
</tr>
</tbody>
</table>

Five tillers (*L. perenne* and *B. hordeaceus*) or five whole plants (*Rytidosperma* species) were collected and separated into leaf blade and stem portions. In the case of *R. racemosum*, which began to form seed heads, the seed heads were considered to be part of the stems (Poorter and Remkes 1990). All other species were in the vegetative stage of growth. Leaf and stem portions, and the remainder of the shoots, which were excised from the roots at the crown, were oven dried at 70 °C for 48 hours. The aboveground leaf mass ratio (ALMR) was calculated as the proportion of leaf blade relative to the whole shoot on a mass basis.

Phosphorus content, critical internal P concentration, PPE PUE\(_{c}\) and PUE\(_{s}\) were determined as per Experiment 1. The YEB P\(_{area}\) was determined as the YEB leaf P concentration multiplied by the LMA.

Relationships among the species for P concentration, P content and shoot morphology traits were explored using ANOVA and linear regression using GenStat (15th edition;
VSN International UK). Where there was no significant species × treatment interaction for a given shoot structure, the factors were considered to be independent and a t-test was performed to test for differences (Moore and McCabe 2003).

Results

Shoot yield and response to phosphorus supply

There was a 2.5-fold difference between species with the highest- (L. perenne) and lowest- (R. erianthum) maximum shoot yields (Table 2) in Experiment 1. All species responded initially to P application, but some failed to continue responding and this led to their lower maximum yields. These species (e.g. R. auriculatum, R. carphoides and R. erianthum) were, consequently, judged to be intrinsically slow-growing (Waddell et al. 2015). Although there were differences in maximum shoot yield among the fast-growing species (B. hordeaceus, L. perenne, R. duttonianum, R. racemosum and R. richardsonii) in Experiment 1, there was no significant difference in maximum shoot yield between B. hordeaceus, L. perenne, or R. duttonianum in Experiment 2. For R. richardsonii, maximum shoot yield was significantly less than that of L. perenne; however, this difference was small (6%). Rytidosperma racemosum was notable as having the highest shoot yield in Experiment 2. However, seed heads emerged for this species in Experiment 2 and its shoot yield response was very different to that observed in Experiment 1.
Table 2: Shoot yields and measures of phosphorus (P) utilisation efficiency including: maximum shoot yield as determined from the asymptote of the Mitscherlich non-linear curve; critical external P requirement (amount of P required for 90% maximum shoot growth), from Waddell et al. (2015); critical internal P concentration of whole shoots and the youngest fully-expanded blade (YEB) corresponding with 90% of maximum shoot yield; physiological P efficiency (PPE); shoot mass per unit P when measured at a common (4 mg) shoot P content (PUEc); and shoot mass per unit P when measured at a common (0.7) relative yield (PUEs). For each experiment, within each column, treatments marked with different letters were significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Species</th>
<th>Maximum shoot yield (g)</th>
<th>Critical external P requirement (mg P pot⁻¹)</th>
<th>Critical internal P concentration (mg P g⁻¹ DW plant tissue)</th>
<th>Physiological P efficiency of shoots (g shoot DW g⁻¹ shoot P)</th>
<th>PUEc at 4 mg P content of shoots (g DM g⁻¹ P)</th>
<th>PUEs at 0.7 rel. shoot yield (g DM g⁻¹ P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole shoots</td>
<td>YEB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>3.61 ± 0.10a</td>
<td>20.6 ± 2.0de</td>
<td>2.82 ± 0.01a</td>
<td>294 ± 24a</td>
<td>538 ± 14a</td>
<td>506 ± 22a</td>
</tr>
<tr>
<td>Bromus hordeaceus</td>
<td>3.24 ± 0.07b</td>
<td>26.4 ± 1.4f</td>
<td>3.28 ± 0.01b</td>
<td>287 ± 12a</td>
<td>445 ± 11b</td>
<td>424 ± 28b</td>
</tr>
<tr>
<td>Rytidosperma duttonianum</td>
<td>2.78 ± 0.06c</td>
<td>22.4 ± 1.6ef</td>
<td>3.18 ± 0.01b</td>
<td>267 ± 17ac</td>
<td>438 ± 11b</td>
<td>380 ± 21bcd</td>
</tr>
<tr>
<td>R. richardsonii</td>
<td>2.37 ± 0.06d</td>
<td>16.3 ± 1.7cd</td>
<td>3.15 ± 0.01b</td>
<td>264 ± 23ac</td>
<td>415 ± 11b</td>
<td>433 ± 26b</td>
</tr>
<tr>
<td>R. racemosum</td>
<td>2.13 ± 0.07c</td>
<td>24.7 ± 2.5ef</td>
<td>3.84 ± 0.01c</td>
<td>218 ± 19bcd</td>
<td>358 ± 9c</td>
<td>340 ± 21cd</td>
</tr>
<tr>
<td>R. fulvum</td>
<td>1.90 ± 0.05f</td>
<td>16.8 ± 1.8cd</td>
<td>3.98 ± 0.01cd</td>
<td>205 ± 17bd</td>
<td>324 ± 9d</td>
<td>314 ± 27d</td>
</tr>
<tr>
<td>R. setaceum</td>
<td>1.80 ± 0.04fg</td>
<td>13.9 ± 1.5bc</td>
<td>3.40 ± 0.01b</td>
<td>242 ± 22ad</td>
<td>351 ± 9c</td>
<td>421 ± 32b</td>
</tr>
<tr>
<td>R. pilosum</td>
<td>1.75 ± 0.04g</td>
<td>11.0 ± 1.3ab</td>
<td>3.13 ± 0.01b</td>
<td>262 ± 25ab</td>
<td>363 ± 9c</td>
<td>464 ± 39ab</td>
</tr>
<tr>
<td>R. carphoides</td>
<td>1.58 ± 0.03h</td>
<td>17.1 ± 1.5cd</td>
<td>4.20 ± 0.01cd</td>
<td>194 ± 15d</td>
<td>318 ± 8d</td>
<td>323 ± 25cd</td>
</tr>
<tr>
<td>R. auriculatum</td>
<td>1.54 ± 0.04hi</td>
<td>14.6 ± 1.5bc</td>
<td>3.42 ± 0.01b</td>
<td>256 ± 21ab</td>
<td>322 ± 7d</td>
<td>369 ± 28bcd</td>
</tr>
<tr>
<td>R. erianthum</td>
<td>1.45 ± 0.04i</td>
<td>7.9 ± 1.4a</td>
<td>3.29 ± 0.01b</td>
<td>271 ± 32ab</td>
<td>306 ± 7d</td>
<td>422 ± 43bc</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. racemosum</td>
<td>2.66 ± 0.05a</td>
<td>15.0 ± 1.2ab</td>
<td>2.53 ± 0.01a</td>
<td>343 ± 21a</td>
<td>488 ± 8a</td>
<td>693 ± 33a</td>
</tr>
<tr>
<td>B. hordeaceus</td>
<td>2.25 ± 0.05b</td>
<td>23.3 ± 1.4c</td>
<td>3.42 ± 0.01d</td>
<td>275 ± 12b</td>
<td>399 ± 6c</td>
<td>474 ± 18b</td>
</tr>
<tr>
<td>L. perenne</td>
<td>2.20 ± 0.04b</td>
<td>14.1 ± 1.0ab</td>
<td>2.89 ± 0.01c</td>
<td>304 ± 16ab</td>
<td>406 ± 7bc</td>
<td>449 ± 18bc</td>
</tr>
<tr>
<td>R. duttonianum</td>
<td>2.19 ± 0.04b</td>
<td>15.6 ± 1.0b</td>
<td>2.80 ± 0.01bc</td>
<td>330 ± 17a</td>
<td>421 ± 7b</td>
<td>435 ± 18bc</td>
</tr>
<tr>
<td>R. richardsonii</td>
<td>2.06 ± 0.03c</td>
<td>12.2 ± 1.0a</td>
<td>2.60 ± 0.01ab</td>
<td>337 ± 23a</td>
<td>416 ± 7bc</td>
<td>400 ± 16c</td>
</tr>
</tbody>
</table>
Phosphorus concentration and uptake into YEB and whole shoots

The P concentration of the YEB increased with increasing P supply for all species in Experiments 1 and 2 (Figs. 1a, b, c). There was a significant difference in P concentration of the YEB among species, with slower-growing species (Fig. 1a) having up to 1.9-fold higher YEB P concentrations at high P supply than the faster-growing species, *B. hordeaceus*, *L. perenne*, *R. duttonianum* and *R. richardsonii* (Figs. 1b, c).

Total shoot P content was increased for all species when grown with high P supply (Fig. 1d, e, f). Faster-growing species contained up to 2.8 times more P than the slowest-growing species at no applied P, and up to 1.8 times more P at high P supply (e.g. 42 mg applied P per pot). Significant positive correlations (*P*<0.001) were evident between whole shoot P content and shoot DM at both high (P42; $R^2=0.77$) and at low (P0; $R^2=0.76$) P supply (data not shown). When no P had been applied, *B. hordeaceus* had the lowest shoot yield and P content of all species, but at high P supply only *L. perenne* contained more P and produced more shoot DM than *B. hordeaceus*. All fast-growing species, except for *R. racemosum*, had higher shoot P content in Experiment 1 than in Experiment 2. *Rytidosperma racemosum* had a similar shoot P content in both experiments, however *R. racemosum* formed seed heads in Experiment 2.
Fig. 1. Phosphorus (P) concentration of the youngest fully expanded leaf blade (YEB) (a-c) and whole shoot P content (d-f) at six levels of P supply. Shown is (a,d) the slow-growing species: *Rytidosperma auriculatum* (Δ), *R. carphoides* (◊), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+), *R. setaceum* (○), and (b,e) and the fast-growing species: *Bromus hordeaceus* (♦), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×), and *R. richardsonii* (▲) in Experiment 1 and (c,f) the fast-growing species used in Experiment 2. *Bromus hordeaceus* and *L. perenne* are depicted with dashed lines, all *Rytidosperma* species are depicted with solid lines. Error bars associated with the data points are 2 x standard error and least significant difference bars (P<0.05) for each panel are for the species x P interaction. The shaded areas represent the range of critical external P requirement of the species as determined by the amount of P required to achieve 90% of maximum shoot yield (see Table 2).
The critical internal P concentration of the whole shoots and YEB differed 1.5-fold among species with the highest concentrations found in two of the slower-growing *Rytidosperma* species (*R. carphoides* and *R. fulvum*) and the lowest concentrations often exhibited by fast-growing species (*B. hordeaceus*, *L. perenne*, *R. duttonianum* and *R. richardsonii*) (Table 2). However, some slow-growing *Rytidosperma* species (*R. auriculatum* and *R. erianthum*) also had relatively low critical internal P concentrations. In both Experiments 1 and 2, *R. richardsonii* consistently had amongst the lowest whole shoot and YEB critical internal P concentration. There was a significant positive correlation ($P<0.001; R^2=0.89$) between critical internal P concentration of whole shoots and YEB in both experiments.

**P utilisation efficiency**

Physiological P efficiency of shoots (PPE: shoot dry mass per unit shoot P) differed significantly among some species (Table 2). The fast-growing species, *B. hordeaceus*, *L. perenne*, *R. duttonianum* and *R. richardsonii*, had amongst the highest PPE. However, some slow-growing species (*R. auriculatum* and *R. erianthum*) also exhibited high PPE. The ranking among the fast-growing species for PPE was similar in Experiments 1 and 2, except for *R. racemosum* which formed seed heads in Experiment 2.

Shoot DM per unit P content was plotted against relative shoot yield (Fig. 2a, b) to examine how PUE was affected by the degree of P stress experienced by each species. For all species, the shoot DM per unit P content across all levels of P supply was high when relative shoot yield was low (high P stress), but declined rapidly when relative shoot yield increased above about 0.5 (Fig. 2a, b). When graphed relative to shoot P content, shoot DM per unit shoot P content across all levels of P supply was similarly high when shoot P content was low and declined exponentially as shoot P content increased (Fig. 2c, d).
Fig. 2. Shoot mass per unit shoot P content across all levels of P supply, plotted against (a, b) relative shoot yield and shoot P content (c, d). Shown is the slow-growing species: *Rytidosperma auriculatum* (△), *R. carphoides* (◊), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+) and *R. setaceum* (○) (a, c), and the fast-growing species: *Bromus hordeaceus* (♦), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) (b,d). Insets (a, b) are whole shoot P content plotted against relative shoot yield. Data are means and error bars are 2 x standard error.

Neither PPE \( y = 0.278x + 246.3; \ R^2=0.002 \) nor critical internal P concentration of whole shoots \( y = 0.01x + 3.4; \ R^2 = 0.005 \) were correlated with the critical external P requirements of the species (independent variable). Likewise, PPE \( y = 26.6x + 192.5; \ R^2 = 0.35 \) and critical internal P concentration \( y = -0.31x + 4.1; \ R^2 = 0.29 \) were not correlated with the maximum shoot yield of the species (independent variable).

Rose *et al.* (2015) have proposed that PUE of species should be compared at a common shoot P content (PUE\(_c\)). It was initially unclear at which level of shoot P the species
should be compared because the choice of any one P level would inevitably result in fast- and slow-growing species being compared at differing degrees of P stress (see inset to Fig. 2a, b). Consequently, comparisons among the species were initially made at three levels of shoot P content (2, 4 and 7 mg P pot\(^{-1}\)) which corresponded with relative shoot yields of about 0.37, 0.59 and 0.77 for the fast-growing species, and 0.55, 0.79 and 0.98 for the slow-growing species, respectively (Fig. 3a). The PUE\(_c\) was positively correlated \((P<0.001; R^2 = 0.77-0.91)\) with maximum shoot yield at all three of the shoot P contents at which comparisons were made. Subsequently, the PUE\(_c\) of the species were compared at shoot P content of 4 mg P pot\(^{-1}\) (Table 2) where the relative shoot yield among the species ranged between 0.59-0.79. The PUE\(_c\) of the fast-growing species (\(L.\ perenne\), \(B.\ hordeaceus\), \(R.\ duttonianum\) and \(R.\ richardsonii\)) were 1.3- to 1.8-fold higher than that of the slowest-growing species (\(R.\ auriculatum\), \(R.\ carphoides\) and \(R.\ erianthum\)). Across all species, PUE\(_c\) (at 4 mg P pot\(^{-1}\)) was not significantly correlated with critical external P requirements (independent variable; \(y = 6.23x + 231.8; R^2 = 0.23\)).
Fig. 3. Shoot DM per unit shoot P content at a three common shoot P contents (PUE\textsubscript{c}; a) and shoot DM per unit shoot P content at a common level of P stress (relative yield = 0.7) (PUE\textsubscript{s}; b) relative to maximum shoot yield. Shown are *Bromus hordeaceus* (♦), *Lolium perenne* (■), *Rytidosperma auriculatum* (△), *R. carphoides* (◇), *R. duttonianum* (●), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+), *R. racemosum* (×), *R. richardsonii* (▲) and *R. setaceum* (○). Error bars associated with the data points are 2 x standard error and the solid lines indicate significant ($P<0.001$) linear correlation; a dashed line indicate the regression was not significant ($P<0.05$).
Shoot DM per unit P content had been shown to increase with increasing P stress (Figs. 2a, b), so it was not clear whether this would confound interpretation of PUEc. Consequently, shoot DM per unit P content was also compared among the species at a common relative shoot yield of 0.7 (PUEs). Unlike PUEc, PUEs was not correlated with maximum shoot yield (Fig. 3b). It was also not correlated with critical external P requirements \( (y = 5.1x + 311; R^2 = 0.23) \). However, variation in PUEs (independent variable) explained 72\% of the variation in PPE \( (y = 0.46x + 0.67; P < 0.001) \).

