Cluster-root Formation and Functioning as Dependent on Phosphorus Supply, Plant Phosphorus Status and Relative Growth Rate in a Range of *Lupinus* Species

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Plant Biology
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The University of Western Australia

2014
SUMMARY

Some *Lupinus* species produce cluster roots in response to low plant phosphorus (P) status; these specialised root structures, together with their functioning, are considered a desirable trait as we face the era when rock P reserves for fertilisers are being depleted. Much research has been conducted on the mechanism of cluster-root functioning in both *Lupinus*, as crop plants, and some Australian/Chilean Proteaceae species. However, the cause of variation in cluster-root formation among cluster-root forming *Lupinus* species is unknown. This PhD research project investigated whether the variation in biomass allocation to cluster roots is an environmentally or genetically regulated process among *Lupinus* species. I aimed to study the relationship between cluster-root formation, plant P status, medium P concentration and plant relative growth rate. I determined if cluster-root-forming *Lupinus* species with a similar shoot P status and relative growth rate had similar cluster-root investment (Chapter 2). Furthermore, I investigated if cluster-root-bearing *Lupinus* species grown with a similar P availability but with a higher relative growth rate, as dependent on light availability, showed a greater investment in cluster roots (Chapter 3). Finally, whether cluster-root formation and carboxylate release from cluster roots is increased at higher internal sugar and/or auxin concentration, irrespective of P supply, was investigated in *L. albus*; the alternative that carboxylate exudation depends on a local low-P status of the roots was also explored (Chapter 4).

Phosphorus treatments caused major effects on cluster-root allocation (Chapter 2), with a significant but incomplete suppression in *L. albus* and *L. pilosus* when P supply exceeded 15 mg P kg$^{-1}$ sand. Complete suppression was found in *L. atlanticus* at the highest P supply; this species never invested more than 20% of its root weight in cluster roots. For *L. pilosus* and *L. atlanticus*, cluster-root formation was decreased at high internal P concentration, irrespective of RGR. For *L. albus*, there was a trend in the same direction, but this was not significant. Multiple regression analysis showed that cluster-root formation in all three *Lupinus* species was suppressed at high leaf P concentration, irrespective of RGR. Variation in cluster-root formation among the three species cannot be explained by species-specific variation in RGR or leaf P concentration.
Cluster-root-forming *Lupinus* species grown at a similar P availability in the root environment, but with a higher growth rate, as dependent on day-length, showed a greater investment in cluster roots (Chapter 3). Multiple regression analysis showed that cluster-root formation was strongly correlated with plant P status, and not with plant RGR for both *L. albus* and *L. pilosus*. For *L. atlanticus*, there was a trend in the same direction. I conclude that plants with a high P status invariably down-regulate cluster-root formation, irrespective of growth rate. However, variation in cluster-root formation among these *Lupinus* species, again, cannot be explained by species-specific variation in RGR or in leaf P concentration.

When *L. albus* was grown in split-root pots (Chapter 4), cluster-root investment decreased following removal of the shoot apex, and thus the source of shoot auxin production. Removal of the shoot apex resulted in a doubling in leaf sucrose concentration, but no increase in root sugar concentration was found and hence no sugar-induced cluster-root formation was to be expected. Exogenous supply of a synthetic auxin, 1-naphthalene acetic acid (NAA), to the roots led to more cluster-root production for both root halves; however, the functioning of cluster roots, *i.e.* carboxylate release, was only enhanced in the –P root halves. It is concluded that cluster-root formation in P-deprived *L. albus* is controlled by a systemic factor. However, the functioning of cluster roots, *i.e.* carboxylate release, depends on a local low-P status of the cluster roots when auxin is supplied.

In conclusion, in the three *Lupinus* species, cluster-root production and functioning was reduced at higher leaf P concentrations, irrespective of relative growth rate and leaf sugar concentrations. In addition, cluster-root formation in the investigated *L. albus* plants was predominantly regulated by an auxin signal, with no evidence for sugar signalling, because sugar concentrations in the roots were unaffected by the removal of the shoot apex. Carboxylate release was not affected by auxin, but dependent on a local low-P signal. Future studies on variation in cluster-root formation and its functioning among cluster-root bearing *Lupinus* species at both physiological and transcriptional levels are necessary to explain variations among *Lupinus* species.
STATEMENT OF AUTHORSHIP

This thesis is, in accordance with postgraduate and research scholarships regulations 33(1) of the University of Western Australia (UWA), presented as a series of scientific papers that resulted from the study.

I declare that the research presented in this thesis is an original contribution to the field of Plant Ecophysiology. The hypotheses and experiments presented and discussed in this thesis are my own work with feedback from my supervisors and co-authors.

People who have made important contributions to this research in addition to those acknowledged in chapters 2, 3 and 4 are:

- Hans Lambers and Stuart Pearse, who were the supervisors of my PhD study and who have guided me through the processes of forming hypotheses, designing experiments and writing up the manuscripts for submission.
- Erik Veneklaas provided invaluable input with my experimental design and the manuscript in Chapter 2.
- David Strack provided great help with my experiment in Chapter 3.
- Greg Cawthray provided technical support with HPLC analysis of carboxylates and sugar detection in Chapter 1 and 3.

This thesis has been completed during the course of enrolment in a PhD degree at UWA, and has not been submitted elsewhere for a degree or diploma at any other institution.

Xing Wang
July 2014
PUBLICATIONS ARISING FROM THIS THESIS

Primary authored manuscript: published


Primary authored manuscript: submitted


Primary authored abstracts published in refereed conference proceedings


Wang X, Pearse SJ, Lambers H. Cluster-root formation and carboxylate release vary among Lupinus species under different phosphorus supply. *In proceedings of:* The 3rd International Rhizosphere Conference on Root-Soil Interaction, RHIZOSHERE 3 2011, Perth, Western Australia. (Chapter 2)
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(Igor Florez-Sarasa, Hans Lambers, Xing Wang, Patrick Finnegan, Miquel Ribas-Carbo; Plant, Cell and Environment:37, 922-928, 2014; Co-authored paper)
ACKNOWLEDGEMENTS

I would like to acknowledge the University of Western Australia (UWA) and the China Scholarship Council (CSC) for UWA-China SIRF scholarships and by the School of Plant Biology (UWA) for giving me this opportunity and supporting me with my PhD study and research in Perth, Australia. Thanks also to many people who helped me during the application of my scholarships. Support for travel to the 4th International Symposium on Phosphorus Dynamics in the Soil-Plant Continuum (ISPSPC 2010) was provided by UWA Graduate Research Travel Award and a School of Plant Biology Travel Award.

My sincere and deepest gratitude goes to Hans Lambers, my dear coordinating and principal supervisor, for giving me this great opportunity to study and work in his lab. Hans has been a fantastic and very accessible supervisor, great mentor, friend and role model. Hans has shown kindness, patience, forgiveness and goodness and has had a positive influence in both my study and my life. Hans not only encouraged and supported me in all aspects of the project, but also guided me to better planning and ways to stay on top of what needed to be done to ensure an efficient and productive research life. With his positive attitude, worthy character and as a respected scientist with high reputation, Hans sets a great example for me and many who know him. Completing a PhD is not only about the project; it is about knowing oneself better and providing experience in effective and successful communication in words and actions. I met and interacted with many people along my PhD journey, and my skills in presenting high-quality papers and oral and poster presentations improved considerably. I also gain much from other fellow researchers and scientists. I feel greatly indebted to for his very helpful guidance, great inspiration, invaluable support and unfailing encouragement in my study at UWA.

Thanks to my co-supervisor, Stuart (Stu) Pearse, who was also awesome in providing invaluable help with various aspects of the project and manuscript preparation. Stu was both approachable and skillful, and he taught me some invaluable hands-on lab and Excel skills, some tricks on figure graphing in PowerPoint and also in our manuscripts. Stu became my external supervisor from the beginning of my third year study; however, he always made himself available to discuss experimental design, problems, data issues and other matters. Thanks so much, Stu.

My thanks also go to Erik Veneklaas, who although not my PhD supervisor, provided invaluable input, critical insights into one of our manuscripts and helped with some aspects of the project, especially when I desperately needed help when neither Hans nor Stu was around at the university; Erik’s door was always open, and he always kindly and patiently provided invaluable advice on how to overcome difficulties in my research. Thanks a lot, Erik.
Many thanks to Barbara (Barb) Jamieson, Pandy du Preez, Natalie Jagals and Alan Luks at the School’s General Office. You all made me feel the warmth of the School family. I truly appreciated Barb’s and others’ caring, invaluable friendship and support, which has gladdened my heart no end.

Thanks to Megan Ryan and Tim Colmer, also Barb, for being so kind and patiently going through my paper work. Many thanks go to Joanne (Jo) Edmondston, from the UWA Graduate Research School (GRS), who has been my mentor beyond the supervisory team, and also my friend; Jo’s valued help with my study and also English of earlier versions of parts of Chapter 2 and Chapter 3 is greatly appreciated. Thanks also to Sato Juniper and other staff from GRS. Sian Boyle for her valued help and great encouragement through the final nine months of my study. Thanks also go to my M.Sc. supervisor Xinlian Tang and others in the Guangxi University, Runmin Ma and Jiangang Kang from the Education Office, Embassy of P. R. China on behalf of the CSC for their kind help and consideration.

I am really grateful to the various people who provided great help for this PhD project: Bevan Buirchell, Richard Snowball and Jon Clements for kindly providing the *Lupinus* seeds; Ray Scott at the former UWA Combined Workshop with experimental equipment; Rob Creasy, Bill Piasini, Daniel Prestage and the team at the School’s Plant-Growth Facilities for their great help with my glasshouse experiments; Susan Barker for providing cardboard boxes, Hai Ngo for providing glassware, Greg Cawthray for help with the freeze dryer, HPLC and also invaluable tips on root-exudate collection and sugar analyses; Craig Atkins for his generosity in showing us how to collect phloem sap from lupin plants; Padmaja Ramankutty, Laura Firth, Guijun Yan, Pieter Poot, François Teste and Allah Ditta for my statistical analyses; Jeremy Foster, Brad Muir, Anna O’Connell and Pauline Yeung for their time and kind help with purchasing and delivery of chemicals; the FNAS and FoS IT Team for the prompt response and help with my computer; the UWA Security Team for kindly giving me a few lifts home when I stayed a bit late at the university. Of course, a big thanks to David Lindsay and Hans, again, for the inspiration through their great books and workshops on Scientific Writing.

My university mates: Jessie Moniodis, Sandra Kerbler, Fazilah Abd Manan, Hazel Gaze, Melinda Trudgen, Yupin Li, Jing Zhang, Yan Liu and Marina Borges Osorio for assisting with the harvests; Mabel Fabiola Delgado Torres and Hiroaki Matsuoka for helping with experiments, to Evonne Walker for helping with plant P analyses in my first experiment; David Strack for invaluable help with my experiments for Chapter 3 and Patrick Hayes for sugar analyses. Thanks to Sheng Chen, Xuanli Ma, Mingpei You, Junmei Wang and Marion Cambridge for their kindness and encouragement; Michael Shane, Chris Jones and Matthew Nelson, and also
many others for friendly chats whenever at the lab or in the Tea Room. Diane Tan, Jessie
Moniodis, Annaliese Mason, Chai Tsun-Thai, Fazilah Abd Manan, Yingjun Chi, for the fun
times, especially at Rottnest; Arbarb Ahmad, Christine Allan, Sonja Jakob, Lalith Suriyagoda,
Honghua He, Kenny Png, Renu Saradadevi, Chandima Ranawana, Hazel Gaza, Bahram
Mirfakhraei, Karlia Meitha, Cahya Prihatna, Hieu Sy Tran and Nathan Craig and, of course, all
people from the School of Plant Biology; Paul Damon, Hossein Khabaz-Saberi, Khalil Kariman,
Basu Del Regmi, Martha Orozco Aceves, Bede Mickan, Leila Heidarvandat and many others in
Soil Science in the School of Earth and Environment.

My church friends: my auntie, Caixia Sophie Li and her family for the love and great help,
Yan Li, Margaret Reid for being my wonderful English teacher and encourager - a BIG thanks
Margaret, again, for your unfailing support and invaluable help with my English, Ian Reid,
Allan Smith, Margaret Donovan, Ilona and Hubert Day, Win and Bob Robert, Lihyu and John
Kuo, Blandine and Jeff Lam, Margaret and Ian Robison, the Pallathils family, Noeline and John
Bloomfield, Sharon and Chris Sorensen, the Francis family and many others at the Nedlands
Uniting Church; and also Mei Li and her family, Zhongwei Chen, Wan Chen and Hengbao
Zhang. My gratitude also goes to my neighbours Jen Zhao and her family, my flat mates
Dandan Wu, Xinxin Geng, Xixi Li and later Wenjing Bai; and other neighbours in the block.
Special thanks to my best friends Margaret Reid, Allan Smith, Diane Tan, Jessie Moniodis and
Yupin Li for your thoughtfulness, accompany and great friendship.

I am richly blessed by my beloved parents Lixia Li and Qingli Wang, who always
supported me and provided a good education, my dear brother Yuan Wang, and also my
grandparents for their unconditional love and support, understanding and sacrifices throughout
my life, especially during my PhD study! My sincere apologies for the fact that I haven’t
visited you in the past three years.

I believe that all the nice people who I have met have made my life more beautiful. I will
keep on staying positive, no matter what happens. Many thanks again to all my family,
relatives and friends for your support and encouragement.
CHAPTER 1

Literature Review and Thesis Outline
1.1 Literature Review

1.1.1 The importance of phosphorus (P) for plants and agricultural systems and the need for improving P-acquisition efficiency

Phosphorus (P) is an essential macronutrient required for plant growth (Holford, 1997, Lambers et al., 2008a, White and Brown, 2010). It is a component of nucleic acids, phospholipids and many intermediates in carbon metabolism. Phosphorus not only acts as a structural element, but is also involved in energy metabolism in plants, and hence plays a key role in plant production and quality (Theodorou and Plaxton, 1993, Lambers et al., 2013).