Shoot morphology traits

In Experiment 2, shoot morphology traits of the YEB and whole shoots were examined on the five fastest-growing species from Experiment 1 (\( B. hordeaceus, L. perenne, R. duttonianum, R. racemosum \) and \( R. richardsonii \)). There were significant differences between species in leaf area, SLA and \( P_\text{area} \) (Fig. 4a, b, c). All species had smaller leaf area and \( P_\text{area} \) at no applied P than at 42 mg applied P per pot. In contrast, SLA was mostly conserved across all levels of P supply. The exception was \( R. duttonianum \) which had up to 12\% larger SLA at no applied P relative to all higher levels of applied P; and \( B. hordeaceus \), which had a 9\% smaller SLA at all P levels under 21 mg applied P per pot relative to that at 42 mg applied P per pot. \( B. hordeaceus \) was notable for having amongst the largest leaf area at 42 mg applied P per pot, but the smallest leaf area at no applied P. \( B. hordeaceus \) and \( L. perenne \) had up to 3-fold larger leaf area and 1.5-fold higher SLA than the \( Rytidosperma \) species. However, \( R. duttonianum \) had up to 2-fold higher \( P_\text{area} \) than \( B. hordeaceus, L. perenne \) and \( R. richardsonii \).
Fig. 4. Leaf area (a), specific leaf area (b), area based leaf P content (c), leaf dry matter content (d) and leaf thickness (e) of the youngest fully expanded blade (YEB) for *B. hordeaceus* (♦), *L. perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) at six levels of applied P in Experiment 2. *Bromus hordeaceus* and *L. perenne* are depicted with dashed lines, all *Rytidosperma* species are depicted with solid lines. Error bars associated with the data points are 2 x standard error and, for each panel, least significance difference (LSD) bars (*P*<0.05) for the species x P interaction are shown (a, c) or, where the interaction was not significant, LSD bars (*P*<0.05) for the main effects for species and level of applied P are shown (b, d, e).
Most species had similar LDMC and leaf thickness at no applied P and 42 mg applied P per pot (Fig. 4d, e). Exceptions were *B. hordeaceus* and *L. perenne* which had higher LDMC at no applied P (by 21% and 11% respectively); and *L. perenne* and *R. duttonianum* which had lower leaf thickness (19% and 15% lower, respectively) at no applied P. Across all levels of P applied, the *Rytidosperma* species had up to 1.7-fold higher LDMC than *B. hordeaceus* and *L. perenne*, whilst the Mediterranean species had up to 1.6-fold thicker leaves than the *Rytidosperma* species. Leaf mass per unit area, the inverse of SLA, was positively correlated ($P<0.001$) with LDMC and negatively correlated ($P<0.001$) with leaf thickness. However, less variance in the data was accounted for by leaf thickness ($R^2 = 0.15$) than by LDMC ($R^2 = 0.59$).

![Graph of Aboveground leaf mass fraction for different species](image)

**Fig. 5.** Aboveground leaf mass fraction for *B. hordeaceus* (♦), *L. perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) at six levels of applied P in Experiment 2. *Bromus hordeaceus* and *L. perenne* are depicted with dotted lines, all *Rytidosperma* species are depicted with solid lines. Error bar associated with the data points are 2 x standard error and the least significance difference bars ($P<0.05$) for the species x P interaction are shown.

The aboveground leaf mass fraction (ALMF) varied by only 1.2-fold among species across most levels of P supply (Fig. 5). *Rytidosperma racemosum* had the lowest ALMF at all levels of applied P, and *B. hordeaceus* and *L. perenne* had the highest. All species,
except for *R. richardsonii*, increased ALMF only at the lowest levels of P, below 10.5 mg P per pot (*B. hordeaceus* and *R. racemosum*) or 4.5 mg P per pot (*L. perenne* and *R. duttonianum*). However, the adjustment in ALMF was only marginal (7-11%), with *R. richardsonii* having the same ALMF at all levels of applied P.

**Discussion**

Phosphorus utilisation

Shoot P concentrations and P content of all species examined in Experiments 1 and 2 increased in response to increased P supply. Shoot P content was positively correlated with shoot yield, reflecting the positive concomitant responses of shoot yield (Table 2, Waddell *et al.* 2015) and P concentration to P supply. However, relative to fast-growing species, slower-growing species stopped increasing their growth rates in response to P at lower levels of applied P (Waddell *et al.* 2015) and typically had a lower shoot P content but a higher P concentration reflecting luxury P uptake (Asher and Loneragan 1967; Clarkson 1967; Christie and Moorby 1975; Chapin and Bieleski 1982).

Whole shoot critical internal P concentration was generally higher than that of the YEB critical internal P concentration with the two measures positively and linearly correlated (*P*<0.001; *R*² = 0.9). It is often expected that the YEB critical internal P concentration will be higher than that of the whole shoot because YEB leaves are physiologically the most active on the plant (Reuter *et al.* 1997). It is likely that the lower critical internal concentration of the YEB relative to that of the whole shoots in the present experiment was due to the plants being grown as micro-swards, with the YEB inevitably grown in shade until it reached full expansion. Shading has been reported to reduce shoot P concentrations (Jackson and Caldwell 1992).

There was a range in critical internal P concentrations observed among the species with the highest concentrations in *R. carphoides* and *R. fulvum*, and the lowest in *L. perenne*, *R. erianthum*, *R. duttonianum*, *R. pilosum* and *R. richardsonii*. There were no obvious groupings of species, especially with respect to the potential growth rates. Ranking of the few species that had been examined previously (*R. richardsonii* ≤ *Lolium* spp. < *B. hordeaceus*) was consistent with the earlier report by Hill *et al.* (2005). Rose *et al.* (2011; 2015) argue that the critical internal P concentration is a misleading measure of
PUE because the value is influenced by the nutrient foraging characteristics of a species. Shoot P concentrations often increase with increasing concentrations of available soil P. Plants with high P uptake efficiency (as a result of superior P foraging by roots; Richardson et al. 2011) will achieve maximum growth rates at relatively low soil P concentrations and can, consequently, also have a relatively low critical internal P concentration. Nevertheless, there was no relationship found between critical internal P concentration and critical external P requirement (i.e. P uptake efficiency) or with maximum shoot DM (i.e. growth rate potential).

Physiological P efficiency (PPE) is a commonly used measure of internal PUE (e.g. Baligar et al. 2001; Hammond et al. 2009; Veneklaas et al. 2012). Some of the species varied significantly, with high PPE recorded for both fast- (e.g. B. hordeacues, L. perenne, R. duttonianum and R. richardsonii) and some of the slow-growing species (e.g. R. auriculatum and R. erianthum). However, PPE was not correlated with the critical external P requirement of the species or their potential growth rates.

Shoot DM per unit P content is the only measure of PUE that can be examined in relation to P application treatments, the P content of a species, or the degree of P stress being experienced. The shoot DM per unit P content varied with relative shoot yield (i.e. P stress) and P content of shoots. It was highest in plants growing in low P supply and high P stress (relative yield less than ~0.5). Shoot DM per unit P declined rapidly in all species as P stress decreased with improved P supply. It was also highest when shoot P content was low and declined exponentially when it was graphed relative to shoot P content of the species (Fig. 2). Both assessments of shoot DM per unit P supported the hypothesis proposed by Rose et al. (2011) that there is a relationship between the internal utilisation of P and the P status of the plant.

These observations also demonstrated that PPE was effectively estimating the average PUE achieved by each species between the high level of P stress experienced in unfertilised soil, and no P stress near the critical level of P supply. There is a risk that when species differ in their critical external P requirements (due to different P uptake efficiencies), they may also differ in the degree of P stress that they experience in soil to which no P was applied. This may mean that estimates of PPE do not compare the efficiencies of P utilisation by the species at comparable levels of P stress. Despite these potential concerns, we found that variation in PUEs (i.e. PUE compared at a common
level of P stress) explained 72% of the variation in PPE. In practical terms, measuring
PPE is considerably easier to achieve than either PUEc or PUEs which require measures
of shoot DM per unit shoot P over a range of P supply rates.

The critical external P requirements of the species were also not correlated with PUEc
(4 mg shoot P content pot\(^{-1}\)) or PUEs. On this basis, the data seemed to indicate that
PUE was not a consistent factor determining the external P requirements of the species.
However, PUEc differed from the other measures of PUE because it was highly
correlated with maximum shoot yield (Fig. 3), suggesting, on face value, that potential
growth rate of the species explained a lot of the variation in PUEc. The potential
problem with this hypothesis is that shoot DM per unit P may not be completely
independent of potential growth rate because it is estimated using maximum shoot yield.
The hypothesis would be more convincing if it were possible to demonstrate an equally
strong correlation between PUEc and some other measure of a plant’s potential growth
rate (e.g. leaf appearance and/or expansion rates). Nevertheless, Hammond et al. (2009)
have also found that increased shoot yields were associated with high PUE in Brassica
oleracea L. accessions. The implications of the relationship between potential growth
and PUEc is that selection of genotypes for improved PUEc (e.g. Rose et al. 2011; Rose
and Wissuwa 2012) will effectively select for intrinsically faster-growing plants. The
methodology required to measure PUEc is experimentally demanding (e.g. Rose et al.
2015) and it may be easier to select for high growth rates. Unfortunately, the present
results did not clarify whether species should be compared using PUEc (PUE at a
common shoot P content) or PUEs (PUE at a common P stress level). When
comparisons were made at a common level of P stress, there was no longer any apparent
relationship between plant growth rate and PUE.

It is widely observed when species from natural communities are compared, that faster-
growing species also have a relatively high capacity for nutrient acquisition in low
nutrient soils. This is usually attributed to superior nutrient foraging (Clarkson 1967;
Chapin 1980; Campbell et al. 1991). The faster-growing Rytidosperma species also
yielded comparatively well in low P soil (Waddell et al. 2015). These species had
amongst the highest PUE regardless of which PUE measure was used (Table 2). This
suggested that under P-limiting conditions, fast-growing species may also benefit from
more effective use of the P that is taken up. However, in an ecological context superior
nutrient acquisition and utilisation does not guarantee persistence in nutrient-poor

55
environments. Slow-growing species are more prevalent in these environments, presumably because of their conservative nutrient requirements (e.g. Clarkson 1967; Grime and Hunt 1976). There was less consistency among the measures of PUE in the efficiency with which slow-growing species utilised P for growth. While PUE$_c$ indicated that slow growth was associated with low PUE, all other PUE measures were not correlated with the growth rate of the species and indicated a range in the internal P efficiencies of the slow-growing species.

Shoot morphology of the five highest-yielding species

It was notable that four of the five fast-growing species (\textit{B. hordeaceus}, \textit{L. perenne}, \textit{R. duttonianum} and \textit{R. richardsonii}) had a lower YEB P concentration at most levels of applied P than most slow-growing species. Further, these four species had amongst the highest PUE, regardless of which measure was used. Given the similarities in their use of P for shoot growth, we examined the shoot morphology of the five fastest-growing species to determine whether they also had shoot morphology traits that are considered to enable efficient use of P: (i) a greater ability to distribute P over a large leaf area (i.e. a low P$_{area}$; Ryser \textit{et al.} 1997) and (ii) a large leaf area per unit DM (i.e. high SLA; Poorter and Remkes 1990; Lambers and Poorter 1992; Poorter and van der Werf 1998).

The shoot morphology traits of the YEB varied among the five fastest-growing species. The YEB leaf area and P$_{area}$ were smaller for the \textit{Rytidosperma} species than for \textit{B. hordeaceus} and \textit{L. perenne}. All species had a lower leaf area at low P supply than at high P supply, which coincided with reduced shoot yields at low P supply (Waddell \textit{et al.} 2015). This is consistent with most studies of how P deficiency restricts plant growth, which indicate that leaf area is affected by P deficiency in the first instance, with constraints to photosynthesis only occurring at very low P supply (Rao and Terry 1989; Plénet \textit{et al.} 2000). \textit{Bromus hordeaceus} was notable for its relatively large decrease in YEB leaf area (2.8-fold) and shoot mass (8.8-fold) between high and low P supply.

For all species, the values of P$_{area}$ were at the lower end of the range observed for grasses in the global leaf trait dataset reported by Wright \textit{et al.} (2005). Ryser \textit{et al.} (1997) hypothesised that leaf P productivity is largely determined by the ability to distribute P over a large area. However, in our study when all fast-growing species
received similar incident radiation, the maximum shoot yield and PUE for
*R. duttonianum*, *R. richardsonii*, *B. hordeaceus* and *L. perenne* were mostly similar,
despite differences in *P*$_{area}$ and the YEB leaf area. Phosphorus utilisation efficiency
could potentially be influenced by differences in *P*$_{area}$ or SLA among the species
because the P concentration of leaf DM is the product of *P*$_{area} \times$ LMA. It is interesting to
compare the fast growing species, *R. duttonianum* and *R. richardsonii* in this respect
because they differed significantly in *P*$_{area}$ and SLA. However, in these species
variations in the leaf traits counteracted each other to the extent that PUE (i.e. shoot DM
per unit P) was not different.

*Lolium perenne* and *B. hordeaceus* (except in the unfertilised soil) also had higher SLA,
lower LDMC and higher ALMF than the *Rytidosperma* species. *Lolium perenne*, but
not *B. hordeaceus*, also had thicker leaves than the *Rytidosperma* species. The stability
of these species rankings for each trait across the six levels of applied P has also been
noted for other species (e.g. Garnier *et al.* 2001a; Pontes *et al.* 2007). The SLA values
reported for *L. perenne* in this study are higher than those reported in other studies (e.g.
Poorter and Remkes 1990; Garnier 1992; Ryser and Urbas 2000). In the published
studies, SLA was determined for whole shoots of single plants grown with minimal
self-shading. In the present study, plants were grown as micro-swards, which resulted in
self-shading of the expanding leaves, which increases SLA (Sobrado and Medina 1980;

It has been suggested that shoot productivity is generally correlated with the amount of
leaf area that a plant realises per unit mass, (i.e. SLA; Poorter and van der Werf 1998),
although there are exceptions (Halsted and Lynch 1996; Villar *et al.* 1998). Species that
invest more in chemical and structural compounds per unit leaf area tend to have slower
growth rates (Lambers and Poorter 1992; van Arendonk and Poorter 1994). The lower
SLA, lower YEB leaf area, higher LDMC and smaller ALMF of the high-yielding
*Rytidosperma* species are consistent with shoot morphology traits that are associated
with resource conservation and slow growth (Lambers and Poorter 1992). However,
*R. duttonianum*, *R. racemosum* and *R. richardsonii* have retained these nutrient-
conservation traits without any obvious trade-off with shoot productivity at high or low
P supply. We concluded on the basis of these comparisons of leaf morphology traits
among the fast-growing *Rytidosperma* species that neither *P*$_{area}$ or SLA alone explained
how these species achieved high PUE or were intrinsically faster-growing species.
An alternative hypothesis is that herbaceous plants can have a large amount of plasticity between SLA and NAR in order to maintain relative growth rate (RGR) across a range of incident radiation (Shipley 2002; 2006). The lower SLA of the *Rytidosperma* species was likely a result of the higher LDMC, rather than due to thinner leaves. Hence, the *Rytidosperma* species may have achieved similar shoot yields to *B. hordeaceus* and *L. perenne* due to increased investment in photosynthetic compounds per unit leaf area such as Rubisco (Evans 1998; Poorter and van der Werf 1998). This is worthy of future study. Over the longer-term the conservative shoot morphology traits of fast-growing *Rytidosperma* species (i.e. low SLA and high LDMC) may be an advantage for persistence by a grassland species. Species which possess shoot traits such as these, are abundant in grasslands subjected to both high and low P supply and lax- and heavy-grazing pressures as they are associated with reduced loss of scarce nutrients through herbivory and leaf turnover (Laliberté *et al.* 2012).

Conclusions

Several measures of PUE were examined to determine whether the way *Rytidosperma* species utilised P for shoot growth influenced their different responses to applied P or their contrasting external P requirements for growth. No measure of PUE explained differences in the external P requirements for growth. Indirectly, this indicates that differences in root foraging are likely to be important. Only PUE$_c$ appeared to be associated with growth rate of the species. However, given that some slow-growing species had similar high critical internal P, PPE and PUE$_a$ to fast-growing species, it was not clear that slow-growing species could be generally categorised as less efficient in their utilisation of P for shoot growth. It was notable that the fast-growing *B. hordeaceus, L. perenne, R. duttonianum* and *R. richardsonii* had high PUE regardless of which measure was used. Shoot morphology traits differed substantially among these fast-growing species. The fast-growing *Rytidosperma* species had low YEB leaf area, low SLA, high P$_{area}$, and relatively high LDMC (traits usually associated with resource conservation and slow-growing species) and were distinctly different, in these respects, to *B. hordeaceus* and *L. perenne*. 
Acknowledgements

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Chapter 4. Root morphology and its contribution to a large root system for phosphorus uptake by *Rytidosperma* species (wallaby grass)
Abstract

**Background and aims** *Rytidosperma* species are native Australian grasses which differ in their growth rate and phosphorus (P) requirements. This study examined the role of root morphological traits in response to P supply.

**Methods** Nine fast- and slow-growing *Rytidosperma* species were examined along with *Lolium perenne* and *Bromus hordeaceus*. Plants were grown in a glasshouse for 47 days in soil supplied with six levels of P between 0 and 60 mg P per pot. Root mass, length and diameter, root hair length and density, and extent of mycorrhizal colonisation were measured.

**Results** Across all species there was a positive correlation (*P*<0.001) between P uptake and root mass, length and root hair cylinder volume (RHCV) at all levels of P supply. An exception was the RHCV of *B. hordeaceus*, where expected P uptake was not achieved due to a markedly reduced root length at low P supply. For the *Rytidosperma* species, morphological plasticity for specific root length, root mass fraction and root hair length ranged from 1.5-fold to 2.7-fold between high and low P supply. However, across all species and P levels no single root morphological trait was identified for universally increasing root size and P uptake.

**Conclusions** Fast-growing species took up more P as a result of an overall larger root mass, greater root length and larger RHCV.
Preface

Chapter 4 follows on from Chapters 2 and 3 in that it reports the results of one of the experiments (Experiment 1 conducted over 47 days) also reported in Chapters 2 and 3. Experiment 2 is not reported as it was conducted to confirm the asymptotic growth of the five fastest-growing species and examine their shoot morphological traits. Chapter 4 examines the root morphological traits and relates these to the growth rates (Chapter 2) and shoot morphological traits (Chapter 3).

Introduction

*Rytidosperma* species (wallaby grass) are native grasses that can form the dominant component of grasslands in southern Australia (Garden et al. 2001; Garden et al. 2003). Many of the soils in this region are low in plant-available phosphorus (P) (Handreck 1997; Stephens and Donald 1959) and it is generally assumed that plants that have evolved on these soils are well adapted to low-P stress. A common adaptation of plants that have evolved on low-P soils is slow growth (Lambers and Poorter 1992). However, broad generalisations about growth rates (Donald 1970), response to P-stress (Waddell et al. 2015) and other adverse soil conditions such as soil acidity (Waters et al. 2009), can be misleading. For example, Waddell et al. (2015) have shown that *Rytidosperma* species differ markedly in their ability to respond to P-fertiliser application. Most slow-growing and poorly P-responsive *Rytidosperma* species have a low critical external P requirement, as determined by the amount of P required to achieve 90% of maximum growth. In contrast, the fast-growing *Rytidosperma* species were more P responsive and tended to have a high critical external P requirement (Waddell et al. 2015) and high P utilisation efficiency (Chapter 3). However, *R. richardsonii* was notable as having a low critical external P requirement (i.e. equivalent to that of some of the slow-growing species) but shoot yields similar to that of *Lolium perenne*, an introduced pasture grass that yields well at both high and low P supply.

There is limited information on the role that root morphological traits have on the growth and P-requirements of *Rytidosperma* species. For example, *R. richardsonii* has been reported to have a small root length and root mass relative to nine other grass, legume and forb pasture species (Hill et al. 2006). In contrast, the slow-growing species,
\textit{R. erianthum} has been recorded as having a similar root mass to \textit{L. perenne} when grown in a soil with low plant-available P (O’Dwyer and Attiwill 1999).

A large root mass, long root length and a large root hair cylinder volume (RHCV) enables greater soil exploration and are recognised as adaptations for increasing P uptake and fast growth (Brown et al. 2013; Robinson and Van Vuuren 1998; Wissuwa and Ae 2001). It has also been suggested that fast-growing species from fertile habitats display greater root morphological plasticity than slow-growing species from infertile habitats (e.g., Chapin 1980; Christie and Moorby 1975; Grime 1979; White 1972). However, the broad applicability of the concept that plasticity of root traits is associated with fast-growth may be limited, as it depends on plant habit, the root morphological trait examined and spatial distribution of P within the soil (Hodge 2004; Robinson and Van Vuuren 1998).

Plants that develop a large root system with minimal overall carbon (C) cost or with low root respiration costs, often yield better under low-P conditions (Nielsen et al. 2001). Root traits that increase soil exploration at minimal C cost include; increased specific root length (SRL) (Eissenstat 1991), increased root hair length and density (Gahoonia et al. 1997), mycorrhizal colonisation (Jakobsen et al. 2005; Koide and Mosse 2004), and decreased root tissue density (RTD) through the formation of aerenchyma and/or decreased cell wall thickness and a lower proportion of stele and sclerenchyma (Lynch and Ho 2005; Wahl and Ryser 2000). It is also important for plants to balance resource allocation between roots and shoots (i.e. the root mass fraction (RMF) and root P fraction (RPF)), to maximise root soil foraging with minimal trade-off against shoot photosynthetic potential (Nielsen et al. 2001; Snapp and Lynch 1996; Wissuwa et al. 2005).

In this paper, root morphological traits and plant P-contents of nine slow- and fast-growing \textit{Rytidosperma} species native to Australian temperate grasslands were compared with two fast-growing introduced grasses, \textit{Bromus hordeaceus} and \textit{L. perenne}, of Mediterranean origin. We hypothesised that fast-growing \textit{Rytidosperma} species such as \textit{R. duttonianum} and \textit{R. richardsonii} (Waddell et al. 2015) would have a large root mass, root length and RHCV and display greater morphological plasticity in response to P supply, whereas slow-growing species would show more conservative root morphological traits with less plasticity.
Methods

Plant material and growth conditions

Eleven grass species were examined: *Bromus hordeaceus* L. (synon. *B. molliformis*), *Lolium perenne* L. cv. Victorian, *Rytidosperma auriculatum* (J.M.Black) Connor & Edgar, *R. carphoides* (F.Muell. ex Benth.) Connor & Edgar, *R. duttonianum* (Cashmore) Connor & Edgar, *R. erianthum* (Lindl.) Connor & Edgar, *R. fulvum* (Vickery) A.M.Humphreys & H.P.Linder, *R. pilosum* (R.Br.) Connor & Edgar, *R. racemosum* (R.Br.) Connor & Edgar, *R. richardsonii* (Cashmore) Connor & Edgar cv. Taranna and *R. setaceum* (R.Br.) Connor & Edgar. Seeds for most *Rytidosperma* species were collected from naturally occurring plants from a single site at ‘Yeumville’ near Hall, New South Wales (35° 4´ S, 19° 5´ E) as part of a field experiment (Garden et al. 2000). Exceptions to this were *R. fulvum* which was a minor component at the site where most *Rytidosperma* seed was collected, and hence seed was collected from ‘Wyreela’ near Dalgety, New South Wales (36° 28´ S, 148° 51´ E); and *R. richardsonii*, which was obtained from a commercial source. *Bromus hordeaceus* was collected from Ginninderra Experimental Station, Canberra, Australian Capital Territory (35° 10´ 30.0´ S, 149° 02´ 33.4´ E, elevation 597 m), and *L. perenne* was obtained from a commercial source (CleanSeeds Pty Ltd.).

Plants were grown as micro-swards in a naturally lit glasshouse in Canberra, Australia from June to August, the southern hemisphere winter. Air temperature was regulated to permit a range of temperatures from 23°C (day) and 12°C (night). Pots were arranged in a randomised block design with each block rearranged twice per week. Each cylindrical pot, 90 mm width × 200 mm depth, contained 1.3 kg of dry soil collected at 20-150 mm depth from an unfertilised pasture at the CSIRO Ginninderra Experimental Station, Australia. The soil was a yellow chromosol (Isbell 2002) and had bicarbonate extractable P (Colwell 1963) of 9 mg kg⁻¹ and P buffer index of 40 (Burkitt et al. 2002).

Micro-swards were established by sowing each species at a rate of 25 mg of germinable seed per pot evenly across the soil surface. As the seedlings emerged, pots were wrapped with reflective aluminium sleeves, the height of which was increased in line with plant growth to ensure that the plants in each pot experienced a similar level of
incident radiation. The plants were watered daily with deionised water to achieve 80% field capacity (approximately 23% moisture content) in the soil.

Phosphorus treatments were established by applying P as KH₂PO₄ at six rates, nil, 4.5, 10.5, 21, 42 and 60 mg P per pot. The P was added once at the beginning of the experiment as an aqueous solution, sufficient to wet the top 50 mm of soil, reflecting the natural distribution of P in field soils. A total of five replicates per treatment were established. All other nutrients were applied at regular intervals as an aqueous solution, which consisted of 2 mM MgSO₄, 7.5 mM CaSO₄, 20 mM KNO₃, 2.5 mM (NH₄)₂SO₄, and the following micronutrients; 23 µM H₃BO₃, 46 µM MnCl₂, 15 µM ZnSO₄, 1.6 µM CuSO₄, 0.7 µM (NH₄)₂MoO₄, 1.0 µM CoCl₂, and 0.05 mM FeNaEDTA. Nutrients were initially applied to pots at 30 ml per week and this was increased over time to provide a total of 382 ml of the P-free solution by the end of the experiment.

Root harvest

All plants were harvested 47 days after germination. Shoots were separated from the roots at the crown and shoot dry mass was reported by Waddell et al. (2015). For assessment of root morphological traits, soil from each pot was obtained as an intact core and sliced lengthwise with a sharp knife to obtain three root samples, two of which were approximately one-eighth each of the soil core. Roots from each section were washed gently to remove soil using 1 mm mesh sieves. The two one-eighth subsamples of roots for analysis of root morphology were stored in 50% (v/v) ethanol at 4 °C until assessed. The remaining roots were dried at 70 °C for 48 hours and weighed. Root mass from all soil sections were added for determination of total root mass.

Mass of roots stored in ethanol solution were corrected for dry matter loss (Crush et al. 2010) by assessment of additional plants- B. hordeaceus, L. perenne, R. carphoides and R. richardsonii grown with nil, 10.5 and 42 mg applied P per pot. Root mass was measured before and after storage in 50% (v/v) ethanol and with no storage in ethanol to determine a correction factor (CF) where:

\[
CF = \left( \frac{\text{fresh mass before ethanol storage}}{\text{dry mass after ethanol storage}} \right) \times \left( \frac{\text{dry mass no ethanol storage}}{\text{fresh mass no ethanol storage}} \right)
\]
The CF was similar for all species and was not affected by soil P level, so a single correction factor (CF = 1.33) was applied to all treatments.

The mass of root radicle and cotyledon fractions of seedlings was determined by germinating 55 g of seeds for each species. Seeds were germinated in a petri dish moistened with an aqueous solution containing a fungicide (thiram, 800 g kg⁻¹, Barmac Industries, Queensland, Australia) with three replicates per species. The cotyledon and radicle fractions were excised once the cotyledon was fully pigmented, typically 4-5 days after germination. Excised cotyledon and radicle portions were dried at 70 °C for 48 hours before being weighed.

Assessment of root morphology

Root length, diameter and volume were measured on one of the root subsamples after staining roots with 0.05% (v/v) toluidine blue solution for at least 5 minutes and scanning at 600 dots per inch with a flatbed scanner (Epson, Sydney, Australia). The scanned images were analysed using the automatic threshold for root distinction from the background in WinRhizo software (Régent Instruments, Quebec, Canada) (Bouma et al. 2000). Root tissue density (RTD) was determined by root dry mass divided by the volume of the same root sample. Eleven root diameter classes of 0.08 mm diameter were analysed. Fine roots were defined as those below 0.24 mm as there was an obvious peak in the occurrence of roots (on a length basis) below these diameter classes for all species. In particular, all species had a prominent peak in the 0.16-0.24 mm diameter class. This definition is similar to that used by Ryser (1998).

Root hair length was determined for ten lateral roots per replicate. For each lateral root, the zone of fully-expanded root hairs was photographed using a Leica DMR upright microscope (Leica, Wetzlar, Germany) fitted with a digital camera. The length of at least ten fully-expanded root hairs on each section was then measured using Image J (U.S. National Institutes of Health, Bethesda, USA).

Root hair density was determined by visually scoring photographs of the same lateral roots used for root hair length measurements by the method of Haling et al. (2010). A photographic reference scale ranging from 0 (no root hairs) to 5 (highest root hair density) was established on at least two lateral root samples that were representative of
each root hair density class. These samples (approximately 15 mm long) were set in 4% (v/v) agar with 0.1 M KOH buffer and dissected into 200 µm transverse sections using a vibrating microtome (Vibratome series 100, The Vibratome Company, USA). The number of root hairs was then determined using a dissecting microscope (Leica Microsystems, Wetzlar, Germany). A single calibration equation \( y = 9.12x + 15.67 \) where \( y \) is the number of root hairs per mm and \( x \) is the density score; \( R^2 = 0.96 \) was subsequently used to estimate root hair density.

The volume of the soil potentially explored by roots was estimated by calculating the root hair cylinder volume (RHCV). The RHCV of 11 root diameter classes (as derived from WinRhizo) was determined using the mid-point root radius for the root class, the average root hair length for the species and P treatment, and the length of root in the diameter class (Gahoonia et al. 1997). These were then summed to give the total RHCV of the root system.

The proportion of roots colonised by arbuscular mycorrhizal fungi (AMF) was determined at two levels of P (nil and 42 mg P per pot) for the grasses and for *Trifolium subterraneum* L. cv. Woogenellup. *Trifolium subterraneum* was included as a control species as it usually highly colonised by AMF and gains a P nutrition benefit from colonisation when grown in a low-P soil (Abbott and Robson 1977). *Trifolium subterraneum* was grown and harvested under the same conditions as the grasses, except three plants were sown per pot. The portion of roots colonised by AMF was determined on the second one-eighth root samples that were stored in 50% ethanol. Roots were cleared in 10% (v/v) KOH at room temperature for 5 days, rinsed with deionised water and treated with 0.1 N HCl for ~30 seconds (Grace and Stribley 1991). Roots were then dyed with Shaeffer black ink (Vierheilig et al. 1998; Vierheilig et al. 2005) and stored in a solution containing glycerol: lactic acid: water (50: 25: 25 v/v/v). Colonisation by AMF was subsequently determined using the gridline intersect method with a minimum of 100 root/gridline intersections recorded per treatment (Giovannetti and Mosse 1980).