Phosphorus is also crucial to agricultural production; however, it is one of the most inaccessible macronutrients for plants to acquire, and the low-P availability has become a global problem (Raghothama, 1999, Marschner, 2012). Plants take up P from the rhizosphere solution as the most oxidised inorganic P (P$_i$) in the form of H$_2$PO$_4^-$ and HPO$_4^{2-}$ (Vance et al., 2003, Hammond and White, 2008). Generally, the P$_i$ concentration in the soil solution is at most 2-10 µM, even in fertilised soil (Schachtman et al., 1998), whereas the P$_i$ concentrations within the cytosol can be greater than 2 mM (Bieleski, 1973, Marschner, 2012). The P$_i$ concentration in the soil solution is $10^3$ times of that in the plant’s cytosol. Uptake of P$_i$ by plant roots takes place against a steep electrochemical potential gradient. Plants usually can only acquire P$_i$ in the soil solution at a distance of 1-4 mm from root surface, but P$_i$ is moved to the root surface mainly by diffusion. The diffusion rate is slow, even in moist soil, due to a low diffusion coefficient of P$_i$ (0.3-3.3 $\times$ $10^{-13}$ m$^2$ s$^{-1}$) in the soil (Clarkson, 1981, Schachtman et al., 1998). The diffusion rates of P$_i$ in the soil solution are largely affected by the length of the flow path and sorptivity of the
environment. The major control of the length of path of diffusion is the water content of the soil, with drier soils having greater tortuosity of path and slower diffusion (Lambers et al., 2008a); and the sorptivity of the environment is regulated by rhizosphere soil solution pH, clay mineralogy, i.e., most P is sorbed onto soil clay minerals (Coleman, 1944, Low and Black, 1950, Hemwall, 1957, Zhou and Li, 2001), and metal hydroxides, like Fe$^{2+}$ and Al$^{3+}$ hydroxides and oxides in acid soils (Sanchez and Uehara, 1980, Sanchez et al., 1997). Some minor control is the combination of soil temperature, sesquioxides, organic matter, and microbial activity (Brady and Weil, 1996, Lambers et al., 2013).

Most crop plants do not have ready access to P, because even when soils may contain substantial levels of P, this occurs in forms that are not readily available for most plants (Holford, 1997, Simpson et al., 2011). There are many reasons that account for the slow diffusion, low bioavailability and acquisition of P by plants. Also, organic P comprises up to 30-80% of the total soil P, with a dominant amount of monoesters (up to 90%) and less diesters, teichoic acids, and phosphonates (Newman and Tate, 1980, Hawkesd et al., 1984, Condron et al., 1990). In order to make P available to plants, inorganic P must be desorbed or solubilised, and organic P must be mineralised, from total soil P pools to release P (in the forms of H$_2$PO$_4^-$ and HPO$_4^{2-}$) into the soil solution. In many parts of the world, P is becoming a nutrient increasingly limiting food, feed and fibre production, due to its low bioavailability for plants.

Phosphorus availability is particularly problematic in many highly-weathered and volcanic soils from the humid tropics and subtropics, and also in the sandy soils from the semiarid tropics, where crop production is largely limited due to the dearth
of available P (Raghothama, 1999). Some issues associated with P bioavailability and its nature of lack of mobility show problems globally (Stutter et al., 2012). It is reported that approximately 30-40% of the world’s arable land has a low P bioavailability because of the strong reaction of P with soils (Runge-Metzger, 1995). In many acidic soils, especially weathered tropical and subtropical soils, plants demonstrate reduced rooting due to Al toxicity combined with limited P bioavailability where $P_i$ is bound to $Al^{3+}$, $Fe^{2+}$, or $Mn^{2+}$ hydroxides and oxides (Sanchez et al., 1997, Li et al., 2009). Under drought stress, P application significantly alleviated the negative effects of drought of moth bean (*Vigna aconitifolia* Jacq. Marechal) on plant water potential, relative water content, net photosynthesis rate, chlorophyll concentration, soluble protein content and nitrate reductase activity, particularly in the late flowing genotype of CAZRI moth-1 and Jawala (Garg et al., 2004). Drought led to diminished plant uptake of P and K and to greater recalcitrance of these mineral nutrients in soil (Sardans and Peñuelas, 2007, Beebe et al., 2008). Micronutrient (Zn and Cu) deficiencies coupled with low-P stress in maize and cotton plants limit agricultural production (Bingham, 1963, Warnock, 1970, Murphy et al., 1981, Cakmak and Marschner, 1986).

Thus, P application is recognised as critically important for crop production (Holford, 1997, Lambers et al., 2006). World-wide, the demand for P for crop production continues to increase, whilst the non-renewable global P reserves mined for production of phosphate fertilisers are being depleted; this as a whole sets a daunting challenge to a growing population world (Cordell et al., 2009, Gilbert, 2009, Marschner, 2012, Fixen and Johnston, 2012, Scholz and Wellmer, 2013). Modern agricultural systems have intensified greatly over the past few decades, and in recent years a concomitant increase in losses of N and P from farming land have an
increasingly detrimental effect on water quality, food security, agricultural sustainability and the environment (Hart et al., 2004). To sustain high crop yields and our world, it is crucial to improve the P-acquisition efficiency of crops in future farming systems.

1.1.2 Plant adaptations and acclimations to P limitation

1.1.2.1 Modification of root architecture

Plants have evolved various traits to acquire P in P-limiting environments, including modification of root architecture, *i.e.*, the spatial configuration of the whole root system, including its shape and structural development (Lynch, 1995, Lynch and Brown, 2001, Fitter et al., 2002, Postma and Lynch, 2012, Niu et al., 2013), increased biomass allocation to roots as a common short-term response to low-P conditions (Neumann and Martinoia, 2002), inhibited growth of primary roots (Williamson et al., 2001, Péret et al., 2011), increased root length and the growth of lateral roots to explore a larger soil volume (Gahoonia and Nielsen, 1996, Bates and Lynch, 2001, Lambers et al., 2008a), enhanced root-hair development and root elongation (Williamson et al., 2001, Li et al., 2007), and the formation of cluster roots to gain access to poorly-available P in the rhizosphere (Neumann and Martinoia, 2002, Lambers et al., 2006, Tomasi et al., 2009, Lambers et al., 2012).

1.1.2.2 Expression of high-affinity phosphate transporters under P deficiency

Inorganic phosphate uptake from soil and P$_i$ transport within the plant depends on P$_i$ transport proteins (P$_i$ transporters, PHT). These transporters, powered by ATP-dependent H$^+$ efflux, can actively take up P$_i$ from the rhizosphere against a steep electrochemical potential gradient. To date, there are four PHT families recognised in *Arabidopsis thaliana*, based on their location: PHT1 (in the plasma membrane),
PHT2 (in the plastid inner envelope), PHT3 (in the mitochondrial inner membrane) and PHT4 (mostly in the plastid envelope and one Golgi-localised transporter) (Rausch et al., 2004, Guo et al., 2008, Gaza, 2012). The nine-member PHT1 multigenic family in Arabidopsis consists of several genes encoding high-affinity P\textsubscript{i} transporters with $K_m$ values ranging from 2.5 to 12.3 $\mu$M (Dunlop et al., 1997, Nussaume et al., 2011). While all the nine members are involved in P\textsubscript{i}-starvation responses, tissue-specific variation in their expression occurs, with some in epidermal and root hair cells while others are in root stelar cells (Plaxton and Tran, 2011). In contrast, PHT2 (H\textsuperscript{+}/P\textsubscript{i} symporter) and PHT4 (Na\textsuperscript{+}/P\textsubscript{i} symporter) families are composed mainly of low-affinity transporters with a $K_m$ from 0.45 to 0.74 $\mu$M (Daram et al., 1999, Guo et al., 2008).

Many high- and low- affinity transporters exist; however, changes in their expression will not significantly impact P uptake of plants from soil, due to diffusion rates to the root surface limiting supply to a greater extent than kinetic properties of the transporters (Mudge et al., 2002). Hydroponic experiments in Arabidopsis thaliana under limiting P supply, demonstrated enhanced P uptake by increased expression of genes that encode specific P\textsubscript{i} transporter proteins, unlike what is expected for plants grown in soil (Dong et al., 1999). Internal P\textsubscript{i} concentration largely controls the transcriptional regulation of AtPHT1 gene expression (Hammond et al., 2003, Wu et al., 2003, Misson et al., 2005, Morcuende et al., 2007, Bustos et al., 2010, Thibaud et al., 2010). Sugars and cytokinins are also involved in the expression of some members of the AtPHT1 gene family (Franco-Zorrilla et al., 2005). In white lupin (L. albus), the high-affinity P\textsubscript{i} uptake gene (LaPT1) from cluster roots is highly expressed specifically under P deficiency (Liu et al., 2001), while another identified high-affinity LaPT2 gene is uniformly expressed at toxic
aluminium (Al) concentrations, as well as when nitrogen (N), manganese (Mn), iron (Fe) or P are in short supply (Liu et al., 2001).

Plants increased expression of phosphate transporters and thus enhanced accumulation of P in leaves, developed symptoms of P toxicity as the effect of poor down-regulation; these P-toxicity symptoms were dependent on transpiration rate (Delhaize and Randall, 1995). Ubiquitination of multiple high-affinity P$_i$ transporters with variable expression patterns reflects the importance of these P$_i$ transporters with specific individual functions (Wu et al., 2003). In barley (Hordeum vulgare L.), two P$_i$ transporters from the Pht1 family characterise a diverse function and kinetic properties among members, with HORvu;Pht1;1 functioning in P$_i$ uptake at the root surface, while HOPvu;Pht1;6 is involved in remobilising P$_i$ from leaves (Rae et al., 2003). The same group found over-expression of the gene encoding a P$_i$ transporter in transgenic barley plants does not account for enhanced P$_i$-uptake rates, but P$_i$-transporter activity may be influenced by post-transcriptional control of the transporter gene (Rae et al., 2004).

Certain members of a microRNA (miRNA) family act as an intermediate component of a signalling cascade in regulating expressions of P$_i$-starvation-induced genes (Sunkar and Zhu, 2004, Chiou, 2007). The up-regulation of miRNA399 leads to the down-regulation of its target gene, UBC24/PHO2, encoding ubiquitin-conjugating E2 enzymes as ubiquitination of P$_i$ transporters (Fujii et al., 2005, Aung et al., 2006, Bari et al., 2006, Chiou et al., 2006). MiR399 functions as a systemic signal to activate P$_i$ uptake and translocation through suppression of PHO2 by reciprocal grafting experiments (Lin et al., 2008, Pant et al., 2008). Under P-limited conditions, a group of miRNAs account for regulating the network of downstream
genes which control morphological and metabolic processes in P$_i$-deficient plants (Chiou et al., 2006, Branscheid et al., 2010, Zhu et al., 2010).

1.1.2.3 Arbuscular mycorrhizal associations

Symbiotic associations between roots of vascular plants and mycorrhizal fungi are common, and these mycorrhizas (mycorrhiza = fungus plus root) include arbuscular (AM), ectomycorrhizas (EM), ericoid, orchid and mycoheterotrophic mycorrhizas (Brundrett, 2002). The AM symbioses in natural habitat is found for the vast majority (about 70-80%) of higher plant species, with increased total surface area in acquiring P and other poorly mobile nutrients under nutrient limitation (Brundrett, 2002, Smith et al., 2011). Mycorrhizal hyphae improve P acquisition by plants, as they enable plants to acquire P from the soil solution, which is beyond the zone available to plant roots and root hairs (Bolan, 1991, Smith and Read, 2010). The AM associations are important for P acquisition in many plants that inhabit low-P environments, including exploring larger soil volume; moving P into mycorrhizal hyphae faster (Bolan, 1991, Koide, 2000, Smith et al., 2011).

Mycorrhizal symbioses are not inevitably of benefit, and mycorrhizal fungi can also have some potential parasitic effect and can be detrimental to their host plants (Johnson et al., 1997). Mycorrhizal infection is suppressed at high P application in onion (*Allium cepa* L.) (Son and Smith, 1988). At high P supply, host plant growth was suppressed by fungal colonisation, possibly due to the carbon-costly process (Peng et al., 1993, Siqueira and Saggin-Júnior, 2001).

Mycorrhizas possibly play a compensatory role in P uptake in root-haired plants, compared with hairless mutants only at low P levels (Schweiger et al., 1995,
Jakobsen et al., 2005). These results suggest that increased mycorrhizal activity in
the non-root-hair mutants may compensate for no hairs and reduced root surface and
also root-soil interface under P deficiency, and the relationship between plant, fungi
and root hairs needs further exploration (Brown et al., 2012, Brown et al., 2013).

In the last decade, the physiology and molecular mechanisms of $P_i$ transport in
the AM symbiosis has provided significantly increased understanding. The
symbiosis development happens with alterations in patterns of the expression of root
$P_i$ transporters and $P_i$ movement in the mycorrhizal root which includes $P_i$ uptake
through either the root epidermis or the symbiotic pathways. In most mycorrhizal
symbioses, for some, $P_i$ is transported via the fungus, but in some other cases, plants
appear to totally depend on $P_i$ transported by the association (Javot et al., 2007). An
AM symbiosis-related signal increases the expression of miRNA399 and decreases
the activity of $PHO2$ (Branscheid et al., 2010). Moreover, miRNA399, *per se*, is not
adequate to enhance mycorrhizal colonisation; however, in mycorrhizal roots,
increased miRNA399 expression is essential to maintain the low expression and
activity of $MtPho2$ which would otherwise increase according to symbiotic $P_i$ uptake
(Branscheid et al., 2010). Altered expression of some distinct groups of miRNA is
essential to $P_i$-starvation-induced responses and plant-microbe interaction namely,
AM symbiosis (Gu et al., 2010). A recent report shows that in soybean (*Glycine max*
L.) a high-affinity $P_i$ transporter, GmPT5, regulates $P_i$ movement from roots to
nodules, which is essential for maintenance of sufficient $P$ in nodules, and
subsequently controls soybean nodulation and growth (Qin et al., 2012).
1.1.2.4 Root hairs

Plants achieve effective absorbing root surface by abundance of root hairs. Root hairs differ in length from 0.2 to 2 mm, depending on plant species. The length of root hairs may increase from 0.1 to 0.8 mm under N or P deficiency (Bates and Lynch, 1996). Soil experiments in *Arabidopsis thaliana*, where only root hairs had access to penetrate $^{32}$P-labelled soil, provided evidence that root hairs can substantially satisfy the plant’s P > 60% demand from soil (Gahoonia and Nielsen, 1998).

Plants with increased length and density of root hairs sustain high yields at a low P supply, whereas in high-P conditions, plants may not develop many root hairs (Gahoonia and Nielsen, 1997, Bates and Lynch, 2001, Gahoonia and Nielsen, 2004). A study of ten temperate pasture species demonstrated root-morphology adjustments of a species maintain root length under low-P conditions, and in consequence, improve the potential for plant P uptake from P-limited soils; however, the intrinsic root morphological trait with extensive, fine roots in particular, as opposed to the competence to adjust their root morphology to P limitation, was most crucially determining of a low P input (Hill et al., 2006). Present knowledge shows that root morphology, root-hair development and rhizosheath formation on some perennial grass species is linked to acid-soil resistance (Haling et al., 2010). The presence of root hairs in barley sustains yield under P-deficiency conditions *per se* and also when combined with drought conditions (Brown et al., 2012). A recent conceptual model proposed that root-hair ideotypes combined with increased root hair length and longevity rather than increased density are efficient to take up P at low-P availability, and have potential to sustain future crop production (Brown et al., 2013).
1.1.2.5 Formation of non-mycorrhizal specialised root structures

Some plant species that occur in severely nutrient-impoverished environments, particularly in severely P-limiting conditions, have evolved non-mycorrhizal specialised roots involved in P acquisition: cluster roots or proteoid roots and dauciform roots (Lambers et al., 2006).