Phosphorus analysis

Dried root and samples were puck-milled to a fine powder and 20-50 mg of each sample was ashed at 550 °C for five hours. The ash was dissolved in 2 M HCl at 1 ml HCl per
10 mg dry weight plant material before being analysed using a modified malachite green method in which disodium phosphate (Na₂HPO₄) was used as a P standard (Motomizu et al. 1983). Shoot P contents are reported in Chapter 3.

Statistical analysis

Relationships between root morphology, species and P supply were examined using two-way ANOVA and linear regression using Genstat (15th edition; VSN International, UK). Where there was no significant species × P-treatment interaction for a given root morphological trait, a t-test was performed.
Results

Root foraging

All species had a radicle fraction smaller than the cotyledon fraction (Table 1). Across most species the radicle fraction was typically about 0.35 mg mg\(^{-1}\). However, *R. auriculatum*, *R. pilosum* and *R. setaceum* had a radicle fraction between 0.24 and 0.29 mg mg\(^{-1}\) and *B. hordeaceus* had the smallest radicle fraction at 0.11 mg mg\(^{-1}\).

Root mass was maintained across P levels by all *Rytidosperma* species, except *R. fulvum* and *R. richardsonii* (Fig. 1a, b). For these two species, root mass was similar between 60 mg P per pot and 4.5 mg P per pot, but was significantly (*P*<0.001) lower at no applied P. *Bromus hordeaceus* and *L. perenne* had up to 4-fold greater root mass at 42 mg applied P per pot relative to that at no applied P. Similarly, for total root length, all *Rytidosperma* species maintained or increased (up to 1.7-fold for *R. carphoides*, *R. racemosum* and *R. setaceum*) root length at lower P supply (Fig. 1c, d). In contrast, *B. hordeaceus* and *L. perenne* had up to 3-fold greater root length (*P*<0.001) when grown in high-P soil compared with that at P-levels less than 10.5 mg P per pot. However, at most levels of P supply, *B. hordeaceus* and *L. perenne* generally maintained significantly larger root lengths than the *Rytidosperma* species. The exception was at no applied P, where *R. richardsonii* and *R. setaceum* had similar root lengths to *L. perenne*, whilst *B. hordeaceus* had amongst the smallest total root length.
Table 1 Root morphological traits including seedling cotyledon fraction and seedling radical fraction and fine root fraction for all species. Fine roots were defined as roots with a diameter less than 0.24 mm. Values are means ± standard error; n=3 (seedling cotyledon and radicle fractions), n=5 (fine root fraction). Within each root morphological trait the least significant difference at \( P=0.001 \) (cotyledon and radicle fractions) and \( P=0.05 \) (fine root fraction) are presented.

<table>
<thead>
<tr>
<th>Species</th>
<th>Seedling cotyledon and radicle fractions (mg mg(^{-1}))</th>
<th>Fine root fraction (m m(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cotyledon</td>
<td>Radicle</td>
</tr>
<tr>
<td><strong>Bromus hordeaceus</strong></td>
<td>0.89 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td><strong>Lolium perenne</strong></td>
<td>0.64 ± 0.03</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td><strong>Rytidosperma auriculatum</strong></td>
<td>0.71 ± 0.03</td>
<td>0.29 ± 0.03</td>
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<td><strong>R. carphoides</strong></td>
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<td>0.36 ± 0.01</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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<td><strong>R. racemosum</strong></td>
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<td>0.34 ± 0.01</td>
</tr>
<tr>
<td><strong>R. setaceum</strong></td>
<td>0.76 ± 0.05</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td><strong>l.s.d.</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) low P – no applied P

\(^b\) high P – 42 mg P per pot
Fig. 1 Root mass of (a) Rytidosperma auriculatum (Δ), R. carphoides (○), R. erianthum (□), R. fulvum (▼), R. pilosum (+) and R. setaceum (○) and (b) Bromus hordeaceus (♦), Lolium perenne (■), R. duttonianum (●), R. racemosum (×) and R. richardsonii (▲) at six levels of P supply; (c) and (d) total root length for the same species. Bromus hordeaceus and L. perenne are depicted with dotted lines, all Rytidosperma species are depicted with solid lines. Data points are the mean for each species ± standard error (SE). For each panel, the least significant difference (LSD) at P=0.001 is given. Note different scales on the y-axis.

The fine root fraction, as defined by diameter classes below 0.24 mm, across all Rytidosperma species (with the exception of R. erianthum) differed significantly (P<0.05) between high (42 mg applied P per pot) and low (no applied P) P supply, whereas B. hordeaceus, L. perenne and R. erianthum were not affected (Table 1). Rytidosperma richardsonii in particular showed the largest adjustment in fine root fraction in response to low-P soil with a 1.3-fold increase. At no applied P, R. auriculatum, R. carphoides and R. pilosum had amongst the largest (up to 0.70 m m⁻¹) fine root fraction based on a total root length basis, whilst B. hordeaceus had the smallest (0.50 m m⁻¹).
Root mass fraction (RMF), expressed as a portion of total plant mass, was increased by all species at low-P supply (Fig. 2a, b). At no applied P, *R. auriculatum* and *B. hordeaceus* had the largest RMF. Across all species RMF increased by 1.5-fold to 2.7-fold at lower P supply. Across all species, the root P fraction (RPF) was similarly increased at low-P supply (Fig. 2c, d). Four species, *B. hordeaceus*, *L. perenne*, *R. auriculatum* and *R. duttonianum* were notable as having up to 2-fold higher RPF than all other species.

Fig. 2 Root allocation traits including root mass fraction of (a) *Rytidosperma auriculatum* (Δ) *R. carpoides* (◊), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+) and *R. setaceum* (○) and (b) *Bromus hordeaceus* (♦), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) at six levels of P supply; and (c,d) root phosphorus fraction of the same species. *Bromus hordeaceus* and *L. perenne* are depicted with dotted lines, all *Rytidosperma* species are depicted with solid lines. Data points are means ± SE and for each panel least significant difference (P=0.05) bars are shown.

All species generally showed a significant (P<0.05) increase in SRL by up to 1.6-fold at low P supply, with the exception of *R. auriculatum*, *R. duttonianum* and *R. pilosum* (Fig. 3a, b). These *Rytidosperma* species had similar SRL at no applied P and 42 mg
applied P per pot. At low P supply *R. carphoides, R. racemosum* and *R. setaceum* showed the highest SRL, whilst *R. duttonianum* showed amongst the lowest. Of the species that showed a greater SRL, the largest adjustment occurred below the lowest level of applied P, 4.5 mg P pot\(^{-1}\).

Root tissue density (RTD) for most species was similar when plants were grown with high-P supply (42 mg P per pot) and no applied P (Fig. 3c,d). However, *L. perenne* was an exception as at above 10.5 mg P per pot, it had a significantly (*P* < 0.001) higher RTD (0.12 mg mm\(^{-3}\)), by up to 2.3-fold, than the *Rytidosperma* species. Across all P levels, *Rytidosperma* species differed in RTD from 0.05 mg mm\(^{-3}\) (e.g. *R. setaceum*) to 0.07 mg mm\(^{-3}\) (e.g. *R. carphoides*).

**Fig. 3** Specific root length of (a) *Rytidosperma auriculatum* (Δ) *R. carphoides* (○), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+) and *R. setaceum* (○) and (b) *Bromus hordeaceus* (●), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) at six levels of P supply; and (c,d) root tissue density of the same species. *Bromus hordeaceus* and *L. perenne* are depicted with dotted lines, all *Rytidosperma* species are depicted with solid lines. Data points are means ± SE and for each panel least significant difference (*P*=0.001) bars are shown.
Root hair length differed by up to 2.4-fold among species at no applied P with the longest and shortest root hairs being observed for *B. hordeaceus* and *R. racemosum*, respectively (Table 2). Most species maintained a similar root hair length between 42 mg applied P per pot and no applied P. By contrast, three species, *B. hordeaceus*, *R. erianthum* and *R. pilosum*, increased root hair length by up to 1.7-fold (*P*<0.05) in response to low P supply. All species maintained a similar root hair density with decreasing P supply, with the exception of *R. pilosum*, which increased root hair density by 1.2-fold at no applied P (Table 2). Mycorrhizal colonisation was low for all species, with *T. subterraneum* having only 17% of root length colonised at no applied P and close to no colonisation at 42 mg applied P per pot (Table 2). For the grasses there was similarly an absence of mycorrhizal colonisation at high-P and low colonisation (0.7 to 10.8% of root length) at no applied P. No grass species had higher colonisation than *T. subterraneum*.

Root hair cylinder volume was greater for *B. hordeaceus* and *L. perenne* than that of all of the *Rytidosperma* species at most levels of P supply (Fig. 4). At no applied P, *R. richardsonii* and *R. setaceum* had a higher RHCV that was similar to that of *B. hordeaceus* and *L. perenne*. Three *Rytidosperma* species, *R. auriculatum*, *R. duttonianum* and *R. fulvum*, had similar RHCV at no applied P as observed at 42 mg applied P per pot. All other *Rytidosperma* species increased RHCV up to 1.2-fold between high and low P supply. In contrast, *B. hordeaceus* decreased and *L. perenne* maintained RHCV between 42 mg applied P per pot and no applied P.
Table 2 Root morphological traits at high- and low-P supply including root hair length, root hair density and mycorrhizal colonisation for all species. Values are means ± standard error; n=5. Within a root morphological trait least significant difference is presented at $P=0.05$ (root hair length and mycorrhizal colonisation) and $P=0.001$ (root hair density).

<table>
<thead>
<tr>
<th>Species</th>
<th>Root hair length (mm)</th>
<th>Root hair density (number mm$^{-1}$)</th>
<th>Mycorrhizal colonisation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-P$^a$</td>
<td>High-P$^b$</td>
<td>Low-P</td>
</tr>
<tr>
<td>Bromus hordeaceus</td>
<td>0.86 ± 0.11</td>
<td>0.56 ± 0.04</td>
<td>39.0 ± 1.9</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>0.54 ± 0.02</td>
<td>0.57 ± 0.04</td>
<td>40.8 ± 1.1</td>
</tr>
<tr>
<td>Rytidosperma auriculatum</td>
<td>0.41 ± 0.03</td>
<td>0.32 ± 0.05</td>
<td>39.4 ± 1.8</td>
</tr>
<tr>
<td>R. carphoides</td>
<td>0.39 ± 0.06</td>
<td>0.32 ± 0.04</td>
<td>37.9 ± 2.7</td>
</tr>
<tr>
<td>R. duttonianum</td>
<td>0.52 ± 0.06</td>
<td>0.56 ± 0.06</td>
<td>47.0 ± 1.8</td>
</tr>
<tr>
<td>R. erianthum</td>
<td>0.68 ± 0.03</td>
<td>0.41 ± 0.02</td>
<td>41.2 ± 0.8</td>
</tr>
<tr>
<td>R. fulvum</td>
<td>0.49 ± 0.02</td>
<td>0.44 ± 0.03</td>
<td>43.0 ± 1.2</td>
</tr>
<tr>
<td>R. pilosum</td>
<td>0.51 ± 0.04</td>
<td>0.37 ± 0.04</td>
<td>40.8 ± 1.3</td>
</tr>
<tr>
<td>R. richardsonii</td>
<td>0.36 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>41.9 ± 1.7</td>
</tr>
<tr>
<td>R. setaceum</td>
<td>0.47 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>42.7 ± 1.8</td>
</tr>
<tr>
<td>Trifolium subterraneum</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

l.s.d interaction: 0.10 interaction: n.s. species: 3.8 P applied: 1.5

$^a$ low P – no applied P

$^b$ high P – 42 mg P per pot

nd – not determined
Fig. 4 Natural log of root hair cylinder volume of (a) *Rytidosperma auriculatum* (∆) *R. carphoides* (○), *R. erianthum* (□), *R. fulvum* (▼) and *R. setaceum* (○) and (b) *Bromus hordeaceus* (●), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲) at six levels of P supply. Data points are means ± SE and for each panel least significant difference (P=0.05) bars are shown

Phosphorus uptake

For all species total plant P uptake was determined by root P content (reported here) and shoot P content (reported in Chapter 3) as shown in Figure 5. Total P content was significantly (P<0.001) correlated with root mass and root length at all levels of P
supply, including at no applied P (Fig. 5a, d); 21 mg applied P per pot (Fig. 5b, e), which corresponded to the critical external P supply required for near maximum growth for the highest requiring species (Waddell et al. 2015); and 42 mg applied P per pot (Fig. 5c, f), a luxury level of P supply for all species. *Bromus hordeaceus* was notable as having the lowest P uptake and smallest root mass and root length when P was not applied, but amongst the largest P uptake and root mass and length at 42 mg applied P per pot. For estimated RHCV, there was also a significant (*P*<0.05) positive correlation with P uptake for most levels of P supply (Fig. 5g, h, i). In the unfertilised soil, P uptake by *B. hordeaceus* was again notable in that its P uptake was markedly impaired, irrespective of its relatively large RHCV. Total P uptake in the unfertilised soil was positively correlated (*P*<0.05) with RHCV when *B. hordeaceus* was removed from the correlation.
Fig. 5 Total plant P uptake relative to (a-c) root mass, (d-f) root length and root hair cylinder volume (g-i) for *Rytidosperma auriculatum* (Δ) *R. carphoides* (◊), *R. erianthum* (□), *R. fulvum* (▼), *R. pilosum* (+) and *R. setaceum* (○) and (b) *Bromus hordeaceus* (♦), *Lolium perenne* (■), *R. duttonianum* (●), *R. racemosum* (×) and *R. richardsonii* (▲). The levels of P-supply are given in each panel. Data points are mean ± SE. Solid regression lines denotes all species included in correlation, dotted regression line (panel g) denotes *B. hordeaceus* removed from correlation.
Discussion

Root system size and P uptake

At all levels of P supply there was a positive correlation between plant P uptake and root mass and length. There was also a positive correlation for plant P uptake and RHCV at all levels of P supply, except no applied P as *B. hordeaceus* was an outlier with a large RHCV relative to P uptake. However, when *B. hordeaceus* was removed from the correlation, the remaining species showed a positive correlation between P uptake and RHCV. The relationship between P uptake and size of roots has been demonstrated for a wide range of crop and pasture species (Jungk and Barber 1974; Otani and Ae 1996; Wissuwa and Ae 2001). It is likely that the large RHCV relative to P uptake of *B. hordeaceus* was attributable to the long root hair length for this species at no applied P (Table 2). It was also notable that at 42 mg P per pot, *B. hordeaceus* and *L. perenne* had a large root mass, length and RHCV relative to P uptake. At 42 mg applied P per pot, *R. richardsonii* had a similar P uptake as *B. hordeaceus* despite having a smaller root mass, length and RHCV. It has been hypothesised that species may also have a large root system due to competition with other species (O'Dwyer and Attiwill 1999; Snaydon and Howe 1986) and the capture of more mobile nutrients such as nitrogen (Hodge et al. 1999).