Cluster roots, originally known as proteoid roots, were described in the Proteaceae family by Purnell (1960). After the ground-breaking work on cluster roots in *Lupinus albus* conducted by Gardner and Parbery (1982), cluster-root formation and their function in some *Lupinus* species and also in most Proteaceae species has been well documented in the literature (Keerthisinghe et al., 1998, Watt and Evans, 1999b, Neumann and Martinoia, 2002, Vance et al., 2003, Shane and Lambers, 2005, Tomasi et al., 2009, Tang et al., 2013). In the rhizosphere of white lupin cluster roots, high levels of P solubilised from Al- and Fe-P compounds, and from organic compounds have been detected (Gardner et al., 1983a, Gerke et al., 1994, Kämh et al., 1999). Cluster-root formation is usually associated with the release of carboxylates, especially from the mature rootlets of these cluster-root-bearing species (Zhu et al., 2005). Following the release of carboxylates from cluster roots, there is also a large up-regulation of phosphate transporters (Liu et al., 2001, Lambers et al., 2006) and an exudative burst of acid phosphatase enzymes in the rhizosphere under P deficiency (Neumann et al., 1999, Gilbert et al., 1999, Wasaki et al., 2003, Wasaki et al., 2009, Tran et al., 2010). Potentially, the pattern of carboxylate release can be optimised, and this will be important for the success of future agriculture as we progress towards “peak phosphorus” (Lambers et al., 2012).
Phosphorus is abundant in the environment (ranked as the 11th most abundant element (Nussaume et al., 2011); however, P is neither readily accessible nor evenly distributed in most soils for plant uptake because of adsorption, precipitation, or conversion to organic forms (Holford, 1997, Richardson, 2001). A large fraction of P in soils is found in the organic form, with the major components of phytic acid - inositol hexaphosphate (Richardson et al., 1994). However, organic P can be an important source to P-deficient plants, especially those that form cluster roots.

Cluster roots also release phosphatases to liberate $P_i$ from soil organic P (Gilbert et al., 1999, Miller et al., 2001, Wasaki et al., 2003, Tran et al., 2010). Enhanced production of cluster roots with the release of phosphate allows plants to mobilise soil P from organic P (Turner et al., 2002, George et al., 2006). Organic P can also be sorbed onto soil particles, similar to $P_i$; hence, carboxylate release is as important for liberating organic P, especially when compared with direct mobilisation of $P_i$ from soils under P deficiency (Lambers et al., 2006, Giles et al., 2012). Plant roots may also release extracellular phytases hydrolysing inositol hexakisphosphate, which generally constitutes a large proportion of the total organic P fraction in soil (Turner et al., 2002).

The capacity of *Lupinus* species to access relatively unavailable P, largely credited to their release of carboxylates from cluster roots, cluster-like roots or non-cluster roots, is considered a desirable trait (Richardson et al., 2011). Young volcanic soils and P-limited Arenosols contain large P reserves, but have a low-P availability due to a low pH and large amounts of $Fe^{3+}$ and $Al^{3+}$ oxides which greatly sorb P (Borie and Rubio, 2003, Gweyi-Onyango et al., 2010). Therefore, *Lupinus* species are ideal crop candidates for liberating P in order to achieve a high yield on such
strongly P-sorbing volcanic soils, e.g., in Chile and West Africa (Baer et al., 2006, Gweyi-Onyango et al., 2010).

Lambers et al. (2012) demonstrated that cluster-rooted species grown on volcanic soils with much total P but low P availability can act as ecosystem engineers in providing P in leaf litter for neighbouring non-cluster-rooted plants. The traits of cluster-root-bearing species to access the relatively unavailable P for other plants is of ecological importance in young volcanic soils (Lambers et al., 2013). Southwestern Australian Proteaceae species, growing on the world’s most ancient and P-impoverished soils, develop foliar P toxicity symptoms when the soil P levels just above those in their natural habitat (Lambers et al., 2002, Shane et al., 2004b), *Hakea prostrata* showed a low capacity to down-regulate P uptake (Shane et al., 2004b). A South American Proteaceae species *Embothrium coccineum*, native on young, relatively P-rich volcanic soils in Chile and Argentina, regulates its P-uptake capacity, preventing P toxicity when grown at a range of P levels (Delgado et al., 2014).

In the short term, P recycling from dead organic matter is the main direct source of plant-available P to soils. Organically-bound P must be released by decomposition. Seasonal patterns implied P stored as organic matter and P and K in soil microbial tissue during winter and then P was significantly greatly released from the microbial biomass and also from the organic matter in summer (Perrott et al., 1990). In the rich organic matter soils, plants explore nutrient by a biological mineralization, by releasing P$_i$ from organic P forms through enzymatic catalysis external to the cell membrane (Aguiar et al., 2013).
Due to low leaf P resorption of *Embothrium coccineum* (Diehl et al., 2003, Lambers et al., 2012) and a short leaf lifespan, leaf litter contributes significant amounts of P to neighbouring plants (Lambers et al., 2012). Southern South American Proteaceae, which produce leaf litter rich in P, may act as ecosystem engineers to provide P for plants lacking specialised roots, to access sorbed P (Lambers et al., 2012). The abundance of labile components (watersoluble and NaHCO$_3$-extractable P) and high activities of microbial and plant phosphatases plays a pivotal role in accessing root and leaf litter during seasonal P recycling in both grassland and forest ecosystems (Polglase et al., 1992, Yanai, 1998, Ross et al., 1999, Chen et al., 2002, Chen et al., 2003, Chen et al., 2008).

Similar to cluster roots, dauciform roots are another specialised form of root structure, which is defined as carrot-shaped small structures on short laterals covered with many long root hairs (Davies et al., 1973, Lamont, 1974, Shane et al., 2005). Dauciform-root formation is inhibited under sufficient P supply, indicating it is an adaption to low-P conditions; dauciform roots are produced only in two tribes of the Cyperaceae (Shane et al., 2005).

Both cluster roots (Watt and Evans, 1999a, Neumann and Martinoia, 2002, Shane et al., 2004a) and dauciform roots (Shane et al., 2005, Playsted et al., 2006) release carboxylates in an exudative-burst. This enhances P acquisition when there is very little P in the soil solution (Lambers et al., 2008b).

### 1.1.2.6 Root exudates in the rhizosphere

Under P deficiency, plants release various exudates into the rhizosphere, including carboxylates, in particular citrate and malate (Jones, 1998, Ryan et al.,
2001); protons (Gardner et al., 1983b, Neumann and Römheld, 1999, Lamont, 2003); phosphatases (Duff et al., 1994, Li et al., 1997, Wasaki et al., 2003, Marschner, 2012); phenolic compounds (Juszczuk et al., 2004, Neumann and Römheld, 2007); mucilage (Nagaraja et al., 1970, Gaume et al., 2000, Grimal et al., 2001); water (Burgess et al., 2000), and allelochemicals (Bertin et al., 2003). These root exudates modify rhizosphere soil pH, increase the mobilisation of soil P, Fe, Zn, Cu and Mn, and, in particular, enhance the availability of soil P (Lambers et al., 2008a).

1.1.3 Formation and functioning of cluster roots and their regulating factors

The formation of cluster roots and exudation of carboxylates from roots of some species, including some *Lupinus* species, are adaptations to acquire sparingly soluble forms of P under conditions of P limitation. *L. albus* is presently the model species, and *Lupinus* species are the only known annual crop species that produce these structures. Cluster roots are also produced by most species of the Proteaceae family and by several other plant species, including some species belonging to Betulaceae, Casuarinaceae, Eleagnaceae, Fabaceae, Myricaceae and Restionaceae (Lamont, 1972, Louis et al., 1990, Dinkelaker et al., 1995).

The functions of cluster roots are: (1) modifying the surface area of roots to enhance uptake of nutrients; (2) releasing acid phosphatases (Gilbert et al., 1999, Miller et al., 2001, Wasaki et al., 2003, Tran et al., 2010) to hydrolyse organic P in soil; (3) exuding phenolics (Neumann et al., 2000), which may have antibiotic traits; (4) releasing large amount of carboxylates to mobilise P in soils (Dinkelaker et al., 1989, Keerthisinghe et al., 1998, Vance et al., 2003, Shane and Lambers, 2005, Lambers et al., 2012). The ability to form cluster roots allows plants to access P effectively from some of the world’s most P-impoverished soils, such as the soils of
south-western Australia and South Africa, and also in soils with high total P but where P availability is low, due to low soil pH such as the soils of Chile (Vance et al., 2003, Lambers et al., 2006, Lambers et al., 2012). Therefore, cluster-root formation is a desirable agronomic trait for crop production systems where P is a significant constraint.

It is well known that cluster-root formation is not only affected by the P-nutritional status but also by hormonal balances (such as auxins and cytokinins) (Gilbert et al., 2000, Neumann et al., 2000, Hocking and Jeffery, 2004, Chiou and Lin, 2011, George et al., 2011) and in some species by Fe nutrition (Dinkelaker et al., 1995, Arahou and Diem, 1997, Hagström et al., 2001). Plant P status is regarded as the most influential factor controlling cluster-root formation (Neumann and Martinoia, 2002, Lambers et al., 2006). Auxins play a role in cluster-root formation (Gilbert et al., 2000, Skene and James, 2000, Hocking and Jeffery, 2004), and hence there might be differences in the strength of this hormonal signal among Lupinus species. However, shoot-derived sugar signals (sucrose, glucose and fructose) also control plant P-starvation responses, including cluster-root formation (Liu et al., 2005, Müller et al., 2007, Zhou et al., 2008), and hence the strength of this signal might vary among lupin species. Possibly, strigolactones play a role as well in cluster-root formation, given their role in lateral root formation (Kapulnik et al., 2011) and response to P deficiency (Yoneyama et al., 2007a, Yoneyama et al., 2007b, López-Ráez et al., 2008, Kohlen et al., 2011). Several researchers have also reported that a family of microRNAs (miRNAs), including miR399, miR156, miR169, miR395 and miR398 acting as systemic signals are responsive to low-P supply, and these miRNAs coordinately regulate a downstream gene network controlling morphological and metabolic processes in P-deficient plants (Valdes-Lopez et al., 2008, Vance, 2010,
Zhu et al., 2010, Chiou and Lin, 2011). Moreover, nitric oxide (NO) plays a role in signalling P deficiency, inducing cluster-root formation in white lupin (Wang et al., 2010, Meng et al., 2012).

Recent advanced and updated genomic and genetic studies have revealed that plant accumulation of P in P-deficient environments involves a network of cross-talk between these signalling components mentioned so far; further exploration in next-generation sequencing in identifying specific genes regulating acclimation to P-stress is warranted (Vance, 2010). O’Rourke et al. (2013) presented the first L. albus gene index (LAGI 1.0) of P-deficient and -sufficient plants by a RNA-seq analysis (Illumina GA-IIx Platform), and identified 12 out of 2128 transcripts in total variably expressed responding to P-deficiency in Arabidopsis thaliana, potato (Solanum tuberosum), and white lupin. By using the RNA-seq data and also quantitative real-time polymerase chain reaction (RT-qPCR), Wang et al. (2014) introduced a model for the gene network regulating cluster-root initiation, maturation and function; a transcriptome sequencing analysis of different cluster-root developmental stages in L. albus confirmed many reports on physiological and metabolic changes linked to cluster-root function and also some on gene expression during cluster-root development. In mature cluster roots, citrate catabolism was down-regulated with citrate accumulation; the phenylpropanioid pathway was up-regulated following accumulation of phenolics; specific transcript expression of transporter genes ALMT and MATE associated with citrate and flavonoid exudation; transcripts expression linked to nucleotide degradation and APases with re-mobilization and hydrolysis of organic phosphate (Wang et al., 2014). Transcripts related to abscisic acid (ABA) and jasmonic acid were up-regulated in mature cluster roots, whereas auxin- and brassinosteroid-related genes and cytokinin receptors were largely expressed during
initiation of cluster roots (Wang et al., 2014). Ethylene plays a central role demonstrated through the hormone-related gene expression, which was associated with the up-regulation of the Fe-deficiency regulated network that regulates ethylene-induced expressions of Fe-deficiency responses in other plant species (Wang et al., 2014).

1.1.4 Some *Lupinus* species as model crop

*Lupinus* (Fabaceae) species are an ancient annual or perennial crop, both in Mediterranean Basin and in South America (Gladstones, 1970). *Lupinus*’s morphology ranges from acaulescent or small prostrate herbs to small trees, woody shrubs which can reach 8 m high (Dunn, 1984, Turner, 1995). In this thesis, three cluster-root-bearing *Lupinus* species, *L. albus*, *L. pilosus* and *L. atlanticus* were investigated. These species are all Old World species (clade D and C) and are able to form true cluster roots, whereas some Old World species (clade B) and most New World *Lupinus* species (clade E) either do not form cluster roots or no information of cluster roots is available about this trait in the literature (Hocking and Jeffery, 2004, Lambers et al., 2013).

*Lupinus albus*, white lupin, soft-seeded, is well known as a model plant species in understanding the non-mycorrhizal phosphorus-acquisition strategy. This strategy is largely due to this lupin’s adaptive traits of classic cluster-root formation and carboxylate exudation (Liu et al., 2001, Neumann and Martinoia, 2002, Watt and Evans, 2003, Shen et al., 2003, Lambers et al., 2006, Tomasi et al., 2009, Weisskopf et al., 2009, Lambers et al., 2013). Recent work (O’Rourke et al., 2013) set a milestone for the first *L. albus* gene index (LAGI 1.0) based on next-generation sequencing and RNA-seq analysis. Also the investigation in molecular mechanisms
behind adaptive responses at the transcriptome level (Wang et al., 2014), allows the researchers to present a hypothetical model for the gene network regulating cluster-root development and function in *L. albus*. With these advances in our understanding of these genes as molecular determinants of nutritional acquisition, especially P uptake, we can be sure *Lupinus albus* and also some other cluster-root-forming *Lupinus* species are regarded as potential suitable crop plants while we move towards “peak phosphorus” (Lambers et al., 2013).

*Lupinus pilosus*, known as greater purple lupin, rough-seeded, is alkaline-tolerant and grows well in calcareous soils even when free CaCO$_3$ is greater than 50% (White, 1990, Tang et al., 1995a, Tang et al., 1995b, Brand et al., 2002). A hydroponic study showed that the adaptation to calcareous soils in alkaline-tolerant *L. pilosus* attributed to its enhanced proton release, cluster-root formation and citrate exudation; P deficiency-induced proton release may be linked to the reduced anion uptake, whereas high pH-induced proton release may be related to increased cation uptake (Wang et al., 2006).

*Lupinus atlanticus*, rough-seeded, is also alkaline-tolerant species. Based on the sensitivity to calcareous soils, *L. pilosus* and *L. atlanticus* are more tolerant than *L. albus* and *L. angustifolius* (Tang et al., 1993, Kerley et al., 2001).

Many nutrient-mobilising plant species are legumes; and many cluster-rooted or dauciform-rooted species are also efficient at nutrient mobilisation (Lambers et al., 2008b). There is a plasticity in the ability to produce these structures among different Old World *Lupinus* species (Hocking and Jeffery, 2004, Ligaba et al., 2004, Pearse et al., 2006, Pearse et al., 2009, Abdolzadeh et al., 2010), and also diversity in the
development and function of the cluster roots (Ligaba et al., 2004, Pearse et al., 2006, Wang et al., 2006). Lupin species differ in their sensitivity to P additions with respect to cluster-root percentage, with *L. atlanticus* and *L. pilosus* showing greatest sensitivity, compared with *L. albus* and *L. micranthus* (Pearse et al., 2006, Abdolzadeh et al., 2010). Lupin species also differ in the percentage of cluster roots under low-P conditions, with a large cluster-root investment in *L. albus* and *L. micranthus* and much less in *L. atlanticus* and *L. cosentinii* (Pearse et al., 2006, Abdolzadeh et al., 2010). In addition, a wide variation exists in the amounts of carboxylates released among legumes (Pearse et al., 2006).

The success of some legumes in habitats with poorly available P (Lambers et al., 2013) highlights that their nutrient-mobilisation mechanisms play important roles in crop quality and yield in a range of natural and semi-natural plant communities (Li et al., 2014). I suggest that future research on the variation in both plasticity and diversity of cluster-root formation and functioning among *Lupinus* species may provide opportunities to explore any implications of diversity of this trait, and also allow us to harness the efficiencies to achieve the goal of more P-efficient farming system and enhance agricultural sustainability.

1.1.5 Literature review - a conclusion

Physiological and molecular responses to P deficiency by plants, including cluster-root initiation and formation, have been the focus of many previous studies. Presently, it is known that differences exist in the regulation of cluster-root formation among species; however, the exact mechanism responsible for these differences is yet to be determined. Attention should be paid to these physiological processes for P-acquisition efficiency by plants in the development of P-acquisition efficient future
farming systems. Investigation of the physiological strategies of P-acquisition and utilisation efficient plants is essential in order to gain critical insights and develop new opportunities for improving P-recovery and reducing P demand in cropping systems.

1.2 Key Research Aims and Thesis Outline

This research project was broadly focused on providing an answer to whether the variation in biomass allocation to cluster roots is a species-specific process among *Lupinus* species. However, it is important to first distinguish if the processes of cluster-root formation is genotypically controlled, before we take a genetic approach.

![Diagram of P uptake rate and shoot P status](image)

Figure 1. Regulation of cluster-root formation/activity and interactions with plant phosphorus (P) uptake and P status. Cluster roots increase P uptake, which enhances P status (black arrows indicate positive effects), but this in turn has a negative effect on cluster root formation and exudation (grey arrow shows negative effect). Figure taken from Pearse *et al.* (2006).

A model introduced by Pearse *et al.* (2006) proposed that cluster-root formation in *Lupinus* species has a positive effect on P uptake, which enhances shoot P status,
but this, in turn, has a negative effect on cluster-root formation and root exudation (Fig. 1). Plant P status is largely balanced by P-uptake and plant growth rate. The capacity of a plant for P-uptake may be influenced by several genetic and environmental factors (Postma and Lynch, 2012, Brown et al., 2012). In addition, the relative growth rate (RGR) of a plant may be affected by many environmental and genetic factors. Faster growth rates are correlated with higher leaf P concentrations (Wissuwa et al., 2005, Pearse et al., 2006, Abdolzadeh et al., 2010); in cluster-forming species, high leaf P concentrations are closely correlated with low cluster-root formation. Hence, we expect cluster-root formation to be correlated with RGR or a combination of both RGR and plant P status. I, therefore, aimed to study the relationship between cluster-root formation, plant P status, and P concentration in the growth medium and plant relative growth rate.

The specific key objectives of this thesis were to:

- Investigate if different *Lupinus* species with similar shoot P status and relative growth rate had similar cluster-root investment (Chapter 2).

- Determine if *Lupinus* species grown with the same P availability but with a higher relative growth rate, as dependent on light availability, showed a greater investment in cluster roots (Chapter 3).

- Investigate if cluster-root formation in *Lupinus albus* was increased at greater internal sugar and/or auxin concentration, irrespective of P supply. However, it was hypothesised that carboxylate release depends on a local low-P status of the roots (Chapter 4).

This thesis comprises five chapters. Chapter 1 is a Literature Review and General Introduction. Chapters 2 to 4 contain the research outcomes to test the
hypotheses proposed above; these chapters have also been presented as manuscripts for publication in international journals. Chapter 2 has been published in *Annals of Botany*, and Chapter 3 has been submitted to *American Journal of Botany*, while Chapter 4 will be prepared for submission to a journal in the future. The thesis concludes with Chapter 5, a General Discussion and Conclusion.

**Literature Cited**


Branscheid A, Sieh D, Pant BD, May P, Devers EA, Elkrog A, Schauser L, Scheible WR, Krajinski F. 2010. Expression pattern suggests a role of miR399 in the regulation
of the cellular response to local Pi increase during arbuscular mycorrhizal symbiosis.

**Molecular Plant-Microbe Interactions**, **23**: 915-926.


**Coleman R. 1944.** Phosphorus fixation by the coarse and fine clay fractions of kaolinitic and monimorillonitic clays. **Soil Science**, **58**: 71-78.


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phosphatase under phosphorus deficiency in *Caustis blakei* (Cyperaceae). *New Phytologist*, **170**: 491-500.


CHAPTER 2

Cluster-root Formation and Carboxylate Release in Three *Lupinus* Species as Dependent on Phosphorus Supply, Internal Phosphorus Concentration and Relative Growth Rate
Cluster-root formation and carboxylate release in three *Lupinus* species as dependent on phosphorus supply, internal phosphorus concentration and relative growth rate

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Received: 10 June 2013 Returned for revision: 5 July 2013 Accepted: 23 July 2013 Published electronically: 22 September 2013

- **Background and aims** Some *Lupinus* species produce cluster roots in response to low plant phosphorus (P) status. The cause of variation in cluster-root formation among cluster-root-forming *Lupinus* species is unknown. The aim of this study was to investigate if cluster-root formation is, in part, dependent on different relative growth rates (RGRs) among *Lupinus* species when they show similar shoot P status.
- **Methods** Three cluster-root-forming *Lupinus* species, *L. albus*, *L. pilosus* and *L. atlanticus*, were grown in washed river sand at 0, 7.5, 15 or 40 mg P kg⁻¹ dry sand. Plants were harvested at 34, 42 or 63 d after sowing, and fresh and dry weight of leaves, stems, cluster roots and non-cluster roots of different ages were measured. The percentage of cluster roots, tissue P concentrations, root exudates and plant RGR were determined.
- **Key results** Phosphorus treatments had major effects on cluster-root allocation, with a significant but incomplete suppression in *L. albus* and *L. pilosus* when P supply exceeded 15 mg P kg⁻¹ sand. Complete suppression was found in *L. atlanticus* at the highest P supply; this species never invested more than 20 % of its root weight in cluster roots. For *L. pilosus* and *L. atlanticus*, cluster-root formation decreased at high internal P concentration, irrespective of RGR. For *L. albus*, there was a trend in the same direction, but this was not significant.
- **Conclusions** Cluster-root formation in all three *Lupinus* species was suppressed at high leaf P concentration, irrespective of RGR. Variation in cluster-root formation among the three species cannot be explained by species-specific variation in RGR or leaf P concentration.

**Key words:** Cluster roots, phosphorus acquisition, relative growth rate, *Lupinus albus*, *L. pilosus*, *L. atlanticus*, net assimilation rate.

**INTRODUCTION**  
The world’s non-renewable phosphorus (P) reserve for P fertilizers is predicted to run out by the end of the century (Cordell et al., 2009; Gilbert, 2009). Whilst new reserves are expected to be found, their quality is likely to be lower and their ease of exploitation is likely to be less, thus increasing P fertilizer prices (Cooper et al., 2011). This presents significant problems for food and fibre production, because crops require P as an essential macro-nutrient for growth (Marschner, 2012). Many soils have accumulated large stores of P from decades of phosphate fertilization; this P is poorly available as it is largely sorbed onto soil particles (Allen et al., 2001). Many currently cultivated crops cannot readily access sorbed P in soil. It is crucial to improve P acquisition and use efficiency in order to sustain high yields in cropping systems to meet global demand for food as P becomes more expensive due to declining P reserves.

Some plants have developed several special characteristics to adapt to low P soils, e.g. decreasing rhizosphere pH (Cartin et al., 1993), changing root morphology, such as increasing the length and surface area of roots (Williamsen et al., 2001; Hu et al., 2010), modifying root architecture, i.e. changing the angle of lateral roots to better explore shallow surface layers (Lynch, 1995), increasing root hair length and frequency (Gaehoorn and Nielsen, 1997), developing mycorrhizal associations (Smith et al., 2011), releasing large amounts of carboxylates into the rhizosphere (Ryan et al., 2001; Shane, 2003) and developing specialized structures such as cluster roots (Parnell, 1960; Gardner et al., 1983b; Dinkelaker et al., 1989) and daisyform roots (Shane et al., 2005). Several researchers have reported that a low availability of P (or Fe, but conditions depend on species) is a major factor that strongly induces cluster-root formation (Keerthisinghe et al., 1998; Shane and Lambers, 2005; Watt and Westton, 2009). When P is limiting, some *Lupinus* species form cluster roots and release carboxylates to enhance P acquisition (Gardner et al., 1983a; Bolland et al., 1999; Lambers et al., 2013).

Cluster-root formation, associated with the release of large amounts of carboxylates in an oxidative burst from mature clusters from *Lupinus* and other cluster-root-forming species (Watt and Evans, 1999; Shane et al., 2003a), is considered a desirable trait under P-poor soil conditions (Lambers et al., 2012a). At sufficient P supply, these plants suppress the initiation of cluster roots, and therefore prevent major loss of carbon from the root system (Keerthisinghe et al., 1998). Several investigators have reported cluster-root formation to be correlated with plant internal P status (Shane et al., 2003b, 2005). However, the
exact location where the signal originates has not been elucidated; it might be the shoot P concentration, the phloem sap P concentration or the root P concentration (Shane et al., 2003b).

A model introduced by Pearse et al. (2006) proposed that cluster-root formation in *Lupinus* species has a positive effect on P uptake, which enhances shoot P status, but this, in turn, has a negative effect on cluster-root formation and root exudation. Plant P status is largely balanced by P uptake and plant growth rate. The capacity of a plant for P uptake may be influenced by several genetic and environmental factors (Brown et al., 2012; Postma and Lynch, 2012).

In addition, the relative growth rate (RGR) of a plant may be affected by many environmental and genetic factors. Faster growth rates are correlated with higher leaf P concentrations (Wissuwa et al., 2005; Pearse et al., 2006; Abdolzadeh et al., 2010); in cluster-forming species, high leaf P concentrations are closely correlated with low cluster-root formation. Hence, we expect cluster-root formation to be correlated with RGR or a combination of both RGR and plant P status. We therefore aimed to assess if cluster-root formation is affected independently by RGR or only by a combination of both RGR and leaf P concentration.

We hypothesized that *Lupinus* species of similar shoot P status and RGR allocate the same proportion of their root weight to cluster roots, and therefore test the model proposed by Pearse et al. (2006). Three cluster-root-bearing *Lupinus* species, *L. albus*, *L. pilosus* and *L. atlanticus*, were grown at a range of P levels. These species are all Old World species and are able to form true cluster roots, whereas most New World *Lupinus* species either do not form cluster roots or no information is available about this trait in the literature (Lambers et al., 2013). The natural habitat of the three *Lupinus* species in the present study was the Mediterranean Basin and North Africa (their origins are: *L. albus*, Egypt; *L. pilosus*, Turkey; *L. atlanticus*, Morocco) (Gladstones, 1970; Käss and Wink, 1997; Lambers et al., 2013).

To test our hypothesis, investment of root dry mass in cluster roots, tissue P concentrations and plant RGR were determined, to evaluate possible relationships of cluster-root investment to leaf P status, RGR or a combination of both.

**MATERIALS AND METHODS**

**Growth of plants**

In early April 2011, three uniformly sized seeds of *Lupinus albus* L. ‘Kiev’ mutant, *L. atlanticus* and *L. pilosus* were sown into 3 kg of sterilized, washed river sand in black plastic pots lined with polyethylene bags in a temperature-controlled glasshouse. Pots were supplied with 0, 7.5, 15 or 40 mg P kg⁻¹ sand as K₂HPO₄ (based on fig. 1 in Pearse et al., 2006). Other basal nutrients were added, based on previous research (Pearse et al., 2007). For the first 4 weeks after sowing, the pots were watered to 70 % of field capacity every second day, and then daily until the end of the experiment. The seedlings were thinned to one plant per pot at 7 d after germination, and cotyledons were removed at day 14. The pots were randomized weekly. The first harvest was carried out 34 d after sowing. Four randomly selected plants from each treatment were harvested 34, 42 and 62 d after sowing.

**Root exudate collection**

A whole plant was removed from each pot, and the plastic bag surrounding the roots in sand was cut away to provide minimum disturbance to the root system. Intact plants were then lifted gently from the sand and shaken lightly to remove bulk sand from the roots. The roots then were gently immersed in either a 100 or a 250 ml vial, depending on their size, and 20–120 ml of 0·2 m CaCl₂ was used to remove as much rhizosphere sand as possible for 15–90 s. The roots were then removed from the vials and the vials were shaken by hand. The pH of the extract was measured. A sub-sample of the ‘rhizosphere extract’ was then filtered through a 0·2 μm syringe filter [13 mm syringe filter with a 0·2 μm Supor (PES membrane) into a 1 ml HPLC vial. These extracts were acidified with a drop of concentrated phosphoric acid and transferred to a ~20 °C freezer until HPLC analysis. The whole root systems were then washed thoroughly with deionized water to remove any remaining sand.

**Analyses of carboxylates**

The carboxylates in the rhizosphere extracts were analysed by HPLC (600 pump, 717 plus autosampler and 996 photodiode array (PDA) detector, Waters, Milford MA, USA) using an Alltech C₁₈ reverse-phase column (Cawthray, 2003) in 50 or 150 μl injections (depending on the expected concentration of carboxylates in the samples). The mobile phase was 93 % 25 mM KH₂PO₄ adjusted to pH 2·5 with concentrated H₃PO₄ and 7 % methanol with a flow rate of 1 ml min⁻¹. The working standards included malic, malonic, lactic, acetic, maleic, citric, cis-aconitic, succinic, fumaric and trans-aconitic acids. Carboxylate exudates were expressed per unit root dry mass.