The *Rytidosperma* species mostly maintained or increased root mass, length and RHCV between 42 mg applied P per pot and low P supply. The exceptions were *R. richardsonii* and *R. fulvum*, which showed a decrease in root mass between 42 mg applied P per pot and no applied P (Fig. 1a, b). *Bromus hordeaceus* and *L. perenne* similarly showed a decrease in root mass, length and RHCV between high and low P supply. For *B. hordeaceus*, this decrease was especially large, with root mass at 42 mg applied P per pot up to 4.4-fold larger than that at no applied P. This large decrease also meant that *B. hordeaceus* had amongst the smallest root mass and length at no applied P, despite having amongst the largest root mass and length at 42 mg P per pot. *Lolium perenne*, by contrast, maintained amongst the largest root mass and length at both high and low P supply. It has been hypothesised that species from infertile habitats have less root morphological plasticity than species from high-fertile habitats (Grime 1979; Robinson and Van Vuuren 1998). However, support for this hypothesis is sometimes weak (particularly amongst monocotyledons, Hodge 2004; Van de Vijver et al. 1993), and
indeed in the present experiment root morphological plasticity did not explain differences in growth rate amongst *Rytidosperma* species.

Whilst the size of the root system explained differences in plant P content among species, it did not explain differences in the P requirement for near maximum growth (Waddell et al. 2015). For example, the two fastest-growing *Rytidosperma* species, *R. duttonianum* and *R. richardsonii* had similar shoot yields (Waddell et al. 2015) and plant P contents at high and low P supply. These species also had similar soil exploration by their roots as measured by the RHCV and mycorrhizal colonisation. We have previously shown that the different critical external P requirements for these species is not related to any measure of P utilisation efficiency including critical internal P concentration (i.e. amount of P in shoots or YEB at 90% of maximum growth), physiological P efficiency of shoots (i.e. amount of shoot yield per unit shoot P) or shoot mass per unit shoot P when shoot P content compared at a common P content (Chapter 3). This suggests that differences in critical external P requirement of the fast-growing *R. duttonianum* and *R. richardsonii* might be related to either root exudates or more precise root foraging in the topsoil. *Rytidosperma richardsonii* is reputed to exude citrate (Barrett and Gifford 1999), but it is not known whether *R. duttonianum* also exudes organic anions. Alternatively, as the P was applied as an aqueous solution in the top 50 mm of the soil only, there may have been differences among the *Rytidosperma* species in the ability to respond to the this heterogeneous P distribution. Whilst some species increase root proliferation within a band of P (Drew 1975; Flavel et al. 2014), it is unclear whether such localised morphological plasticity can determine a species’ critical external P requirement.

**Contribution of root traits to large root systems**

There was no distinct grouping of fast- and slow-growing species with respect to specific root morphological traits which implies that the greater P uptake of the fast-growing species could not be attributed to a single trait. The low levels of mycorrhizal colonisation and similarity of root hair density across species and P-treatments suggests these traits were not of major importance. Of the other root morphological traits, RMF, RPF, SRL, proportion of fine roots, RTD and root hair length, adjustment between high- (42 mg applied P per pot) and low-P supply (no applied P) ranged between 1.2-fold to 2.7-fold for all species. These root morphological traits contribute to increased P uptake.
through an increase in soil exploration, with some traits such as an increased root hair length having a lower carbon cost than other traits such as an increase in RMF (Lynch and Ho 2005). The lack of a single morphological trait, which explained a large root size was demonstrated by the SRL adjustment of the fast-growing species, *L. perenne* and *R. richardsonii*. These species had the longest root length at low P supply and *L. perenne* increased SRL by 1.5-fold between high and low P supply, whilst *R. richardsonii* increased SRL by 1.7-fold (Fig. 3). An increase in SRL is considered to be a carbon-efficient adaptation for increasing soil exploration through an increase in root length (Eissenstat 1992; Wahl and Ryser 2000). For *L. perenne*, the adjustment in SRL was specifically a result of a decrease in RTD (Fig. 3), whilst for *R. richardsonii* the increase in SRL was a result of an increase in the proportion of fine roots (Table 1).

Root hairs were present on all species, with three species, *B. hordeaceus*, *R. erianthum* and *R. pilosum* having longer root hairs at no applied P relative to 42 mg applied P per pot. *Bromus hordeaceus* had the longest root hairs of all species at no applied P. Root hairs can increase the soil exploration through an increased root surface area in contact with the soil, and further, relative to other root morphological traits, root hairs have very low carbon construction and maintenance costs (Bates and Lynch 2000; Fohse et al. 1991; Lynch and Ho 2005). However, root hair length should not be considered in isolation of other root traits, especially root length. This was demonstrated by the large RHCV relative to P uptake of *B. hordeaceus* at no applied P (Fig. 5g). Whilst the long root hairs of *B. hordeaceus* increased the RHCV, this species had amongst the shortest total root length. Long root hairs on a short root system did not help *B. hordeaceus* to fully explore the soil and acquire P. These results indicate that efforts to increase soil exploration need to also consider the total root length rather than only the root hair cylinder volume per unit root length as proposed by Brown et al. (2013).

An increase in RMF also contributes to a larger root system in low-P soils, although this can incur substantial carbon costs (Lynch and Ho 2005). All species increased RMF up to 2.7-fold between high- and low-P supply. After 47 days of growth, *B. hordeaceus* had amongst the smallest shoot mass (Waddell et al. 2015), root mass and root length at no applied P. Despite these low yields, *B. hordeaceus* also had amongst the highest RMF (0.42 g g⁻¹) at no applied P (Fig. 2). This species also had the smallest seedling radicle fraction at 0.11 mg mg⁻¹. It is likely that in the low-P soil, *B. hordeaceus* grew poorly because it did not have a large root system to explore the soil quickly and take up
P at the seedling stage. The high RMF at no applied P after 47 days of growth indicates *B. hordeaceus* over-invested in root growth at the expense of its shoots and also failed to convert the high allocation of plant mass to the roots into a long root length. Bean plants (*Phaseolus vulgaris* L.) grown in low-P soils use a greater fraction of their whole-plant available carbon for root respiration than plants grown in high-P soils, resulting in less carbon available for the growth of other organs including shoots (Nielsen et al. 2001). In contrast, *L. perenne, R. duttonianum* and *R. richardsonii*, which were fast-growing at both high- and low-P supply (Waddell et al. 2015), had RMF values (0.35 g g⁻¹) at low-P supply similar to the seedling radicle fractions (up to 0.37 mg mg⁻¹). Nielsen et al. (2001) hypothesised that P-efficient plants that retain a similar ontogenetic allocation of C between shoots and roots (i.e. RMF) under low-P conditions are able to maintain a larger root system for P-acquisition with less root respiration C costs, thus enabling these species to retain more photosynthetic C for plant growth.

**Conclusion**

Amongst the *Rytidosperma* species there was similar adjustment of all root morphological traits at high and low P supply which indicates that root morphological plasticity did not explain differences between fast- and slow-growing species. However, for all species, root mass, length and RHCV were positively correlated with P uptake. Species that had larger root systems explored more soil, took up more P and grew faster. Several root morphological traits were examined; however, no single trait was notable for contributing to a large root system for all fast-growing species. Rather, different species utilised different root morphological traits. These morphological traits should not be considered in isolation of the size of the root system, as demonstrated by the inability of long root hairs to compensate for the short root length of *B. hordeaceus*. *Bromus hordeaceus* was also notable for investing a large portion of its mass as cotyledon at the seedling stage which may have compromised soil exploration and P-uptake, but had amongst the largest RMF at low-P stress which may have compromised shoot photosynthetic potential. In contrast, the fast-growing species, *L. perenne, R. duttonianum* and *R. richardsonii*, balanced resource allocation between their roots and shoots to maximise soil exploration for P-uptake with minimal trade-off against shoot photosynthetic potential.
Acknowledgements

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Chapter 5. Root organic anion exudates and the critical external phosphorus requirements of fast- and slow-growing *Rytidosperma* species (wallaby grass)
Abstract

Among fast-growing *Rytidosperma* species, there are differences in the critical external phosphorus (P) requirement for growth, that is, the amount of P required for 90% of maximum growth. In particular, *R. duttonianum* consistently has a high critical external P requirement for growth, whilst *R. richardsonii* has a low critical external P requirement. These differences in critical external P requirements may be related to differences in root exudates, especially organic anions, some of which mobilise sorbed P from the soil. To test this hypothesis, four *Rytidosperma* species differing in critical P requirements for growth, *Bromus hordeaceus*, *Lolium perenne*, *Trifolium subterraneum* and *Lupinus albus* were grown at high and low P supply for collection of root exudates. All species exuded total carbon at similar rates at high and low P supply. Four species, *L. albus*, *R. carphoides* *R. duttonianum* and *R. richardsonii* exuded more organic anions at low P supply than at high P supply. *Rytidosperma erianthum* exuded similar quantities of organic anions at low and high P supply. This suggests that low-P stress may stimulate the exudation of organic anions for some *Rytidosperma* species, as has been shown for *L. albus*. Further, whilst citrate and malate were detected in the root exudates of *R. duttonianum*, lactate was the major exudate collected for *R. richardsonii*. Hence, the role of organic anions in P uptake and the critical external P requirement for growth of *Rytidosperma* species remains uncertain.
Preface

Chapter 5 follows on from Chapter 4 in that it examines some physiological root adaptations of slow- and fast-growing *Rytidosperma* species to applied P. Chapter 4 failed to identify morphological traits which may explain the different P requirements for growth amongst *Rytidosperma* species (Chapter 2) and hence Chapter 5 examines some physiological traits. It is a different experimental set up to that reported in Chapters 2, 3 and 4.

Introduction

*Rytidosperma* species are Australian native perennial grasses common in cool-temperate grasslands (Garden et al. 2001). Some species were identified as slow- or fast-growing in response to six levels of P-supply ranging from high- (46 mg P per kg soil) to low (no added P) P (Chapter 2). There was also a range of P-requirements for growth as measured by the critical external P requirement, the amount of P required to achieve 90% of shoot growth (Chapter 2). In particular, two fast-growing *Rytidosperma* species, *R. duttonianum* and *R. richardsonii*, yielded as well as *Lolium perenne* at high and low P supply. Compared with the other *Rytidosperma* species, *R. richardsonii* had amongst the lowest critical external P requirement, whilst *R. duttonianum* had amongst the highest critical external P requirement. Both fast-growing *Rytidosperma* species have similar critical internal P concentrations of the whole shoot (i.e. the concentration of P in shoots at 90% maximum growth), physiological P efficiencies (i.e. the amount of shoot mass produced per unit shoot P), shoot mass per unit P when measured at a common shoot P content and shoot mass per unit P when measured at a common relative shoot yield, suggesting the way in which they utilise P for growth is similar (Chapter 3). Further, both *R. duttonianum* and *R. richardsonii* have similarly large root systems, especially root hair cylinder volumes, a measure of potential soil exploration which incorporates root length, diameter and root hair length (Chapter 4). Given this similarity in shoot and root morphological traits, it was hypothesised that differences in critical external P requirements of *R. duttonianum* and *R. richardsonii* may be due to differences in root functional traits. One possibility is that the two species may differ with respect to root exudates, which may impact their capacity to mobilise sorbed P from the soil.
Root exudates containing carbon (C) compounds are an important mechanism through which the plant interacts with, and modifies, the rhizosphere. Carbon exuded by the roots is mostly derived directly from shoots as photosynthates (Johnson et al. 1996) and commonly accounts for around 5-10% of plant fixed C, although this can vary with plant age and species (Grayston et al. 1997; Jones et al. 2009). Johnson et al. (1996) demonstrated that Lupinus albus L. plants grown under low-P conditions have higher rates of total C-fixation and C-exudation than plants grown under high-P supply. Quantitatively, the main C-compounds that are exuded include carbohydrates, amino acids and low-molecular-weight organic anions, although this can vary according to species and soil nutrient conditions (Curl and Truelove 1986). Organic anions are of particular interest, as they potentially play a role in improving P availability in the rhizosphere. There are several mechanisms by which this may occur: firstly protons are often concomitantly released with organic anions which lead to the acidification of the rhizosphere and in alkaline soils may increase the solubility of sparingly-soluble inorganic P compounds or affect the kinetics of orthophosphate adsorption-desorption reactions (Dinkelaker et al. 1989; Hoffland et al. 1989; Neumann and Römheld 1999). Reduced sorption of P to soil particles also occurs through the alteration of the surface characteristic of the soil particles due to ligand exchange and ligand-promoted dissolution reactions. Further, tricarboxylates (e.g. citrate) and dicarboxylates (e.g. malate) chelate cations, particularly Al\(^{3+}\), Fe\(^{3+}\) and Ca\(^{2+}\), which immobilise phosphate (Jones 1998; Ryan et al. 2001). Organic anions have also been proposed to increase plant-available P that is bound in humic-metal complexes (Gerke 1993) and solubilise organic forms of P such as phytate thus making it more amenable to dephosphorylation by phosphates (Hayes et al. 2000).

Some plants such as L. albus, exude organic anions from proteoid roots, which are dense clusters of lateral roots (rootlets) covered in root hairs that form in specific regions of the root system, whilst other species exude organic anions from non-proteoid roots (Hoffland et al. 1989; Keerthisinghe et al. 1998). There is interest in the potential applicability of utilising species that exude organic anions in response to low-P stress within agricultural systems to improve the availability of P to species such as wheat (Triticum aestivum L.) and other important agricultural plants that do not have adaptations to access sparingly-available soil P (Lambers et al. 2006; Nuruzzaman et al. 2005).
Rytidosperma richardsonii is reputed to exude citrate at rates similar to that of L. albus when grown under low-P conditions (Barrett and Gifford 1999; Ryan et al. 2001). However, it is not known whether the exudation of organic anions by R. richardsonii plays a role in P-uptake, and whether other Rytidosperma species also exude organic anions. Therefore, this study aimed to examine total C and organic anion exudates by four fast- and slow-growing Rytidosperma species of differing critical external P requirements. The Rytidosperma species were compared with L. albus, as well as three other common pasture species, Bromus hordeaceus, L. perenne and Trifolium subterraneum.

Methods

Eight species were examined: L. albus L. cv. Luxor (Hart Bros Seeds Pty Ltd.), T. subterraneum L. cv. Seaton Park (CleanSeeds Pty Ltd.), B. hordeaceus L. (synon. B. molliformis), L. perenne L. cv. Victorian (CleanSeeds Pty Ltd.), R. carphoides (F.Muell. ex Benth.) Connor & Edgar, R. duttonianum (Cashmore) Connor & Edgar, R. erianthum (Lindl.) Connor & Edgar and R. richardsonii (Cashmore) Connor & Edgar cv. Taranna. Of the Rytidosperma species, R. duttonianum and R. richardsonii are fast-growing species, whilst R. carphoides and R. erianthum are slow-growing (Chapter 2). Lupinus albus was included as it is well known to exude large amounts of organic anions at low P supply (Dinkelaker et al. 1989; Gardner et al. 1983; Keerthisinghe et al. 1998), whilst B. hordeaceus, L. perenne and T. subterraneum are common pasture species and were included to provide a range of pasture species as controls. Seeds of all Rytidosperma species were collected from naturally occurring plants from a single site, ‘Yeumville’, near Hall, New South Wales (35° 4´S, 19° 5´E) as part of a field experiment (Garden et al. 2000), except for R. richardsonii which was obtained from a commercial source. Bromus hordeaceus was collected from Ginninderra Experimental Station, Canberra, Australian Capital Territory (35° 10´S, 149° 02´E, elevation 597 m).