**Plant measurements**

Each plant was separated into stems (including petioles), expanding leaves, most recently matured leaves, oldest mature leaves and senesced leaves. Cluster roots, defined as portions of lateral roots containing bottlebrush-like root clusters of >10 rootlets cm⁻¹ (Johnson et al., 1996), were then separated from the rest of the root system. Each of these components was scanned before drying (see below) and the root mass ratio (root dry mass/total plant dry mass) was calculated. RGRs based on total dry mass were calculated using the following equation (Ricker, 1979):

\[
\text{RGR} = \frac{(\ln M_2 - \ln M_1)}{(T_2 - T_1)}
\]

where \(M_2\) is the plant dry mass at \(T_2\) (day 62) and \(M_1\) is the plant dry mass at \(T_1\) (day 42).

**Scanning and image analysis of roots and leaves**

Each root sample (cluster roots and non-cluster roots) was dispersed in water in a transparent tray (30 x 30 x 3 cm) and scanned (EPSON Expression 1600, Seiko EPSON Corp., Japan) at a resolution of 200 dpi. Also, all leaflets of each sample were separated and spread in a transparent tray (without water) and scanned. For plants with a larger root system or larger number of leaves, up to four trays were used for scanning. The images were analysed with WinRHIZO software by the method of object separation from the background and classification of pixel colours (Regent Instruments Inc., 2002). Leaf area was measured and the leaf area ratio (LAR; m² g⁻¹) was calculated.
Tissue P measurement

Fresh weights of stems including petioles, leaves [expanding leaves, recently fully expanded leaves (RFELa) and mature leaves] and roots (cluster and non-cluster) were determined at each harvest. After drying at 70 °C for 1 week, the plant components were stored in a desiccator until the dry mass was recorded. Each component was then ground with a ball-mill grinder when the dry mass was > 0.4 g. A 0.1-0.4 g sub-sample was taken and digested in concentrated HNO₃/HClO₄ (3:1 or 10:1) at 175 °C. The digests were transferred into 10 mL plastic tubes and these samples were then analysed for total P concentration using the malachite green colorimetric method (Motomizara et al., 1983).

Statistics

Data were compared using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (α = 0.05) using SAS 9.2 statistical software (SAS Institute Inc.). Means are presented with standard errors to indicate the variation of each measurement. Significant differences among means were tested at a significance level of P ≤ 0.05.

All multiple regressions of three-dimensional graphs were carried out using the R statistical environment for statistical computing and graphics (R Development Core Team, 2009) and the Scatterplot3d Package (Ligges and Mächler, 2003) in exploring multivariate patterns between combinations of cluster-root formation and both the P concentration of RFELa and RGR. For statistical comparisons, differences were considered statistically significant at P ≤ 0.05.

RESULTS

Plant growth

Increasing P supply in sand invariably resulted in increased biomass for all three Lupinus species, showing significant differences at day 42 and day 62 (Fig. 1). All Lupinus species produced significantly more biomass at the highest P treatment of 40 mg P kg⁻¹ sand, with approx. 2.4-, 2.6- and 2.5-fold more biomass than that with 0 P supply at the final harvest on day 62 in L. atlanticus, L. albus and L. pilosus, respectively (Fig. 1).

There were major differences among the three Lupinus species in growth in response to P supply. Over the 20 d period from day 42 to day 62, L. pilosus produced most biomass, followed by L. atlanticus, and then L. albus for all P treatments (Fig. 1).

The root mass ratio (root dry mass/total dry mass) varied significantly among species at each harvest, increasing slightly with time for all three species (data not shown). On day 62, the allocation of biomass to roots in L. albus and L. pilosus was significantly higher than that of L. atlanticus; on average it was 0.20, 0.19 and 0.14 g, respectively. However, there was no significant difference in root mass ratio for each Lupinus species under all P treatments.

Cluster-root formation

The highest percentage of cluster roots, based on total root mass, was observed when 0 P was added to the sand, and increasing P supply decreased cluster-root formation in all species (Fig. 2). Cluster-root formation in L. pilosus was suppressed when the P supply exceeded 15 mg P kg⁻¹ sand, while that of L. albus and L. atlanticus was less sensitive to P supply, with significant suppression at the highest P supply of 40 mg P kg⁻¹ sand. The most severe inhibition of cluster-root formation by P supply was observed in L. pilosus, where at day 62 the dry mass of cluster roots decreased from 42 % of the total root mass at 0 mg P kg⁻¹ sand to < 4 % at 40 mg P kg⁻¹ sand (Fig. 2). Total inhibition, at 40 mg P kg⁻¹ sand after day 42 and 62, was observed only in L. atlanticus (Fig. 2). L. pilosus showed the highest percentage of cluster roots of the total root mass, with an average of 25 and 45 % at day 34 and day 42, respectively, followed by L. pilosus (Fig. 2). On day 62, L. pilosus and L. albus had 48 and 47 %...
Cluster-root formation decreased sharply with increasing leaf P concentrations for *L. albus* and *L. pilosus*, and decreased slightly in *L. atlanticus* (data not shown). The strongest inhibition of cluster-root formation by P concentration in the RFELs was in *L. pilosus* at day 62. At this time, cluster-root production decreased to <4% when the P concentration in the RFELs increased to about 3 mg P g⁻¹ dry matter (DM) (Fig. 3). In contrast, for *L. atlanticus*, there was only a slight decrease in cluster-root formation when the P concentration in the RFELs increased. Cluster-root production in this species was as low as 10% at 1 mg P g⁻¹ DM compared with 42 and 40% in *L. albus* and *L. pilosus*, respectively, at the same P concentration in the RFELs (Fig. 3). The differences among species in cluster-root formation in response to plant P concentration were even more pronounced when comparing leaf P concentration, in particular, in RFELs (Fig. 3), showing a reduction of cluster roots to 5% at 4, 3 and 2 mg P g⁻¹ DM in *L. albus*, *L. pilosus* and *L. atlanticus*, respectively.

**Relationship between cluster-root formation and relative growth rate**

Based on linear regression analysis, there was a significant correlation between cluster-root formation and RGR in *L. albus* (P = 0.049) and *L. pilosus* (P = 0.008), but not in *L. atlanticus* (P = 0.379) (Fig. 4). Cluster-root formation decreased sharply with increasing RGR for *L. albus* and *L. pilosus*, while there was only a slight decrease in cluster-root formation with increasing RGR for *L. atlanticus* (Fig. 4).

**Relationship between cluster-root formation and both internal P concentration and relative growth rate**

Based on a multiple regression analysis, at day 62 for *L. pilosus* and *L. atlanticus*, there were significant correlations between cluster-root formation and P concentration in the RFELs, irrespective of RGR (Fig. 5). In *L. albus*, a high P concentration in the RFELs strongly and significantly suppressed cluster-root formation. However, cluster-root formation was not significantly correlated with either P concentration in the RFELs or RGR.

*L. pilosus* showed a strong and significant negative correlation of cluster-root formation with P concentration in the RFELs, but not with RGR, with suppression of cluster-root formation in these two species stronger as the P concentration in the RFELs increased.

In *L. atlanticus*, the correlation of cluster-root formation with P concentration in the RFELs was significant, but not with RGR; cluster-root formation was suppressed as the P concentration in the RFELs increased, irrespective of RGR.

**Rhizosphere carbonates**

The concentration of carbonates accumulated in the rhizosphere was highest when no P was supplied, and decreased with higher P supply up to day 34. The exudation of malate was strongly influenced by the P supply (Fig. 6). At the first harvest, a high P supply reduced malate release; afterwards, there was an increased release of malate with increasing P supply, with a maximum exudation of malate at day 42 at 7 mg P kg⁻¹ and at day 62 at 15 mg kg⁻¹ in *L. pilosus*. However, for...
all species, at later growth stages peak exudation was shifted to one of the treatments with some P supplied (Fig. 6).

**Shoot P concentration and P allocation**

The P concentration in plant tissues was low at 0 P and increased with increasing supply of P for all three species at all three harvests (Fig. 7). At each harvest, the highest concentration of P for all three species was observed at 40 mg P kg\(^{-1}\) sand, and the plant P concentrations were significantly higher for this P treatment than for any other P treatments for all tissue types.

At day 34, the P concentration in expanding leaves of *L. atlanticus* was the highest at 12.1 mg P g\(^{-1}\) DM compared with the other harvest times for all species and plant tissues. However, by day 42, the P concentration in the expanding leaves of *L. atlanticus* dropped to 6.5 mg P g\(^{-1}\) DM, still higher than that of *L. albus* and *L. pilosus* (Fig. 7).
At day 34, the P concentration of expanding leaves, which are the youngest leaves, was highest for all three species. The P concentrations in these expanding leaves at this time were about 1.5- and 2-fold higher than those in the RFELs and mature leaves, respectively (Fig. 7). At day 62, the P concentrations in the expanding leaves remained 2-fold greater than that in the older leaves for all three species. Also, at day 62, compared with the other plant tissues, the P concentration was lowest in senesced leaves for all three species.

There were no significant differences among the three *Lupinus* species in shoot P concentration at each level of P treatments (data not shown).
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Fig. 7. Phosphorus (P) concentration of expanding, recently fully expanded and mature leaves of Lupinus alata and L. pinnatissima at (A) 34, (B) 42 and (C) 62 after sowing; and (D) P concentration of senesced leaves, stems, cluster roots and non-cluster roots at day 62 supplied with either 0, 7.5, 15 or 40 mg P kg⁻¹ sand in the form of K₂HPO₄. Error bars represent the s.e. (n = 4). Treatment means marked with the same lower case letter are not significantly different within each organ group using a one-way ANOVA for each tissue for all the harvests followed by Duncan’s multiple test (P ≤ 0.05).
DISCUSSION

The objective of the present study was to assess if Lupinus species of similar shoot P status and RGR allocate the same proportion of their root weight to cluster roots, and therefore to test the model proposed by Pearse et al. (2006). The results demonstrate major differences in cluster-root allocation, despite a similar internal P status for the three Lupinus species.

Phosphorus treatments induced large species-specific variation in cluster-root formation for three Lupinus species

Phosphorus treatments had significant effects on total plant biomass and leaf P concentrations, but no effect on root mass ratio, as found before for Lupinus species (Shen et al., 2003; Pearse et al., 2006; Abdolzadeh et al., 2010). However, there were major effects on cluster-root allocation.

Cluster-root formation in *L. albus* and *L. pilosus* was significantly but not totally suppressed when P supply exceeded 15 mg P kg⁻¹ sand. Conversely, cluster-root formation in *L. atlanticus* showed a complete suppression at the highest P treatment (Fig. 2). Phosphorus treatments had major effects on carbohydrate release in the three Lupinus species. Peak exudation was observed at the lowest P supply at day 34 for all Lupinus species; however, at later growth stages the peak shifted to treatments with more P supplied (Fig. 6). The exudation of malate from the whole root system was strongly influenced by P supply, where high P supply reduced malate release at the first harvest; afterwards, there was an increased release of malate with increasing P supply in *L. pilosus*. This indicates that *L. pilosus* exuded more malate at later growth stage even at higher P supply.

The highest leaf mass ratio (LMR), the ratio between total leaf mass and total plant dry mass, at a P supply of 15 mg P kg⁻¹ sand at day 62 was observed for *L. pilosus*, followed by *L. atlanticus* and *L. albus*. In the same treatment and harvest, the highest specific leaf area (SLA), the ratio between leaf area and leaf dry mass, was observed for *L. pilosus* followed by *L. albus* and *L. atlanticus*. Relative growth rates increased from *L. albus* to *L. atlanticus*, followed by *L. pilosus* (Table 1). The RGR of the three Lupinus species in this study between day 42 and day 62 was low, compared with the RGR of two dicot species (*Pimpinella saxifraga* L. and *Plantago major* ssp. major L.) out of 11 dicot species tested by Poorter and Remkes (1990) – 171 and 240 mg g⁻¹ d⁻¹, respectively. The two species used in this comparison had a similar LMR and SLA at 0.54 and 0.64 g g⁻¹ and 31.2 and 32.8 m² kg⁻¹, respectively. The low RGR for all three Lupinus species may reflect measurements at a later stage of development than that of the two species measured by Poorter and Remkes (1990). However, values for RGR at an earlier stage (from day 34 to day 42) were actually rather similar for *L. albus* and *L. atlanticus*, but was higher for *L. pilosus* (Table 1), and also similar to values presented in the literature for *L. albus* (Pate et al., 1979; Chen et al., 2001). The low RGR of the Lupinus species in the present study reflects their relatively low net assimilation rate (NAR), which was, from day 42 to day 62 on average, 4.4, 3.2 and 5.4 g m⁻² d⁻¹ as opposed to 10.2 and 11.8 g m⁻² d⁻¹ in the two species used for comparison measured by Poorter and Remkes (1990).

The NAR is largely the balance of carbon gain in photosynthesis and carbon use in respiration of different plant organs and in exudation of carbohydrates (Poorter et al., 1990; Lambers et al., 2008). The net CO₂ assimilation rate of white lupin (Campbell and Sage, 2006) is about 5-fold of that of the fast-growing wild species studied by Poorter et al. (1990). About 30 to 40% of the total carbon gained in photosynthesis in white lupin with a similar RGR is used in shoot and root respiration (Pate et al., 1979; Chen et al., 2001), as opposed to only 15% in the two dicot species used in the comparison (Poorter et al., 1990). Thus, the relatively low NAR in the lupins studied here is partly accounted for by the relatively large fraction of carbon use in shoot and root respiration. In addition, carbon use in root exudation of carbohydrates will play a role (Lambers et al., 2013).

Cluster-root formation in the three Lupinus species is dependent on internal P concentration and relative growth rate

Cluster-root formation was significantly correlated with internal P concentration for all three Lupinus species (Table 1), with strong and negative correlations in *L. albus* and *L. pilosus*, whereas that in *L. atlanticus* was not quite as strong. These negative correlations are consistent with previous findings for *L. albus* (Shane et al., 2003b; Shen et al., 2003; Pearse et al., 2006; Abdolzadeh et al., 2010) and *L. atlanticus* (Pearse et al., 2006; Abdolzadeh et al., 2010). High leaf P concentration and low cluster-root formation are tightly correlated in all three Lupinus species. However, since leaf P concentration is correlated with RGR, these correlations might reflect effects of RGR, as explored below.

There were significant correlations between cluster-root formation and RGR for *L. albus* and *L. pilosus*, but not for *L. atlanticus*, which never invested a large fraction of its biomass in cluster roots (Fig. 4). Faster growth rates are correlated with higher leaf P concentration, so the correlations with RGR might actually reflect correlations with leaf P concentration, as discussed below.

When we took both RGR and leaf P concentration into account, using multiple regressions on cluster-root formation with both leaf P concentration and RGR, the analysis showed that cluster-root investment varied in different Lupinus species dependent on leaf P concentration, irrespective of RGR (Fig. 5). In *L. albus*, there was a trend of cluster-root formation...
decreasing with increasing leaf P concentration, but there was no significant correlation between cluster-root formation and either leaf P concentration or RGR, due to the relatively large variation in *L. albus* plants in the present study. In *L. pilosus*, cluster-root formation was significantly downregulated at greater leaf P concentration at higher P supply, independent of RGR. In *L. atlanticus*, cluster-root formation was never very large, but it increased significantly with decreasing leaf P concentration, again independently of RGR.

We therefore present a new model of the interaction between cluster-root formation, plant P uptake, shoot P status and RGR (Fig. 8). Cluster-root formation increases the plant P uptake rate, which stimulates plant growth and increases plant P status; however, cluster-root formation does not depend on relative growth rate per se.

In summary, in two of the three *Lupinus* species, cluster-root formation was suppressed at high internal P concentration, irrespective of RGR. For *L. albus*, there was a trend in the same direction, but this was not significant. Variation in cluster-root formation among the three species cannot be explained by species-specific variation in RGR or leaf P concentration. We can only speculate on the differences in cluster-root formation among the lupin species which we studied.Auxins play a role in cluster-root formation (Gilbert et al., 2000; Skene and James, 2000; Hocking and Jeffery, 2004), and hence there may be differences in the strength of this hormonal signal among the *Lupinus* species studied here. However, shoot-derived sugar signals (sucrose, glucose and fructose) also control plant P starvation responses, including cluster-root formation (Liu et al., 2005; Mütter et al., 2007; Zhou et al., 2008), and hence the strength of this signal might vary among lupin species. Possibly, striptolactones also play a role in cluster-root formation, given their role in lateral root formation (Kapulnik et al., 2011) and response to P deficiency (Yoneyama et al., 2007a, b; López-Ráez et al., 2008; Kohlen et al., 2011). Future research will be required to characterize the relationship between plant P status and signalling compounds to determine the cause of variation in cluster-root formation that was observed in our study.

**Phosphorus uptake and concentration and internal P allocation**

Phosphorus treatments had a significant effect on plant P status for all three *Lupinus* species. Luxury uptake of P was found at the highest P treatment, which is significantly different from other P treatments in all three *Lupinus* species (Fig. 7). However, there were no significant differences in leaf P concentration in the three *Lupinus* species within the same P treatment at day 62. At low P supply, all *Lupinus* species showed the highest P concentration in their youngest expanding leaves, especially at the later stages (Fig. 7). The P concentration declined during leaf maturation. This may indicate that young expanding leaves have a greater requirement for P, of which, at low P supply, about 30% is required for RNA, which is needed for protein synthesis (Yoneyama et al., 2012). About 20% of leaf P is associated with phospholipids, and at a limiting P supply some of these may be replaced by galactolipids and sulfolipids (Anderson et al., 2005; Fjellström et al., 2008; Lambers et al., 2012). The decline in leaf P concentration during leaf development in *Lupinus* may improve their P use efficiency by optimizing P distribution.

At the final harvest, day 62, all *Lupinus* species demonstrated the lowest P concentration in their senesced leaves, and the highest in their expanding leaves (Fig. 7). Across all P treatments, P concentrations in senesced leaves were 51, 78 and 62% of that in mature leaves (for *L. atlanticus*, *L. albus* and *L. pilosus*, respectively (Fig. 7). This shows that all three *Lupinus* species remobilized a substantial amount (49, 22 and 58%) of P from senescing leaves to other plant parts at low P supply for *L. atlanticus*, *L. albus* and *L. pilosus*, respectively. This P remobilization efficiency is by no means exceptional, when compared with a global average P resorption efficiency of 65% presented in the literature, and is similar to a mean P resorption of 76% in herbaceous forb species (Verguts et al., 2012).

Taken together, *Lupinus* species optimized P distribution by allocating more P to their youngest leaves even at a very low plant P status. Efficient P remobilization from senesced leaves is a desirable trait, which allows the improvement of P distribution within plants, especially under P-limiting conditions.

**Concluding remarks**

The present experimental approach tested the model proposed byPearse et al. (2006). Fast growth rates are correlated with high leaf P concentration, which are tightly correlated with low investment in cluster roots. Therefore, one might expect cluster-root formation to be correlated with RGR, given that plants with fast growth rates, even with a high P-uptake rate, might not accumulate shoot P. However, we have shown that cluster-root formation in all three *Lupinus* species is suppressed at high leaf P concentration, irrespective of RGR (Fig. 8). Variation in cluster-root formation among the three species cannot be explained by species-specific variation in RGR or leaf P concentration.

**Acknowledgements**

X.W. was supported by the University of Western Australia and China Scholarship Council for UWA China scholarships, and by the School of Plant Biology for project funding. We are grateful to Dr Bevan Bairdell, Dr Richard Snowball and Dr Jon...
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Clements for kindly providing the Lupinus seeds, to Jessie Monodis, Sandra Kerbler, Fuzilah Adbi Manan, Hazel Gaze, Melinda Trudgen, Yupin Li and Marina Borges Osorio for assisting with the harvest, to Dawn Walker for helping with plant P analyses, to Dr Joanne Edmondson for her valued help with X.W.'s English in an earlier version of this paper, and to many friends for their support and encouragement.

LITERATURE CITED

Abdolzadeh A, Wang X, Veneklasen EJ, Lambers H. 2010. Effects of phosphorus supply on growth, phosphorus concentration and cluster-root forma-


Bolland MDA, Siddique KHM, Lour SP, Baker MJ. 1999. Comparing responses of grain legumes, wheat and canola to applications of superphos-

Brown L, George T, Thompson J, et al. 2012. What are the implications of vari-
ation in root hair length on tolerance to phosphorus deficiency in combina-


Currie D, Syers J, Bolan N. 1993. Phosphorus sorption by soil in relation to ex-

Dinkelaker B, Römheld V, Marschner H. 1988. Citric acid excretion and precip-


Gilbert GA, Knight JD, Vance CP, Allaway DL. 2008. Phosphate root development of phosphorus deficient lupin is mimicked by auxin and phospha


Hocking PJ, Jeffery S. 2004. Cluster-root production and organic anion exi-

ogy and phosphorus uptake kinetics in Brazilian napo under low phospho

Jahnke JF, Vance CP, Allaway DL. 1996. Phosphorus deficiency in Lupinus albus – altered root development and enhanced expression of phos-


Kerrithingle G, Hocking PJ, Ryan PR, Deluca H. 1998. Effect of phospho-

Koehn W, Chalotukova T, Liu Q, et al. 2011. Sesquiterpenes are transported through the xylem and play a key role in short-architectural responses to phos-


Ligges U, Meicher M. 2003. Scatterplot3D – an R Package for visualizing multi-


terpenes are derived from carotenoids and their biosynthesis is promoted by phosphatase starvation. New Phytologist 178: 863–874.


Purcell HM. 1980. Studies of the family Proteaceae. I. Anatomy and morphol


Shane MW, Yen M, de Roock S, Cawthray GR, Lambers H. 2005a. Effects of external phosphorus supply on internal phosphorus concentration and the


CHAPTER 3

Interactions between Cluster-root Investment, Leaf Phosphorus Concentration and Relative Growth Rate in Two *Lupinus* Species: Can Faster-growing *Lupinus* Plants Maintain Cluster-root Production at Higher Phosphorus Supply?
ABSTRACT

Premise of the study Cluster-root formation in lupins is suppressed as plants attain a higher phosphorus (P) status; however, if increased P uptake enhances plant growth, cluster-root formation might be maintained at the same level. We investigated the interactive effects of cluster-root formation, leaf P status and relative growth rate (RGR) in two Lupinus species whereby variation in RGR was imposed by varying day-length.

Methods Lupinus albus and L. pilosus were grown hydroponically with 6, 5 or 3 \( \mu \)M P (in the form of \( \text{KH}_2\text{PO}_4 \)) at a day-length of 14, 10 or 6 h day\(^{-1}\), respectively. Fresh and dry mass of leaves, stems, non-cluster and cluster roots were measured at 22, 38 or 52 days after sowing. The percentage of cluster roots, leaf P concentrations and RGR were also determined.

Key results Day-length treatments induced differences in RGR. Slower-growing plants grown at a shorter day-length showed reduced biomass allocation to cluster roots in both Lupinus species. Cluster-root percentage in the two Lupinus species decreased with greater leaf P concentrations, but did not increase with a higher RGR. Multiple regression analysis showed that the percentage of cluster roots was strongly and negatively correlated with plant P status, and only marginally and positively correlated with RGR for both L. albus and L. pilosus.

Conclusions The two Lupinus species with high P status invariably down-regulated cluster-root formation, irrespective of RGR. The difference in cluster-root formation between these plants cannot be explained by a species-specific variation in either RGR or in leaf P concentration.

Key words: biomass allocation; day-length; multiple regression analysis.
INTRODUCTION

Phosphorus (P) is an essential macronutrient for plant growth; however, commercially extractable P reserves are being depleted and P may become a more severely factor limiting crop production worldwide (Gilbert, 2009; Marschner, 2012), although probably not as rapid as forecasted previously (Fixen and Johnston, 2012; Scholz and Wellmer, 2013). Many soils contain substantial levels of P, but in forms not readily available for most crop plants, like wheat and canola (Holford, 1997; Simpson et al., 2011). In order to sustain high crop yields, it is critical to improve P-acquisition and P-use efficiency in cropping systems to satisfy world demand for food, feed and fibre as P becomes more expensive due to dwindling and increasingly expensive P resources.

The formation of cluster roots and exudation of carboxylates from these cluster roots of some lupin species (e.g., Lupinus albus L.) are considered adaptations to acquire sparingly-available P under low-P conditions (Bolland et al., 1999; Ryan, Delhaize, and Jones, 2001; Shane and Lambers, 2005). Cluster roots are not only present in L. albus and certain other Lupinus species (Lambers, Clements, and Nelson, 2013), but also occur in most Proteaceae and several other taxa (Dinkelaker, Hengeler, and Marschner, 1995; Lambers et al., 2006). Cluster roots release large quantities of carboxylates in an exudative burst from mature clusters (Watt and Evans, 1999; Shane and Lambers, 2005). This strategy is considered the most effective to access poorly available soil P sources (Lambers et al., 2008). Consequently, in field studies the response to P fertiliser is relatively small for legume species that exhibit a very high phosphorus-acquisition efficiency due to the release of carboxylates from their cluster roots (Bolland et al., 1999).
Cluster-root formation and carboxylate exudation are plastic traits such that plants in high-P soil, with a favourable plant P status, minimise investment in these carbon-costly P-acquisition mechanisms (Richardson et al., 2011; Lambers, Clements, and Nelson, 2013). Split-root experiments have shown that systemic signals associated with shoot P status, rather than P availability in the rhizosphere regulate cluster-root formation in *L. albus* (Shane et al., 2003; Li et al., 2008).

Several researchers have shown in *Arabidopsis thaliana* that an exogenous supply of sugars to the root growth medium induces expression of genes that are also induced by P deficiency, namely *Pht1;1, Pht1;4, PAP, RNase2* and *At4* (Nacry et al., 2005; Karthikeyan et al., 2007; Müller et al., 2007). These genes encode proteins involved in P transport and carbon metabolism, and also play a role in the P-starvation response in *Arabidopsis* (Doerner, 2008). Both a low P supply and exogenous sugars stimulate the transcription of a gene (*LaPT1*) encoding a phosphate transporter and a high sugar supply, irrespective of P supply induces the release of an acid phosphatase (*LaSAP1*) from white lupin (Liu et al., 2001). *LaPT1* encodes P-transport proteins, and both *LaPT1* and *LaSAP1* are involved in cluster-root formation in white lupin (Liu et al., 2005; Zhu et al., 2005; Zinn et al., 2009). Several researchers have also reported that microRNAs, including miR399, miR156, miR169, miR395 and miR398 are responsive to low P supply, acting as systemic signals (Valdes-Lopez et al., 2008; Zhu et al., 2010; Chiou and Lin, 2011). Furthermore, nitric oxide plays a role in signalling P deficiency, inducing cluster-root formation in white lupin (Wang et al., 2010; Meng et al., 2012).

Some P-starvation responses, including inhibition of primary root growth, initiation of lateral root formation and also the formation of root hairs are a result of local signalling (Gilbert et al., 2000; Jungk, 2001; Péret et al., 2011). Phosphorus-
dependent transcriptional changes related to photosynthesis, are associated with growth
of secondary roots; these are short-term responses (Misson et al., 2005; Morcuende et
al., 2007). Longer-term responses involving transcriptional changes include down-
regulation of P-acquisition mechanisms (Hammond et al., 2003; Wasaki et al., 2003;
Wu et al., 2003; Misson et al., 2005; Morcuende et al., 2007). These transcriptional
studies demonstrate that internal P quickly leads to stimulation of growth occurs, and
that down-regulation of P-acquisition mechanisms takes longer.

As summarised by Pearse et al. (2006), cluster-root formation is regulated by a
negative feedback loop, in which cluster roots have a positive effect on P uptake, which
enhances P status, which in turn suppresses cluster-root formation and exudation in
Lupinus species. However, an increased P-uptake rate may not lead to a more
favourable plant P status if growth rate is stimulated to the same extent. In our recent
study (Wang, Pearse, and Lambers, 2013), we presented a revised model: cluster-root
formation increases plant P-uptake rate, which stimulates plant growth and increases
plant P status; however, in that study, we found that cluster-root formation does not
depend on relative growth rate (RGR) per se. To date, all experiments on the effect of P
status on cluster-root formation have achieved a different P status by varying P
availability, causing a parallel effect on growth rate. It is therefore important to
investigate if plants with high P status invariably down-regulate cluster-root formation,
irrespective of growth rate. We hypothesised that Lupinus species grown at a similar P
availability, but with a higher growth rate, as dependent on light availability, show a
greater investment in cluster roots. We also hypothesised that the differences in cluster-
root formation between Lupinus species is associated with differences in both relative
growth rate and internal P concentration. Two cluster-root-forming Lupinus species, L.
albus and L. pilosus, were grown at a similar P availability in the root environment, but at different day-lengths, to impose different growth rates.

Our objective was to investigate if the negative correlation between plant P status and investment in cluster roots is maintained if differences in growth rate are imposed by a factor other than P supply. To test our hypotheses, we determined phenotypic and species-specific relationships between cluster-root formation, leaf P status and plant relative growth rate.