All seeds were germinated in a petri dish moistened with an aqueous solution containing a fungicide (thiram, 800 g kg⁻¹, Barmac Industries, Queensland, Australia) before being transplanted into pots containing washed coarse river sand (see below). Seedlings were thinned to either one plant per pot (L. albus, T. subterraneum, B. molliformis and L. perenne), two plants per pot (R. carphoides, R. duttonianum and R. erianthum) or three plants per pot (R. richardsonii) seven days after transplanting. For the grasses, each pot
had the number of plants required to achieve the equivalent mass of one *L. perenne* seed per pot. The cotyledons of *L. albus* seedlings were removed eight days after transplanting, to minimise the transfer of seed P reserves due to the importance of internal P concentration for the formation of cluster roots (Keerthisinghe et al. 1998; Marschner et al. 1987).

Each cylindrical pot, 90 mm × 200 mm depth, contained 1.72 kg of dried washed river sand. Low-P treatments were established by applying P as an aqueous solution of KH$_2$PO$_4$ in a single dose of 0.2 mg P per pot sufficient to wet the top 40 mm of sand, at the beginning of the experiment; high-P treatments were established by applying 1 mM KH$_2$PO$_4$ as part of a modified Hoagland nutrient solution (Hoagland and Arnon 1950) in which a total of 2.1 mg P per pot was applied by the end of the experiment. The remainder of the nutrient solution consisted of 0.05 mM FeNaEDTA and 2 mM MgSO$_4$, 7.5 mM CaSO$_4$, 20 mM KNO$_3$, 2.5 mM (NH$_4$)$_2$SO$_4$, and the following micronutrients; 23 µM H$_3$BO$_3$, 46 µM MnCl$_2$, 15 µM ZnSO$_4$, 1.6 µM CuSO$_4$, 0.7 µM (NH$_4$)$_2$MoO$_4$ and 1.0 µM CoCl$_2$ which were initially applied at quarter strength and increased to half concentration. Over the course of the experiment each pot received equivalent of a total of 190 ml of the nutrient solution, typically applied at a rate of 10 ml three times per week. A total of six replicates per treatment were established. Plants were otherwise watered daily with deionised water to maintain pots at 80% field capacity.

Plants were grown in a growth cabinet (Conviron CMP6050, Winnipeg, Canada) under the following conditions, day: 12 h, 22°C, quantum flux density at plant height 500 µmol m$^{-2}$; night: 12 h, 14°C. All plants were harvested 37 days after transplanting by gently washing sand from roots and care was taken to minimise damage to the roots. To collect exudates, plants were placed in a known volume (40 to 100 ml) of sterilised deionised water sufficient to submerge the roots and placed in the growth cabinet for a recorded period of time of up to 120 minutes. For short-term incubations of less than two hours there is no difference in organic acid exudation into deionised water compared with that into diluted CaSO$_4$ solution (Neumann et al. 1999). In addition, it was considered that the osmotic adjustment of roots obtained from the coarse river sand would not be adversely affected. Plants were then removed from the solution, separated into root and shoot portions, dried at 70°C for 48 hours and weighed. The solution was sterilised immediately by passing through a 0.22 µm filter, divided into three subsamples and frozen until further analysis. A 10 ml subsample of the exudate solution
at 1 × concentration was used to measure total carbon using a total organic carbon analyser (Vario TOC cube, Elementar Analysensysteme GmbH, Hanau, Germany) in which a thalate standard that was linear for C concentration up to 120 mg l⁻¹ was used. Organic anion concentration and composition was measured by a HPLC (600E pump, 717 plus autosampler and 996 photodiode array (PDA) detector, Waters, Milford, USA) using the methods described by Cawthray (2003). The procedure was quantitative for the detection of a range of organic anions that are commonly found in root exudates (Neumann and Römheld 1999; Pearse et al. 2006) and included acetate, cis-aconite, trans-aconite, citrate, fumarate, lactate, malate, maleate, malonate, shikimate and succinate. Positive identification and concentration of unknown organic anions was achieved by comparing them against the standard retention time and PDA peak spectral analysis of known samples obtained from ICN Biomedicals (Aurora, Ohio, USA).

Concentrations of organic anions determined by HPLC were converted to mass by molecular weight (MW) according to the MW of free (disassociated) anions when in solution. Total organic anion exudation was determined by summation of mass of all detected organic anions that were above the limit of detection (Table 1).

Data were analysed using two-way ANOVA with GenStat (15th edition; VSN International, UK). Where there was no significant species × P treatment interaction, a t-test was performed. Natural logarithm was used to transform data for statistical analysis where there were large differences in variation in the data.

**Results**

Cluster roots were present on *L. albus* roots at low- and high-P, but not in any other species.

At high- and low-P, *L. albus* produced more shoot and root mass than any of the other species (Fig. 1a, b). Of the grasses, *L. perenne* had the highest shoot and root mass. *Trifolium subterraneum* had less shoot and root mass than *L. perenne*, but similar shoot and root mass to all other grasses. Across all species, there was no difference in root mass between high- and low-P treatments. However for *B. hordeaceus*, *R. duttonianum* and *R. richardsonii*, shoot mass was greater at high P supply than at low P supply. For all other species, there was no difference in shoot mass between high and low P supply.
Amongst the *Rytidosperma* species, at high P supply *R. duttonainum* and *R. richardsonii* had more shoot mass than *R. carphoides* and *R. erianthum*.

Total carbon exudation by roots was determined as total C collected in the root exudates solution and expressed per unit of total root mass. There was a significant (*P*<0.05) difference amongst species for total carbon exuded (Fig. 2). *Bromus hordeaceus* had the largest amount of C exuded per gram root dry mass per hour, whilst *L. perenne* had amongst the least amount of C exuded. However, there was no difference between the C exuded at low or high P supply for any species.
Fig. 1 Shoot mass (a) and root mass (b) of eight species at low P (fill) and high P (no fill) supply. Data are means ± SE, and LSD bars at $P=0.05$ are shown.
Total release of organic anions in the root exudates varied considerably with species with *R. carphoides* notable as having a very high level of organic anions compared with other species. Significant amounts of organic anions were also detected for *L. albus*, *R. duttonianum* and *R. richardsonii* at low P supply.

Four species, *L. albus*, *R. carphoides*, *R. duttonianum* and *R. richardsonii* exuded more organic anions per gram of root dry weight at low P supply than at high P supply (Fig. 3). Of these species, *L. albus* was notable in that it exuded eight times more organic anions at low P relative to high P supply, whilst *R. duttonianum* exuded six times more at low P relative to high P supply. All other species exuded similar quantities of organic anions at high and low P supply. Further, at low P supply, *R. duttonianum* and *R. richardsonii* exuded a similar amount of organic anions as *L. albus*, whilst *R. carphoides* exuded more. All other species exuded less organic anions than *L. albus* at low P supply.
Fig. 3 Total release of organic anions in root exudates (µmoles g⁻¹ root dry mass hour⁻¹) for eight species at low P (fill) and high P supply (no fill). Total amount of organic anions was determined by summing of individual organic anions as reported in Table 1. Data are means ± SE, and LSD bars at \( P = 0.05 \) are shown.

The organic anion C concentration of specific root exudates was also expressed per unit of total C in the exudates (Fig. 4). There was a large range in the ratio of organic anion C to total C amongst species. The two-way ANOVA showed a significant interaction across all species and for four species, *L. albus*, *R. carphoides*, *R. duttonianum* and *R. richardsonii* differences between organic anion C to total C fraction at low P supply and high P supply were significant when tested by paired t-tests. All other species had a similar organic anion C to total C fraction at high and low P supply. *Lupinus albus* was notable for exuding 23% of its C as organic anions at low P supply, but just 2% at high P supply. There was a large amount of variability between replicates for the slow-growing *Rytidosperma* species, *R. carphoides* and *R. erianthum*. For reasons unknown, the calculated total amount of organic anion C, measured by HPLC, detected in the root exudates solution for *R. carphoides* exceeded the total carbon content as measured using a total organic carbon analyser. Whether this represents a specific error for *R. carphoides*, or is a more general error across all species (i.e. that total C
determination by HPLC is not quantitatively consistent with that determined by total organic carbon analyser) remains unclear.

Fig. 4 The fraction of total organic anion carbon (as analysed in HPLC) relative to total carbon (measured using a total organic carbon analyser) exuded for eight species at low (fill) and high P supply (no fill). Data are means ± SE and the LSD bar at $P=0.05$ for the interaction are shown. Note, for reasons unknown, total organic anion carbon of *R. carphoides* exceeded total C content.

For most species, the amount and type of organic anions exuded was small and variable. Acetate, maleate, malonate and succinate were tested for, but not detected for any species at high or low P supply. Across all species, the organic anions detected in the greatest amounts were citrate and malate. Most of this was detected in *L. albus* and *R. carphoides* at low and high P supply. Smaller amounts of citrate and malate were also detected in *R. duttonianum* at low P supply and in *R. erianthum* at high P supply (Table 1). Lactate was detected in large quantities for *R. duttonianum* and *R. richardsonii*. However, lactate was only detected in one of the *R. richardsonii* replicates. For *R. richardsonii*, no other organic anion was exuded in similarly high quantities; however, for *R. duttonianum* malate was also exuded at a similar rate as lactate.
Table 1. Quantities of organic anions (µmoles g⁻¹ root dry weight hour⁻¹) detected for eight species. Values were determined by HPLC and are means with standard error presented in parenthesis only for samples where a positive detection above the limit of detection occurred (Cawthray 2003).

nd – not detected

*a* acetate, maleate, malonate and succinate were tested for but not detected for any species.

*b* detection limits were: acetate 24 µM, *cis*-aconite 0.1 µM, *trans*-aconite 0.1 µM, citrate 5 µM, fumarate 0.06 µM, lactate 13 µM, malate 7 µM, maleate 0.05 µM, malonate 8 µM and succinate 15 µM.

<table>
<thead>
<tr>
<th>Exudate&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Lupinus albus</th>
<th>Trifolium subterraneum</th>
<th>Bromus hordeaceus</th>
<th>Lolium perenne</th>
<th>Rytidosperma carphoides</th>
<th>R. duttonianum</th>
<th>R. erianthum</th>
<th>R. richardsonii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low P</td>
<td>High P</td>
<td>Low P</td>
<td>High P</td>
<td>Low P</td>
<td>High P</td>
<td>Low P</td>
<td>High P</td>
</tr>
<tr>
<td>cis-aconite</td>
<td>0.002 (0.002)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>trans-aconite</td>
<td>0.002 (0.002)</td>
<td>nd</td>
<td>nd</td>
<td>0.74 (0.39)</td>
<td>0.15 (0.15)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>citrate</td>
<td>6.32 (3.16)</td>
<td>0.36 (0.36)</td>
<td>nd</td>
<td>0.64 (0.41)</td>
<td>nd</td>
<td>2.0 (2.0)</td>
<td>0.07 (0.07)</td>
<td>17.67 (5.04)</td>
</tr>
<tr>
<td>fumarate</td>
<td>0.03 (0.007)</td>
<td>0.005 (0.005)</td>
<td>0.01 (0.01)</td>
<td>0.01 (0.01)</td>
<td>0.02 (0.02)</td>
<td>0.01 (0.01)</td>
<td>0.20 (0.05)</td>
<td>0.06 (0.06)</td>
</tr>
<tr>
<td>lactate</td>
<td>2.0 (2.0)</td>
<td>nd</td>
<td>nd</td>
<td>4.22 (4.22)</td>
<td>0.61 (0.61)</td>
<td>nd</td>
<td>1.89 (1.89)</td>
<td>5.32 (3.57)</td>
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<tr>
<td>malate</td>
<td>4.13 (1.13)</td>
<td>0.79 (0.79)</td>
<td>nd</td>
<td>0.48 (0.48)</td>
<td>nd</td>
<td>1.13 (0.39)</td>
<td>0.50 (0.50)</td>
<td>23.09 (7.09)</td>
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<tr>
<td>shikimate</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.002 (0.001)</td>
</tr>
</tbody>
</table>
Discussion

Exudates of carbon and organic anions

All species exuded C at high and low P supply. However, there were no differences in the quantity of C detected between high and low P supply. At low P supply, L. albus exuded a large portion of total C as organic anions (23%) and further, the quantity of organic anions exuded at low P supply was eight times higher than that at high P supply. This is consistent with many studies, which have demonstrated that low P stress is a stimulus for organic anion exudations for L. albus (Dinkelaker et al. 1989; Gardner et al. 1983; Keerthisinghe et al. 1998) and that a large amount of C is exuded as organic anions (Johnson et al. 1996). Further, the amount of citrate exuded by L. albus at low P supply, 10.7 µmol g$^{-1}$ root DM hour$^{-1}$, is within the range reported by Ryan et al. (2001).

For all species, there were differences in the amount of C exuded as organic anions. Bromus hordeaceus was particularly notable for having amongst the highest quantity of total C exuded at both high and low P supply, but amongst the smallest amount of organic anions exuded. Trifolium subterraneum and L. perenne also had a small organic anion C to total C fraction. Other carbon-compounds are also known to be exuded under low P stress, including carbohydrates and amino acids (Carvalhais et al. 2011; Hütsch et al. 2002). It has been hypothesised that these other carbon-compounds may play a role in plant acclimation to P-stress through interactions with soil microorganisms, including mycorrhizal fungi (Ratnayake et al. 1978) and rhizobacteria (Richardson et al. 2009). Alternatively, for these species, P may not be a stimulant for organic anion exudation. For example, aluminium toxicity, but not P, stimulates the exudation of organic anions for many species (Ryan et al. 2001).

The ratio of organic anion C to total exuded C was particularly high for R. carphoides, and there was a large degree of variation for other species (Fig. 4). This highlights the difficulties in accurately measuring total exuded C and in particular, the organic anion component. The detection of organic anions and the quantity collected can depend upon the method of collection, time of day and the collection area of the plant root (Shi et al. 2011; Watt and Evans 1999). Whilst many of these factors were controlled, consistent with many other studies, large variability amongst replicates still occurred (e.g. Lipton et al. 1987; Marschner et al. 2002; Shi et al. 2011; Shi et al. 2012). The growing
medium used (river sand) in this experiment may have contributed to this variability. In particular, in the field some *Rytidosperma* species, including *R. erianthum*, are associated with finer-textured soils containing clay and loam, not sand (Waters et al. 2009), although *R. richardsonii* has been recorded as growing in washed quartzite sand under glasshouse conditions (Barrett and Gifford 1999). The accurate collection of root exudates in fine-textured soils whilst minimising root damage and microbial contamination is particularly difficult, although further development of *in situ* methods utilising anion exchange membranes in silt loam soils may help with these challenges (Shi et al. 2011). Despite advances in *in situ* methods, inconsistencies between methods can still remain. In this experiment, the amount of C detected by total organic carbon analysis was less than that measured by HPLC, especially for *R. carpoides*. Whether this error was specific for *R. carpoides* or across all species remains unclear, although the large error bars for other species such as *R. erianthum* could suggest the latter.