MATERIAL AND METHODS

Plant growth

In November 2012, seeds of Lupinus albus and L. pilosus were germinated in pots filled with washed and sterilised river sand in a glasshouse. At 7 days after sowing, uniformly sized seedlings from each of the three Lupinus species were carefully removed from the pots, and the roots washed free of sand. The stem of each seedling was placed in the centre of a grey foam disk, which formed the centre lid of a 4-L black plastic container with continuously aerated nutrient solution of the following composition (µM): 400 NO₃⁻, 200 Ca²⁺, 210 K⁺, 154 SO₄²⁻, 54 Mg²⁺, 0.24 Mn²⁺, 0.1 Zn²⁺, 0.018 Cu²⁺, 2.4 H₃BO₃, 0.03 Mo⁴⁺, 10 Fe-EDTA. Eight seedlings of the different species were used as a block under the same day-length treatment. In total, 96 plants formed 32 blocks and three day-length treatments (14, 10 or 6 h day⁻¹). They were grown in a temperature-controlled glasshouse, with containers half-immersed in a root-cooling tank maintained at 18 – 22 °C. The entire nutrient solution was replaced daily.
Cotyledons were removed 14 days after sowing to reduce the transfer of seed P reserves to seedlings.

At 14 days after sowing, day-length treatments (14, 10 or 6 h day\(^{-1}\)) were commenced. Inverted cardboard boxes, which were partly open at the ends, were used to cover the plants for the 10 h and 6 h day-length treatments. These cardboard boxes were designed with two flaps on the two opposite ends which facilitated airflow and exchange, and also minimised any possible impacts on atmospheric conditions, temperature and humidity in the experiment, but prevent light entering to the boxes. Since the aim was to modify the growth rate of the plants, any effects in addition to those of day-length did not compromise the approach. Natural day-length in November and mid-December in Western Australia is 13.5 – 14.5 h. To restrict the day length to set periods, the boxes were placed at the end of the natural daylight period at 19:00, and removed the next day at 09:00 (for the 10 h treatment) and 13:00 (for the 6 h treatment. For the 14-h treatment, plants were not covered by cardboard boxes. In our preliminary experiment, we used 20-L black plastic tubs with 12 plants in each tub supplied with 10 µM of P (in the form of KH\(_2\)PO\(_4\)) for all day-length (12 h, 9 h and 6 h) treatments. Plants showed different relative growth rates (0.17, 0.15 and 0.12 mg g\(^{-1}\) day\(^{-1}\)), and leaf P concentrations (3.4, 3.9 and 8.4 mg g\(^{-1}\) dry mass at 29 days after germination) at 12, 9 and 6 h, respectively. The higher plant P concentration of slower-growing plants caused by shorter day-length, reduced cluster-root production from 37% to 5% of total root dry mass. Therefore, we estimated the need and availability of P for plants under different day-length treatments, based on the previous experiment, so P in the growth medium was not a major factor that strongly induces cluster-root formation. Thus, P concentrations in the growth medium was adjusted to 6, 5 or 3 µM of P for the
14, 10 or 6 h day\(^{-1}\) treatment, respectively. Four plants of each species from each treatment were harvested randomly at 22, 38 and 52 days after sowing.

**Plant measurements**

Four plants of all of the 96 harvested plants were gently removed from each pot and then rinsed with de-ionised water. For each plant, root clusters were separated from the rest of the root system, and defined as portions of lateral roots containing bottlebrush-like root-clusters of more than ten rootlets per cm (Johnson, Vance, and Allan, 1996). Fresh mass of stems including petioles, leaves and roots (cluster and non-cluster) were determined at each harvest, and the dry mass was determined after drying at 70 \(^\circ\)C for 1 week. Each of these plant parts was weighed before and after drying (see below) and the root mass ratio (root dry mass/ total plant dry mass) was calculated. Relative growth rates (RGR) based on total plant dry mass were calculated using the equation (Ricker, 1979) as follows:

\[
RGR = \frac{(\ln M_2 - \ln M_1)}{T_2 - T_1}
\]

Where \(M_2\) is the plant dry mass (day 52/38), \(M_1\) is the plant dry mass (day 38/22) and \(T\) is the number of days of plant growth.

Dried material was then ground into a fine powder using a stainless steel ball mill. A 10 to 300-mg subsample of recently fully expanded leaves of each *Lupinus* species from each harvest was taken and digested using a hot concentrated HNO\(_3\):HClO\(_4\) (3:1 or 10:1) acid mixture. The digest solutions were clear when the digestion was completed. Samples in digests were then analysed for total P concentration by the malachite green colourimetric method (Motoniz, Wakimoto, and Toei, 1983) using a UV-VIS spectrophotometer at a wavelength of 630 nm (Shimadzu Corporation, Kyoto, Japan).
Statistics

Data were compared using one-way ANOVA followed by LSD test ($\alpha = 0.05$) using Statistix 8.1 (Analytical Software, USA). Means are presented with standard errors to indicate the variation of each measurement. Significant differences among means were tested at a significance level of $P \leq 0.05$.

Multiple regressions were carried out and results were plotted in three-dimensional graphs using the R statistical environment for statistical computing and graphics (R Development Core Team, 2009) and the Scatterplot3d Package (Ligges and Mächler, 2003) in exploring patterns between combinations of cluster-root formation and both P concentration in RFEL and RGR in *Lupinus* species. For statistical comparisons, differences were considered statistically significant at $P \leq 0.05$. 
RESULTS

Plant growth

A longer day-length resulted in more biomass for both *Lupinus* species, showing significant differences at all three harvests (Fig. 1). At the third harvest (day 52), *L. albus* and *L. pilosus* produced significantly more biomass at the longest day-length (14 h), approximately 49% and 21% more than at a day-length of 10 h and 5-fold and 2.5-fold more than that at a 6 h day-length, respectively (Fig. 1).

Day-length treatments induced differences in RGR. Over the 16-day period, between 22 and 38 days after sowing, relative growth rates (RGR) were 141, 140 and 83 mg g\(^{-1}\) day\(^{-1}\) for *L. albus* and 131, 137 and 103 mg g\(^{-1}\) day\(^{-1}\) for *L. pilosus* at a day-length of 14, 10 and 6 h, respectively. Over the 18-day period between 38 and 52 days after sowing, the RGR were 55, 42 and 22 mg g\(^{-1}\) day\(^{-1}\) for *L. albus* and 46, 43 and 16 mg\(^{-1}\) day\(^{-1}\) for *L. pilosus* at a day-length of 14, 10 and 6 h, respectively. The longest day-length (14 h) resulted in a higher RGR, which was 70% and 27% higher, and 150% and 188% higher than that at a day-length of 6 h for *L. albus* and *L. pilosus*, between day 22 to 38 and between day 38 to 52, respectively.
Fig. 1. Total plant dry biomass of *Lupinus albus* and *L. pilosus* at 22 (A), 38 (B) and 52 (C) days after sowing, when grown at a day-length of 6 hours (black), 10 hours (grey) or 14 hours (white); plants were grown in a hydroponic culture supplied with either 3, 5 or 6 µM of P (in the form of KH$_2$PO$_4$) for each day-length treatment, respectively. Error bars represent standard error (*n* = 4, with the exception of *L. pilosus* at day 52, where *n*=2). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA for each harvest followed by LSD at *P* ≤ 0.05.
The root mass ratio (RMR) increased significantly with increasing day-length for
*L. albus* in the last two harvests and for *L. pilosus* in the final harvest; there was a slight
decrease in root mass ratio with time for the two species. On 52 days after sowing, the
RMR at a 14 h day-length was 37% and 26% greater than that at a 10-h day-length, and
100% and 16% larger than that at a 6-h day-length for *L. albus* and *L. pilosus*,
respectively (Fig. 2).

*Cluster-root percentage*—

In contrast to the small effect on total root investment (Fig. 2), the two *Lupinus*
species produced a much greater percentage of cluster roots at the longest day-length
(14 h) at 38 days after sowing (Fig. 3), constituting an average of 16% and 11% of total
root mass for *L. albus* and *L. pilosus*, respectively. On day 52, *L. albus* showed the
highest percentage of cluster roots at a day-length of 14-h, which was 2.3-fold and 10.5-
fold greater than that at a 10-h and at a 6-h, respectively; the percentage of cluster roots
in *L. pilosus* at a 14-h day-length was 2-fold and 14-fold greater than that at 10 and 6 h,
respectively (Fig. 3). Cluster-root investment was suppressed at shorter day-length
treatments.
Fig. 2. Root mass ratio (ratio of root dry mass to total plant dry mass) of *Lupinus albus* and *L. pilosus* at 22 (A), 38 (B) and 52 (C) days after sowing, when grown at a day-length of 6 hours (black), 10 hours (grey) or 14 hours (white); plants were grown in a hydroponic culture supplied with either 3, 5 or 6 µM of P (in the form of KH$_2$PO$_4$) for each day-length treatment, respectively. Error bars represent standard error (*n* = 4, with the exception of *L. pilosus* at day 52, where *n*=2). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA for each harvest followed by LSD at *P* ≤ 0.05.
Fig. 3. Percentage of cluster roots based on the total root mass of *Lupinus albus* and *L. pilosus* at 22 (A), 38 (B) and 52 (C) days after sowing, when grown at a day-length of 6 hours (black), 10 hours (grey) or 14 hours (white); plants were grown in a hydroponic culture supplied with either 3, 5 or 6 µM of P (in the form of KH₂PO₄) for each day-length treatment, respectively. The percentages were calculated as dry mass of cluster roots divided by total root dry mass times 100. Error bars represent standard error (*n* = 4, with the exception of *L. pilosus* at day 52, where *n* = 2). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA for each harvest followed by LSD at *P* ≤ 0.05.
Leaf phosphorus concentration

We aimed to achieve very similar leaf P concentrations in plants for all day-length treatments, by providing slightly more P to plants grown at longer days. However, this was only partly effective. Both *Lupinus* species had significantly lower P concentrations at the longer day lengths at the later harvests. At day 22 days after sowing, P concentrations in recently fully expanded leaves (RFEL) at different day-lengths were the same (Fig. 4). On day 52, RFEL [P] at a day-length of 14 h was 20 % and 56 % lower than those at a day-length of 10 and 6 h in *L. albus*, and 10% and 44% lower in *L. pilosus*, respectively (Fig. 4). Trends of RFEL P concentrations at day 38 were similar to those at day 52 (Fig. 4). Phosphorus concentrations in RFEL at each day-length decreased with time for these *Lupinus* species (Fig. 4).

Relationship between the percentage of cluster roots and leaf P concentration

There was a significant negative correlation between the percentage of cluster roots and P concentration in RFEL for *L. albus* (P ≤ 0.001) and *L. pilosus* (P = 0.015) (Fig. 5). The strongest inhibition of cluster roots by P concentration in the RFEL was in *L. albus* at day 52. At this time, cluster-root percentage decreased to less than 3% when P concentration in the RFEL increased to about 6 mg P g⁻¹ DM (Fig. 5). The percentage of cluster roots in this species was as low as 5% at 3 mg P g⁻¹ DW compared with 15% and 12% in *L. albus* and *L. pilosus*, respectively, at the same P concentration in the RFEL (Fig. 5). Cluster-root allocation was suppressed at a higher internal P concentration.
Fig. 4. Phosphorus (P) concentration of recently fully expanded leaves of *Lupinus albus* and *L. pilosus* at 22 (A), 38 (B) and 52 (C) days after sowing, when grown at a day-length of 6 hours (black), 10 hours (grey) or 14 hours (white); plants were grown in hydroponic culture supplied with either 3, 5 or 6 µM of P (in the form of KH$_2$PO$_4$) for each day-length treatment, respectively. Error bars represent standard error ($n = 4$). Treatment means marked with the same lower-case letter are not significantly different within each organ group using a one-way ANOVA for each tissue for all three harvests followed by LSD at $P \leq 0.05$. 
Fig. 5. Relationship between percentage of cluster roots of total root dry mass and phosphorus (P) concentration in recently fully expanded leaves (RFEL) for Lupinus albus and L. pilosus. The solid lines indicate significant regressions ($P \leq 0.05$).

**Relationship between the percentage of cluster roots and relative growth rate**

According to a linear regression analysis, there was no correlation between the percentage of cluster roots and relative growth rate (RGR) between day 22 and 38 and between day 38 and 52 in *L. albus* and *L. pilosus* (Fig. 6). The percentage of cluster roots did not increase significantly with increasing of RGR for the two *Lupinus* species.
Fig. 6. Relationship between percentage of cluster roots of total root dry mass at day 38 and relative growth rate (RGR, between day 22 and day 38) and that at day 62 and RGR (between day 38 and day 52) for *Lupinus albus* and *L. pilosus*. The broken line indicates no significant regression.

**Relationship between the percentage of cluster roots and both leaf P concentration and relative growth rate** —

Based on multiple regression analyses, there were strong and significant negative effects of P concentration in RFEL (*P* ≤ 0.001 for both *L. albus* and *L. pilosus*), and small positive effects of RGR (*P* = 0.015 and *P* = 0.002 for *L. albus* and *L. pilosus*, respectively) on the percentage of cluster roots for *L. albus* (*P* ≤ 0.001) and *L. pilosus* (*P* ≤ 0.001) (Fig. 7). We plotted these relations using data on the percentage of cluster roots at day 38 and 52 and data on both P concentrations in RFEL and RGR for the respective growth intervals (day 22-38 and 38-52) for these three species. Both *L. albus*
and *L. pilosus* showed a strong and significant negative correlation of the percentage of cluster roots with P concentration in the RFELs, and a marginal and significant positive correlation with RGR (Fig. 7).

**Fig. 7.** Relationship between percentage of cluster roots of total root dry mass and both phosphorus (P) concentration in recently fully expanded leaves (RFEL), between day 22 and day 38. The multiple regressions were made using relative growth rate (RGR, between day 22 and day 38), and RFEL [P] at day 38; and also RGR (between day 38 and day 52) and RFEL [P] at day 52 for *Lupinus albus* and *L. pilosus*. The *** (*P ≤ 0.001*), ** (*P ≤ 0.01*) and * (*P ≤ 0.05*) indicate significant regressions.
DISCUSSION

The objective of the present study was to investigate if the negative correlation between investment in cluster roots and plant internal P status is maintained if differences in growth rate and plant P status are induced by a factor other than P supply, namely day-length. The results demonstrate cluster-root allocation of these *Lupinus* plants was down-regulated at a high shoot P status, irrespective of growth rate.

*Day-length treatments induced species-specific variation in relative growth rate and cluster-root formation for two Lupinus species*—

Our results show major differences in cluster-root formation for the two *Lupinus* species in response to day-length, which induced differences in RGR and also internal P concentrations. *Lupinus albus* and *L. pilosus* accumulated less biomass at shorter day-length; however, the relative allocation to total root mass was decreased only slightly at the final harvest. Similarly, only small responses of root mass ratio to P supply have been reported before for *L. albus* (Keerthisinghe et al., 1998; Nuruzzaman et al., 2006). In striking contrast with the minor differences in biomass allocation to the root system as a whole, *L. albus* and *L. pilosus* showed much greater percentages of cluster roots at longer day-lengths at day 38 and day 52, compared with plants grown at shorter day-length.