*Rytidosperma* species

There were differences in shoot yields for *R. duttonianum* and *R. richardsonii* grown at high and low P supply, but not for *R. carpoides* or *R. erianthum*. At high P supply, *R. duttonianum* and *R. richardsonii* also produced more shoot dry mass than *R. carpoides* and *R. erianthum*. This is consistent with the distinction between fast- and slow-growing *Rytidosperma* species identified in Chapter 2. The fast-growing *Rytidosperma* species also had similar shoot mass as *B. hordeaceus* at high and low P supply, but less shoot mass than *L. perenne*. In Chapter 2, *R. richardsonii* and *R. duttonianum* were identified as having similar shoot mass as *L. perenne*; however, in this experiment, plants were grown in sand rather than finer-textured soils (Waters et al. 2009) and sward conditions were not replicated (Chapters 2,3). Despite these differences in shoot yields amongst fast- and slow-growing grasses, there were no clear differences in the total C exudates detected amongst species or P-treatment. In particular, all *Rytidosperma* species exuded similar quantities of C at both high and low P supply. This result was surprising given that it has been shown that for *L. albus*, the quantity of C exudates is higher at low P supply than at high P supply (Johnson et al. 1996). Further studies are required to examine whether there are differences in the amount of C fixed by shoots amongst *Rytidosperma* species, and the impact this has on the C exudation rate of fast- and slow-growing *Rytidosperma* species in fine textured soils.
Relative to *L. albus*, *R. carphoides* was notable for the quantity and variability in the amount of organic anions exuded. It is likely that the quantity of organic anions exuded by *R. carphoides* was distorted by its small root mass in comparison with that of *L. albus*. Further distorting the amount of organic anions exuded is the lack of knowledge of the sections of *Rytidosperma* roots from which organic anions are exuded. *Lupinus albus* is known to exude organic anions mainly from young proteoid roots 1-3 cm behind the root axis tip (Keerthisinghe et al. 1998), although this can vary between different organic anions. For example, the exudation of malate has been recorded at being greater at the apical region of non-proteoid roots, whilst citrate was greatest at the mature region of proteoid roots (Neumann et al. 1999). In contrast, organic anion exudates of *Brassica napus* L. are known to occur at root tips or more mature regions of its roots, depending on the location of the P (Hoffland et al. 1989; Hoffland et al. 1992). Hence, the expression of root exudates per unit total root dry mass may have distorted the calculations of organic anion exudates for *R. carphoides*.

Three of the four *Rytidosperma* species, *R. carphoides*, *R. duttonianum* and *R. richardsonii*, increased organic anion exudates in response to low P supply. This response was very different from that of *B. hordeaceus*, *L. perenne* and *T. subterraneum*, which consistently had low levels of organic anion exudation. In particular, *R. duttonianum* had a 6-fold increase in organic anion exudates between high and low P supply, in comparison to the 8-fold increase for *L. albus*. This suggests that for some *Rytidosperma* species P may be a stimulant of organic anions released. Further, similar to *L. albus*, citrate and malate were detected in the exudates of *R. carphoides* and *R. duttonianum*. *Rytidosperma richardsonii* is also reputed to exude citrate (Barrett and Gifford 1999), but citrate was not detected in *R. richardsonii* exudates in this experiment. These organic anions are known to play a role in P acquisition through competing with phosphate for binding sites on soil particles and forming stronger complexes with aluminium, iron and calcium in the soil (Ryan et al. 2001). However, further studies are required to verify the role of organic anion exudates from *Rytidosperma* species in P-uptake.

It was previously hypothesised that root exudates were one of two unexplored factors that may play a role in determining the different critical P requirement of *Rytidosperma* species (Chapter 4). This experiment found no evidence of differences in organic anion exudates between *Rytidosperma* species with high or low critical P requirements.
Indeed the two *Rytidosperma* species with the highest critical P requirements, *R. carphoides* and *R. duttonianum*, exuded citrate and malate at low P supply. In contrast, of the *Rytidosperma* species with a low critical P requirement, the main organic anion detected for *R. richardsonii* was lactate in just one of the replicates, which is a monocarboxylate with a weak ability to chelate cations that bind phosphate in the soil (Ryan et al. 2001); and *R. erianthum* exuded significantly less organic anions than *R. carphoides* and *R. duttonianum*. Given that citrate has previously been detected in the exudates of *R. richardsonii* (Barrett and Gifford 1999), the role of organic anions in determining the critical P requirement of these species remains an open question.

Conclusion

Carbon exudates were detected for all species; however, there were no differences in the quantity of C between high and low P supply or between fast- and slow- growing *Rytidosperma* species. The quantity and composition of organic anion exudates from most *Rytidosperma* species was more similar to that of *L. albus* than that of *B. hordeaceus*, *L. perenne* or *T. subterraneum*. This suggests that P may be a stimulant of organic anions for some *Rytidosperma* species. However, the role of organic anions in P-uptake by *Rytidosperma* species, and their role in the critical P requirement for growth are yet to be fully determined.
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Chapter 6. General Discussion
Introduction

Phosphorus (P) is an essential nutrient for plant growth and reproduction and low P availability in soils limits global agricultural production (Vance et al. 2003). Rock phosphate, from which most P-fertilisers are derived, is a non-renewable resource (Scholz and Wellmer 2013). There is a need to improve the efficiency with which P-fertilisers are used to conserve this non-renewable resource and to minimise negative environmental impacts from the over-use of P, especially eutrophication of waterways (Carpenter and Bennett 2011) and oligotrophic systems (Lambers et al. 2013). One strategy to improve the P efficiency of agricultural systems is the use of plants that grow well with limited P availability. Measures of plant P-use efficiency (PUE) include P-utilisation efficiency, which is the incorporation of P into plant mass; and P-uptake efficiency, which is the acquisition of P from the soil (Baligar et al. 2001). These measures of PUE can be used to determine guidelines for effective P fertilisation rates for optimal production in pasture systems (Simpson et al. 2014). Additionally, measures of PUE can help identify species which grow well at low P supply so that desirable shoot and root traits can be identified (Baligar et al. 2001; Föhse et al. 1988; Hammond et al. 2009). An ideal plant is one that has a high P-uptake efficiency, that is, a low critical external P requirement for growth and/or high agronomic P efficiency, and a high P-utilisation efficiency due to a high physiological P efficiency of shoots and/or low critical internal P concentration (Föhse et al. 1988). Hill et al. (2005) identified a native Australian grass species, *Rytidosperma richardsonii*, as having two such characteristics, namely a low critical external P requirement and low critical internal P concentration. However, little is known about other *Rytidosperma* species.

*Rytidosperma* species form the dominant vegetation component of some grasslands in cool-temperate regions of Australia (Garden et al. 2001). Typically, more than three *Rytidosperma* species are present within a site, and up to ten species have been reported (Powells et al. 2012; Waters et al. 2009). Due to difficulties in identifying species using vegetative traits alone (Humphreys et al. 2010; Linder et al. 2010), field-based studies often group all *Rytidosperma* species as one entity (e.g. Garden et al. 2003; Garden et al. 2000). Existing information on the growth response of individual *Rytidosperma* species to varied P supply has been limited by a lack of rigorous statistical analysis of plant growth (Bolger and Garden 1999) and confounding field-based variables (e.g. soil edaphic, climatic and management factors) (Dorrough et al. 2011). This thesis therefore...
examined the response of several *Rytidosperma* species to varying P supply in glasshouse studies. Results from this thesis have highlighted the range of growth responses to P supply amongst *Rytidosperma* species, their interesting shoot morphological traits and the role that a large root system (i.e. large root mass, long root length and large root hair cylinder volume (RHCV)) play in determining the different yield potentials of fast- and slow-growing *Rytidosperma* species.

**Summary of key findings**

Growth response of *Rytidosperma* species to phosphorus supply

There are divergent opinions on the response of native Australian grasses to P supply. This includes the belief that native species are of low value due to their low yield potentials and lack of response to P fertiliser (Donald 1970), through to the belief that native perennial species have a valuable role to play in maintaining the stability of pastures (Sanford et al. 2003). Decreases (Dorrough et al. 2011; Garden et al. 2003) and increases (Garden et al. 2001) in the occurrence and basal cover of *Rytidosperma* species with increasing soil P fertilisation have been observed. Hence, we examined the growth response to P of nine *Rytidosperma* species, *R. auriculatum*, *R. carphoides*, *R. duttonianum*, *R. erianthum*, *R. fulvum*, *R. pilosum*, *R. racemosum*, *R. richardsonii* cv. Taranna, and *R. setaceum* (Chapter 2). The *Rytidosperma* species were compared with two introduced species, *Bromus hordeaceus* and *Lolium perenne*, which were known to have contrasting P requirements for growth (Hill et al. 2005). *Lolium perenne* is a valued pasture species, due to its fast growth rate, high feed value and perennial habit. In contrast, *B. hordeaceus* is a more ‘weedy’ species of low feed value and annual habit. The *Rytidosperma* species were all collected from a single paddock, except for *R. fulvum*, which was present at the site but not in large enough numbers to enable the collection of seed and hence was collected from another site, and *R. richardsonii* cv. Taranna, which is a commercially available cultivar. There was a range of growth responses to P supply amongst the *Rytidosperma* species, including slow-growing (*R. auriculatum*, *R. carphoides* and *R. erianthum*) and fast-growing (*R. duttonianum*, *R. racemosum* and *R. richardsonii*). Two of these fast-growing species, *R duttonianum* and *R. richardonii*, also had similar shoot yields to *L. perenne* at high and low P supply. No species showed evidence of P toxicity at high P supply. Species that grew well at high P supply also grew well at low P supply, except for *B. hordeaceus* which had a
lower shoot yield at low P supply relative to its yield at high P supply. There was also a range in critical external P requirements, that is, the amount of P required to achieve 90% of maximum growth; and agronomic P-use efficiency, that is, shoot yield per unit applied P. For example, of the fast-growing *Rytidosperma* species, *R. richardsonii* had a low critical external P requirement and high agronomic P-use efficiency, whilst *R. duttonianum* had a high critical external P requirement and lower agronomic P-use efficiency.

The differences in shoot yields and P requirements for growth among the *Rytidosperma* species help to explain divergent opinions on the response of native Australian grasses to P-supply. If slow-growing unresponsive species such as *R. auriculatum*, *R. carphoides* or *R. erianthum* are present, then conclusions of a low value for native species within pastures are not surprising. The fast-growth, low critical external P requirement and high agronomic P-use efficiency of *R. richardsonii* is particularly notable. This species could potentially play a role in improving the P-balance efficiency of pastures (ratio of P output to P input) (Simpson et al. 2014) without compromising productivity. Further field studies on the long-term persistence and grazing tolerance of *R. richardsonii* under low-P soil conditions are warranted.

**Shoot P contents and morphology traits**

Chapter 3 aimed to determine whether the internal efficiency with which *Rytidosperma* species utilised P for shoot growth contributed to differences in responses to P applications or contrasting critical external P requirements. Shoot P concentration was used for four measures of P-utilisation efficiency (PUE): (i) critical internal P concentration, which is the concentration of P in the shoots at near maximum growth, (ii) physiological P efficiency of shoots (PPE), which is the amount of shoot mass produced per unit shoot P content (Baligar et al. 2001; White and Hammond 2008) (iii) shoot mass per unit shoot P at a common shoot P content (PUE$_{c}$, 4 mg shoot P) (Rose et al. 2015) and (iv) shoot mass per unit shoot P at a common relative shoot yield (PUE$_{s}$, 0.7 relative yield). Leaf P concentration was also used in conjunction with shoot morphology traits to test the hypothesis that species which have a low area-based leaf P concentration (P$_{area}$) produce more leaf mass per unit leaf P concentration (Ryser et al. 1997) and further species with a larger leaf area per unit leaf mass, that is a high specific leaf area (SLA), grow faster (Lambers and Poorter 1992).
The fast-growing species tended to have a lower shoot P concentration in the youngest fully expanded blade (YEB), which is physiologically the most active leaf, than the slow-growing species. However, the fast-growing species were more responsive to P supply, and hence had higher shoot P contents than slow-growing species. No measure of PUE was correlated with the critical external P requirements of the species and most measures were not correlated with potential maximum growth rates of the species. The exception was the positive correlation between PUE\textsubscript{c} and potential maximum growth rates of the species. Inevitably, the measure PUE\textsubscript{c} may not be completely independent of growth rates, as it was measured using the maximum shoot yield. In contrast to the lack of correlation between all other measures of PUE and potential maximum growth rates, there was a positive correlation between shoot yield per unit P and maximum potential growth rate (Chapter 2 Fig. 3). Hence, for the *Rytidosperma* species growth per unit P may be more related to potential maximum growth rate and the utilisation of P for growth, rather than the efficiency of utilisation.

The PUE varied substantially among the slower-growing species, with *R. carphoides* and *R. fulvum* had amongst the lowest PUE of all species, whilst *R. auriculatum* and *R. erianthum* had amongst the highest critical internal P concentration and PPE of all species. Hence, it was not clear whether slow-growing species could be generally categorised as less efficient in their utilisation of shoot P for growth. In contrast, all of the fast-growing species (*B. hordeaceus*, *L. perenne*, *R. duttonianum* and *R. richardsonii*) had similarly high PUE, regardless of which measure of PUE was used. However, there were key differences in shoot morphology traits between the *Rytidosperma* species and *B. hordeaceus* and *L. perenne*. In particular, the *Rytidosperma* species had a smaller leaf area, lower SLA, higher YEB P\textsubscript{area} and higher leaf dry matter content (LDMC) than *B. hordeaceus* and *L. perenne*. These shoot morphology traits are typically associated with slow-growing species and nutrient conservation (Lambers and Poorter 1992). It was hypothesised that the *Rytidosperma* species may have a better ability to utilise available leaf area for growth through a greater photosynthetic capacity (Evans 1998). Further studies should measure the rate of photosynthesis of the high-yielding *Rytidosperma* species.
Root morphological traits

Given the differences in shoot morphological traits amongst the fast-growing grasses, root morphological traits were also examined (Chapter 4). It has been hypothesised that fast-growing species have a large root system, that is, a large root mass, long root length and large root hair cylinder volume (RHCV) (Brown et al. 2013; Robinson and Van Vuuren 1998; Wissuwa et al. 2005). It has also been suggested that fast-growing species that have evolved in nutrient rich soils have more root morphological plasticity than slow-growing species that evolved in nutrient poor soils (Chapin 1980; Christie and Moorby 1975; Grime 1979; White 1972). However, others have questioned the broad applicability of this hypothesis (Hodge et al. 2009; Robinson and Van Vuuren 1998). It was found that there were significant positive correlations between P uptake (measured as whole plant P content) and root mass, length and RHCV at all levels of P supply. The exception was RHCV at no applied P, as B. hordeaceus was an outlier with less P uptake than predicted given its RHCV. Plants that had a larger root system took up more P and produced more mass. However, there was no single root morphological trait that contributed to a larger root system for all species. For example, L. perenne and R. richardsonii had the longest root length at no applied P and both species had a larger specific root length (SRL) at no applied P relative to 42 mg applied P per pot. For L. perenne, the increase in SRL was related to a decrease in root tissue density whereas in R. richardsonii the proportion of fine roots was increased. The low P uptake relative to RHCV of B. hordeaceus at no applied P was associated with having long root hairs on a very short total root length. The RHCV was therefore very large but ineffective due to poor soil exploration. Hence, root morphological traits should not be considered in isolation of the size of the root system. The small shoot yield of B. hordeaceus at low P supply relative to high P supply (Chapter 2) was related to the lack of balance in resource allocation between roots and shoots. Bromus hordeaceus had the smallest radicle fraction (0.11 mg mg\(^{-1}\)) at the seedling stage. However, at low P supply B. hordeaceus had amongst the highest root mass fraction (RMF) (0.42 g g\(^{-1}\)), but amongst the smallest root mass and root length. Plants must balance resource allocation between roots and shoots to ensure adequate exploration of the soil for P uptake without compromising shoot photosynthetic potential.

The root morphological traits were examined in plants grown in pots in a glasshouse after 47 days. This was considered an adequate period of time to examine root
morphological adaptations to applied P. In Chapter 2, Fig. 1 indicated that for all species most growth occurred between 26 and 47 days, after full canopy closure. It was concluded that the growth responses observed at 47 days reflected each species’ growth response to applied P. The significant differences in most root morphological traits between high and low P supply indicated this time period was adequate for each species to adapt their root morphology traits to the low P soil conditions. It was determined that the experiment would not continue beyond 47 days due to the risk of the species becoming root bound and the observed root morphological traits not reflecting P treatments (Poorter et al. 2012). Whilst it is likely that the results from the glasshouse study reflect species’ growth in the field, if environmental components (e.g. soil moisture content) which affect P availability in the field are accounted for (Hammond et al. 2009), this should be verified for the *Rytidosperma* species. Ideally, this would be tested directly in the field and at several points of time. However, it is more difficult to collect all roots belonging to the target species whilst excluding other non-target species, and hence accurately measure root morphology traits in the field.

Of the fast-growing *Rytidosperma* species, both *R. duttonianum* and *R. richardsonii* had similar root morphological traits, despite different critical external P requirements (Chapter 2). It was hypothesised that the differing critical external P requirements may be due to differences in root exudates or more precise foraging for P by roots in the topsoil where the P was applied. Hence, the root exudates, especially organic anions, were examined in Chapter 5 to determine if they might play a role in P-acquisition and determination of the critical external P requirement of these species. In particular, two of the fast-growing *Rytidosperma* species, *R. duttonianum* and *R. richardsonii* had different critical external P requirements (Chapter 2), similar shoot P-utilisation efficiencies (Chapter 3) and a similarly large RHCV at all levels of P supply (Chapter 4). *Rytidosperma richardsonii* is reputed to exude citrate at a similar rate to *Lupinus albus* (Barrett and Gifford 1999; Ryan et al. 2001). It was found that both *Rytidosperma* species had a similar rate of root carbon exudates at high and low P supply, but had a greater rate of organic anion exudates at low P supply than at high P supply. A greater rate of organic anion exudates at low P supply than at high P supply was also detected for *R. carphoides*, a slow-growing species with a high critical external P requirement; but not for *R. erianthum*, a slow-growing species with a low critical external P requirement. *Rytidosperma erianthum* had similar rates of organic anion exudates at low and high P supply. Further, citrate and malate were detected in the
exudates of *R. duttonianum*, *R. carphoides* and *R. erianthum* but not in those of *R. richardsonii*. It was concluded that low-P stress may be a stimulant for organic anion exudates in some *Rytidosperma* species, but not in others. However, the role of organic anions in determining the critical external P requirement in *Rytidosperma* species remains uncertain and further investigation is warranted. The large release of root carbon exudates relative to organic anion exudates observed in *B. hordeaceus* was also interesting. Further studies are required to determine whether this was due to *B. hordeaceus* interacting with and hence modifying the soil rhizosphere through carbohydrates and/or amino acids.

Phosphorus-use and -acquisition efficiency

This thesis reported many examples of the utilisation and uptake of P by *Rytidosperma* species. The aim of these studies was to understand whether any *Rytidosperma* species have traits that make them particularly desirable for use in low-P agricultural systems. Each measure of P-acquisition and -utilisation efficiency has benefits and shortcomings. No one measure of PUE adequately describes all aspects of P efficiency, because the interaction of plants with their environment is complex and multi-faceted. Hence, it is often useful to examine more than one measure of PUE.

The critical external P requirement is the amount of P required to achieve near maximum (here defined as 90% of maximum) shoot growth. Fast-growing species, which require less P to achieve near maximum growth are more desirable than those that have higher external P requirements. Agronomic P efficiency was measured in two ways: Agronomic Efficiency a (AEa) is the maximum shoot yield (i.e. asymptote) minus minimum shoot yield (i.e. the intercept of the Mitscherlich curve) divided by the critical external P requirement of the species with the largest requirement. This measure is relevant to pastures in which the amount of fertiliser applied is determined by the species with the highest P requirement. An alternative measure that is of physiological interest is Agronomic Efficiency b (AEb). This is the difference in shoot yields at high and low P supply divided by the critical external P requirement of the specific species being assessed. This species-specific measure quantifies the efficiency in which the species is using its recommended rate of P fertiliser for herbage production. For both measures of agronomic efficiency (AE), species with the higher AE indicate a greater ability to convert P fertiliser to shoot mass. Both the critical external P requirement and
AE should be interpreted simultaneously. For example, *B. hordeaceus* has amongst the highest AEa and AEb. However, it also had amongst the highest critical external P requirement and lowest shoot yield at high P. Hence, *B. hordeaceus* was able to efficiently convert P fertiliser to shoot mass, but required one of the largest amounts of P fertiliser. Another example of the caution required when interpreting these measures is illustrated by *R. erianthum*. This species had the lowest critical external P requirement and also had amongst the highest AEb, indicating it was able to efficiently produce shoot mass per unit P fertilisation with a low fertilisation requirement. However, *R. erianthum* also had amongst the lowest shoot yields at high and low P supply and had amongst the lowest AEa. Hence, this species would not be considered desirable in a pasture, due to its poor shoot production and high likelihood of being outcompeted by species with a higher AEa if a pasture was fertilised at the rate required by a species such as *B. hordeaceus*, which has a higher critical external P requirement.

Several measures of P-utilisation efficiency were examined in Chapter 3. Critical internal P requirement is the shoot P concentration at 90% of near maximum growth; physiological P efficiency (PPE) is the amount of shoot mass produced per unit shoot P; shoot dry mass per unit P when measured at a common shoot P content (PUEc) or at a common relative stress level (PUEs). Species that are best able to utilise available shoot P for maximum shoot yield (i.e. low critical internal P, high PPE, high PUEc and high PUEs) are desirable. Some measures such as PPE are too coarse to adequately determine P-use efficiency of several species. As PPE measures the average P-use efficiency between high and low P, species are compared at different levels of P stress, due to their differing critical external P requirements. As a result, species such as *R. erianthum* may appear to have a high PPE, despite their low yields and high shoot P concentrations. Rose et al. (2015) argued that species should be compared at a similar shoot P content as species with a greater ability to forage for and take up P will also grow relatively quickly in low-P soils. Hence, P-utilisation measures are not fully de-coupled from P uptake. This thesis demonstrated that a comparison of species at a similar shoot P content also results in species being compared at different levels of P stress. Whilst there was a positive relationship between PUEc and maximum shoot yield, there was no such relationship between PUEs and maximum shoot yield. However, PUEc may not be completely independent of potential growth rate, as it was estimated using maximum shoot yield. Both measures should be compared with other measures of plant growth
such as leaf appearance and/or expansion rate to better determine which measures of P-utilisation efficiency should be used to compare these species.

Perhaps the most meaningful way of comparing all measures of P-acquisition and -utilisation efficiency is to do so amongst species of similar shoot yield. Whilst this could still result in species of different critical external P requirements (and hence P stress) being compared, it would ensure that the need for pasture systems to maintain or increase yields are not compromised by improvements in P-acquisition or -utilisation efficiency. In this regard, comparisons were made between *R. duttoniaum* and *R. richardsonii*, the species with the highest shoot yields at high and low P supply. These species had similar abilities to convert P fertiliser to shoot mass (AE) and similarly high utilisation efficiency of P, regardless of which measure was examined. However, *R. richardsonii* had a lower critical external P requirement and, when used in a pasture, would require less P fertilisation to reach near maximum yield.

**Rytidosperma richardsonii cv. Taranna**

*Rytidosperma richardsonii* cv. Taranna was included as it is one of the few commercially-available cultivars. This cultivar was selected from a single plant of a naturally occurring population from the New England Tablelands of New South Wales (Lodge 1993). It was selected due to its high seed yield, an important trait for the domestication of plants, although it was also one of the more productive *R. richardsonii* accessions (Lodge and Schipp 1993). This thesis singled out *R. richardsonii* on multiple occasions as one of the more notable *Rytidosperma* species. *Rytidosperma richardsonii* was one of the fastest-growing *Rytidosperma* species at both high and low P supply (Chapter 2). Relative to other fast-growing *Rytidosperma* species such as *R. duttonianum*, *R. richardsonii* had a low critical external P requirement and high agronomic P-use efficiency (Chapter 2). It was also shown that *R. richardsonii* had similarly high yield at low P supply and P utilisation efficiency as *L. perenne*, a fast-growing introduced pasture species (Chapters 2, 3). However, unlike *L. perenne*, *R. richardsonii* had a greater ability to accumulate shoot mass per unit leaf area which suggests a greater photosynthetic capacity or lower respiration cost. Further, relative to *L. perenne* and *R. duttonianum*, *R. richardsonii* had a lower root phosphorus fraction at low P supply. The greater proportion of total internal P in the shoots, where it potentially may be used for photosynthesis, did not come at the expense of root growth
or P-uptake (Chapter 4). This cultivar could therefore play a role in improving the farm-gate P-balance efficiency of pasture systems without compromising productivity (Simpson et al. 2014).

**Future research directions**

This thesis has identified opportunities to further our understanding of the role of *Rytidosperma* species in P-efficient, high-production pastures. Chapter 2 highlighted the diverse growth responses amongst *Rytidosperma* species to applied-P and the importance of understanding which species are present within a pasture. However, unless easy-to-use toolkits are developed to assist farmers to readily identify *Rytidosperma* species within their pasture, challenges to understanding how the pasture will respond to P fertilisers will remain. Ideally, such tool-kits will incorporate recent improvements in genetics-based identification, as well as the ecological and morphological data which are currently the main tool for identifying *Rytidosperma* species. Further studies are also required to determine the long-term persistence of the fast-growing *Rytidosperma* species, *R. duttonianum*, *R. racemosum* and *R. richardsonii*, in the field under a range of grazing densities and P supply. Given that none of the *Rytidosperma* species examined in this thesis suffered from P-toxicity, the role of grazing in the decrease in the occurrence and mass produced by *Rytidosperma* species observed by Dorrough et al. (2011) and Garden et al. (2003), should be examined.

Differences in shoot morphology traits between the fast-growing *Rytidosperma* species, and *B. hordeaceus* and *L. perenne*, were identified in Chapter 3. It was hypothesised that the fast-growing *Rytidosperma* species may have a greater photosynthetic capacity which enabled them to accumulate similar shoot mass as *B. hordeaceus* and *L. perenne*, despite having a lower SLA. Future studies should examine the photosynthetic potential of these fast-growing *Rytidosperma* species and also look at whether this is a trait found in other *Rytidosperma* species. Another area for potential future studies is to determine whether self-shading within the micro-sward played a role in the lack of correlation between SLA and growth rates of these grasses. Shipley (2006) hypothesised that plants have a large amount of plasticity between SLA and net assimilation rate under different quantum radiation conditions to enable them to maintain their relative growth rates. Hence, future studies should examine differences in SLA and net assimilation rate
between plants grown as swards and those grown as individual plants in which self-shading is minimised.

Future studies should also examine the influence the leaf morphological traits that are associated with slow-growing species, but observed in the fast-growing *Rytidosperma* species, namely small leaf area, lower SLA, higher YEB $P_{area}$ and higher LDMC, have on the long-term persistence and abundance in grazed grasslands. The leaf morphological traits observed in the fast-growing *Rytidosperma* species are associated with lower P turnover rates in the shoots and longer leaf lifespan (Aerts and Chapin 2000). These traits may not be desirable under grazing pressure as fast-growing species which rapidly take up and replace nutrients that are lost through grazing are more resilient than slow-growing species which conserve nutrients. However, in a long-term ecological observation and modelling study, Laliberté et al. (2012) found that species with a lower SLA and higher LDMC were more abundant in grasslands subjected to both high and low P supply and lax- and heavy-grazing pressures. They concluded this was related to these species having longer leaf lifespan and hence reduced nutrient turnover. Specific studies are needed to determine the impact of shoot P turnover, grazing and leaf lifespan on the persistence and abundance of fast-growing *Rytidosperma* species under long term grazing pressure. Ideally, these long-term field studies would also examine the impact of other edaphic factors such as pH and soil moisture content. Garden et al. (2003) conducted some field studies examining these factors, however, further long-term studies are required to fully understand how *Rytidosperma* species respond to multiple environmental stresses in Australian grasslands.

Root morphology traits examined in Chapter 4 did not explain differences in the critical external P requirements amongst *Rytidosperma* species identified in Chapter 1. In particular, of the fast-growing *Rytidosperma* species, *R. duttonianum* consistently had a higher critical external P requirement than *R. richardsonii*. However, both species had a similar total soil exploration as measured by the RHCV and mycorrhizal colonisation. Further, in Chapter 2 it was demonstrated that the critical external P requirement was not related to any measure of internal P utilisation efficiency. It was hypothesised that differences in critical external P requirement may be related to either root exudates, especially organic anions, or more precise foraging in the topsoil where most P is located. A clear role for organic anions was not resolved. Citrate and malate were
detected in the root exudates of *R. duttonianum* and *R. carphoides*, both of which had high critical external P requirements (Chapter 5), but were not detected in the root exudates of *R. richardsonii* despite previous reports of this species exuding citrate at similar rates as *L. albus* (Barrett and Gifford 1999). Hence, further studies should be conducted to clarify whether organic anion exudates play a role in P uptake and the critical external P requirements of *Rytidosperma* species. The role of precise foraging by roots in the topsoil (i.e. the ability of the species to exploit nutrient rich soil patches) should also be examined in *R. richardsonii* and *R. duttonianum*. In particular, future studies should examine differences in the root mass, root length and RHCV of these species in the topsoil relative to that at greater depth. Identification of root physiological and morphological traits which correlate with decreased critical external P requirements may contribute to our understanding of how to reduce P requirements of pasture systems without compromising productivity.


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Appendix

As outlined in Chapter 4, a correction factor (CF) was used to correct for the dry mass of roots that were stored in ethanol. *Bromus hordeaceus*, *Lolium perenne*, *Rytidosperma carphoides* and *R. richardsonii* were grown with nil, 10.5 and 42 mg P per pot. All plants were otherwise grown under the same conditions as those presented in Chapter 4. Plants were harvesting by separating the roots from the shoots at the crown. The intact root core was divided into quarters and gently washed to remove soil. Two quarters were used to determine the dry mass no ethanol storage (DM) to fresh mass no ethanol storage ratio (FM), and the fresh mass before ethanol storage (FMa) to dry mass after ethanol storage (DMa). This data formed the basis of the CF, where:

\[
CF = ([FMa / DMa] \times [DM/ FM])
\]

The fresh and dry mass components and CF calculations for each species and P-treatment are presented in Table 1

It was found that a single CF was suitable for all species and P-treatments.
Table 1 Fresh mass before ethanol storage (FMa), dry mass after ethanol storage (DMa), dry mass no ethanol storage (DM) and fresh mass no ethanol storage (FM) for four species at three levels of applied phosphorus. These ratios were used to calculate the correction factor (CF) to account for the storage of roots in ethanol in Chapter 4. A single CF was used based on the mean of all species and P levels. Values are means with standard error presented in parentheses.

<table>
<thead>
<tr>
<th>Species</th>
<th>P level (mg P pot⁻¹)</th>
<th>FMa (mg)</th>
<th>DMa (mg)</th>
<th>FMa / DMa (mg mg⁻¹)</th>
<th>DM (mg)</th>
<th>FM (mg)</th>
<th>DM / FM (mg mg⁻¹)</th>
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1.33