*Cluster-root formation in the two Lupinus species as dependent on leaf P concentration and relative growth rate*—

Cluster-root formation was significantly correlated with leaf P concentration for both *Lupinus* species (Fig. 5), with substantial stronger and negative relationship in *L. albus* and a less strong and negative correlation in *L. pilosus*. These findings are in line
with previous reports for *L. albus* (Shane et al., 2003; Shen et al., 2003; Abdolzadeh et al., 2010). A higher leaf P concentration is closely correlated with low cluster-root allocation in the two species. However, as leaf P concentration is correlated with a plant’s RGR, these relationships might imply effects of RGR, as investigated below.

There were no correlations between cluster-root allocation and RGR for both *Lupinus* species (Fig. 6). Higher growth rates are closely related with greater leaf P concentration, so the non-correlations with RGR might imply correlations with leaf P concentration, as explored below.

When considering both leaf P concentration and RGR, by using multiple regressions on the percentage of cluster roots with both leaf P concentration and RGR, the analysis showed the percentage of cluster roots varied in *L. albus* and *L. pilosus* dependent on leaf P concentration (Fig. 7). The percentage of cluster-root was strongly suppressed at greater leaf P concentration and weakly stimulated at higher RGR.

Taken together, cluster-root formation was down-regulated by higher leaf P concentration, irrespective of RGR. Our results support the finding that cluster-root formation as induced by a low-P supply involves systemic signalling from the shoot to the roots in white lupin (Marschner, Romheld, and Cakmak, 1987; Shane et al., 2003); the same is true for other P-starvation responses in other species (Jungk, 2001; Doerner, 2008; Hammond and White, 2008; Liu, Allan, and Vance, 2010). Variation in cluster-root formation in the two *Lupinus* species cannot be interpreted by species-specific differences in leaf P concentration or RGR. Since we did not investigate signalling at transcriptional level, we can only speculate on the variations in cluster-root allocation in these *Lupinus* species which we investigated. Cluster-root formation in these two
Lupinus species may involve a complex series of signalling cascades controlling transcription and initiating plant responses to P starvation. The exact mechanism accounting for the difference of signalling in regulating cluster-root formation in Lupinus species under P starvation needs further investigation.

**Cluster-root formation in different Lupinus species**

The present results show variation in cluster-root formation among Lupinus species. However, this variation cannot be accounted for by a greater leaf P concentration, because Lupinus species that produce fewer cluster roots do not invariably show greater leaf P concentrations. In the present study and a previous one (Wang, Pearse, and Lambers, 2013), L. albus invested a considerable fraction of its root biomass in cluster roots, as long as the leaf P status was low. This is similar to the findings for the same species in the literature (Shane et al., 2003; Shen et al., 2003; Pearse et al., 2006; Abdolzadeh et al., 2010). Similarly, L. pilosus in the present and previous studies (Wang, Pearse, and Lambers, 2013) showed a total suppression of root biomass investment in cluster roots at greater plant P concentrations, similar to L. luteus (Pearse et al., 2006).

In the present study, rates of cluster-root formation and carboxylate release were highest at a longer day-length. Longer day-length or higher light intensity will enhance photosynthesis, which, probably leads to carbohydrate signalling and sensing as a systemic control of plant responses to P deficiency. This is in agreement with findings by Yang et al. (2010), who showed that in white lupin P-deficiency-induced cluster-root formation and citrate release are stimulated by high light intensity, presumably due to increased carbohydrate availability.
In summary, variation in cluster-root formation in the two *Lupinus* species cannot be interpreted by species-specific differences in leaf P concentration or RGR. For *L. albus*, cluster-root investment was strongly and significantly suppressed at higher leaf P concentrations. In *L. pilosus*, there was also a strong negative correlation between cluster-root allocation and leaf P; the correlation was weaker than that of *L. albus*. An increased leaf sucrose concentration up-regulates genes encoding transport proteins to transfer organic carbon and sucrose to the phloem which helps the movement of these compounds to the root (Hermans et al., 2006; Hammond and White, 2008). Shoot-derived sugar signals (sucrose, glucose and fructose) control plant P-starvation responses, including cluster-root formation (Liu et al., 2005; Müller et al., 2007; Zhou et al., 2008). Hence the strength of this signal might vary among lupin species. In addition, auxins play a role in cluster-root formation (Gilbert et al., 2000; Skene and James, 2000; Hocking and Jeffery, 2004), and hence there may be differences in the strength of this hormonal signal among the present *Lupinus* species. Possibly, strigolactones, whose production increases under P deficiency (Yoneyama, Takeuchi, and Sekimoto, 2007; López-Ráez et al., 2008; Kohlen et al., 2011), play a role as well in differences in cluster-root formation among *Lupinus* species, given their role in lateral root formation and in the regulation of shoot architecture in response to P deficiency (Kapulnik et al., 2011). Also, as discussed above, microRNAs act as a systemic low-P signal (Zhu et al., 2010), and this signal might also vary among *Lupinus* species. Future research is warranted to characterise species-specific variation in systemic signal compounds to determine possible consequences for variation in cluster-root formation among species as demonstrated in our study.
CONCLUDING REMARKS

Our results demonstrate that cluster-root-forming *L. albus* and *L. pilosus*, grown at a similar P availability in the root growth environment, but at a higher growth rate, as dependent on light availability, showed a greater investment in cluster roots. We conclude that plants with high P status invariably down-regulated cluster-root formation, irrespective of growth rate (Fig. 7). The difference in cluster-root formation between the two *Lupinus* species was strongly and negatively associated with leaf P concentration and only weakly and positively correlated with relative growth rate. Variation in cluster-root investment between the two *Lupinus* species cannot be accounted for a species-specific variation in leaf P concentration or RGR.

ACKNOWLEDGEMENTS

X.W. was supported by the University of Western Australia and the China Scholarship Council for UWA China scholarships and by the School of Plant Biology for project funding. We are grateful to Bevan Buirchell, Richard Snowball and Jon C. Clements for kindly providing the *Lupinus* seeds, to Ray Scott at the UWA Combined Workshop and Rob Creasy and Bill Piasini at the School of Plant Biology’s Plant Growth Facility, to Susan Barker for providing cardboard boxes, to Mabel Fabiola Delgado Torres and Hiroaki Matsuoka for helping with experiments, to Hai Ngo for providing glassware, to Jing Zhang and Yan Liu for assisting with the harvests, to Greg Cawthray for help with HPLC, to Laura Firth, François Teste and Allah Ditta for helpful advice on the statistics, to Joanne Edmondston for her valued help with X.W.’s English of an earlier version of the Materials and Methods of this paper, and also to many friends for their support and encouragement.
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LITERATURE CITED


CHAPTER 4

Cluster-root Formation and Functioning in White Lupin

Grown with a Split-root System as Dependent on

Sugars, Auxin and Internal P Concentration
ABSTRACT

White lupin (*Lupinus albus* L.) is a legume that has been considered a model plant for the investigation of cluster-root formation and its functioning under limited phosphorus (P) conditions. This study investigated if cluster-root formation and carboxylate release of white lupin is controlled systemically or locally by low availability of P. To do so, white lupin plants that had their shoot apex removed, plants that were treated with a synthetic auxin, naphthylphthalamic acid (NAA), and control plants were grown hydroponically in a split-root system, with one root half deprived of P (−P) and the other half supplied with 75 µM P (+P). Fresh and dry mass of leaves, stem, cluster roots and non-cluster roots of plants were recorded. The percentage of cluster roots, leaf P concentrations, leaf sugar concentration and cluster-root exudates were determined. Following removal of the shoot apex, and thus the source of shoot auxin production, the investment of root mass in cluster roots decreased. Removal of the shoot apex led to a doubling in leaf sucrose concentration, but no increase in root sugar concentration, and hence no increase in sugar-induced cluster-root formation was to be expected. Exogenous NAA supplied to the roots resulted in a higher percentage of cluster-roots for both root halves; however, the exudation of carboxylates from cluster roots was only enhanced in the −P roots. It is concluded that cluster-root formation in P-deprived *L. albus* is controlled by a systemic factor. The functioning of cluster roots, *i.e.* carboxylate release, was, however, dependent on a local low-P status of the roots when auxin was supplied. Cluster-root formation in the investigated *L. albus* plants was predominantly regulated by an auxin signal, with no evidence for sugar signalling, because root sugar concentrations were unaffected by the removal of the shoot apex.

Key words: *Lupinus albus*, shoot apex removal, 1-naphthylphthalamic acid, cluster-root exudates, P supply

This experiment has been prepared as a thesis chapter only, at this stage, but it is intended for submission to an international journal when additional experiments have been completed.
INTRODUCTION

With an increasing global demand for phosphate fertilisers for food, feed and fibre production, whilst the world’s reserves of phosphorus (P) are dwindling, we may run out of P resources at the end of this century. Phosphorus may become a more severely factor limiting plant production worldwide (Gilbert, 2009, Marschner, 2012), although probably not as rapidly as predicted previously (Fixen and Johnston, 2012, Scholz and Wellmer, 2013). On the other hand, there is a considerable amount of P stored in many soils; however, this P is in forms that are not readily accessible for most crop plants (Holford, 1997, Simpson et al., 2011).

Cluster roots (a specialised root structure) of Lupinus albus are a desirable trait for crop plants, as they exude large amounts of carboxylates (malate and citrate) to acquire sparingly available P in the rhizosphere (Lambers et al., 2013). Several researchers have reported that cluster-root initiation and inhibition is associated with internal P concentrations in the plants (Shane et al., 2003, Shen et al., 2005). Our recent research findings confirmed that higher shoot P concentration strongly suppresses the investment of root biomass in cluster roots in three Lupinus species, without major effect of the plants’ relative growth rate (Wang et al., 2013, Wang et al., submitted). Several investigators have reported that shoot-derived sugar signals (sucrose, glucose and fructose) control plant P-starvation responses, including cluster-root formation (Liu et al., 2010, Müller et al., 2007, Zhou et al., 2008). Auxins also play a role in cluster-root formation (Gilbert et al., 2000, Skene and James, 2000, Hocking and Jeffery, 2004). Possibly, strigolactones, whose production increases under P deficiency, are also associated with cluster-root formation, given their role in lateral root formation and shoot architecture (Yoneyama et al., 2007, López-Ráez et al., 2008, Kohlen et al.,
MicroRNAs are also considered a systemic low-P signal (Zhu et al., 2010). However, which signal(s), *i.e.* shoot-derived sugars, auxin, strigolactones or a local low P concentration, exactly contribute to plant P-starvation responses, including cluster-root formation and carboxylate release, still needs further investigation. It is therefore of interest to investigate the potential role of sugars, auxin and internal P concentration in the formation of cluster roots and their carboxylate release.

A split-root design/system is frequently used to study systemic effects on symbiotic root nodule production (Gentili and Huss-Danell, 2002), mycorrhizas (Vierheilig et al., 2000), plant water relations (Turner et al., 1996) and cluster-root formation as dependent on shoot P status (Shane et al., 2003, Li et al., 2008). It is a valuable approach to differentiate systemic effects originating in the shoot from local effects on the roots.

After excision of a plant’s shoot apex, the source of shoot auxin production is removed, and thus auxin export from the shoot is reduced; this treatment enhances accumulation of photosynthates (*e.g.*, sucrose) in the shoot, and possibly also enhances photosynthate transport to the root. Some researchers found auxin depletion following excision of the apex did not correlate with bud release (Morris et al., 2005, Renton et al., 2012), while other studies showed that removal of the shoot apex and also exogenous feeding of sucrose to leaf petioles promoted rapid bud release or shoot branching of field pea (Beveridge et al., 2000). Sucrose, a crucial component of the apical dominance system, induces rapid bud release; therefore, it acts as a hormone-like signal (Beveridge et al., 2000). In terms of cluster roots, we expect that there would be more cluster-root production in plants that have their shoot apex removed regardless of P supply in root halves, due to enhanced sugars transported to both of the root halves;
however, we also expect that carboxylate release from cluster roots only happens in the root half receiving no P. After the shoot apex is removed, leaf sugar concentrations of plants are expected to increase and auxin concentrations are expected to decline. That is, the two signals that are involved in cluster-root formation, sugars and auxin, are affected in opposite ways by the removal of the shoot apex. Therefore, we hypothesised that \textit{L. albus} plants with their shoot apex removed, show higher root sugar concentrations and, therefore, produce more cluster roots. Furthermore, the auxin signal might be more important than the sugar signal, leading to the hypothesis that removal of the shoot apex will reduce the percentage of cluster roots. We, therefore, hypothesised that the activity of cluster roots, \textit{i.e.} carboxylate release, is specifically dependent on a local low P concentration in the roots, rather than being systemically controlled.

The objective of this study was to investigate how cluster-root formation and carboxylate release in \textit{L. albus} are regulated by sugars, auxin and the local internal P concentration. We compared intact plants of \textit{L. albus} as well as plants with their shoot apex removed, and also exogenous 1-naphthylphthalamic acid (NAA) plus the control, which were grown hydroponically in split-root pots; with one root half receiving no P and the other half sufficient P. Root exudates from a few individual cluster roots of each root half, sugar concentrations and P concentrations in both root halves and also in leaves were determined to evaluate possible associations with cluster-root formation.
MATERIALS AND METHODS

Growth of plants

Seeds of white lupin (Lupinus albus L. cv. Kiev Mutant) were surface-sterilised with 5 % (v/v) bleach for 20 min and washed five times in deionised water. The seeds were sown into pots filled with washed and sterilised river sand in a glasshouse. After six days, seedlings were carefully removed and the roots washed free of sand. Seedlings of uniform size were selected. The tip of the primary root of each seedling was excised to promote lateral-root formation, leaving a remaining primary root of approximately 3 cm in length. The seedlings were transferred to a hydroponic system consisting of a rectangular 20-L black plastic container, with a lid with 12 round holes. The stem of each of the 12 seedlings was positioned in the centre of the grey-foam discs, creating a light-tight seal. The roots were immersed in continuously aerated nutrient solution of the following composition (µM): 400 NO$_3^-$, 200 Ca$^{2+}$, 210 K$^+$, 154 SO$_4^{2-}$, 54 Mg$^{2+}$, 0.24 Mn$^{2+}$, 0.1 Zn$^{2+}$, 0.018 Cu$^{2+}$, 2.4 H$_3$BO$_3$, 0.03 Mo$^{4+}$ and 10 Fe-EDTA (pH 5.8). No P was present in the nutrient solution.

After 14 days, cotyledons were removed. At this stage, primary roots consisting of two groups of three to four lateral roots, which were approximately five cm in length. The plants were assigned to treatment or control split-root plant cultures. The split-root pots were made from two 3.5 L black pots, fastened together by stainless steel rivets and a V-notch cut through the adjacent walls (Shane et al., 2003). Some photographs (Fig. A1) of plants grown in split-root culture can be found in the Appendix to this chapter.
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**Experiment 1- Shoot apex removal treatment**

Six plants were transferred into the treatment split-root pots by dividing the root mass into two groups and placing each group into one side of the split pots. At day 34, the shoot apex of each plant was removed. Any new lateral buds, which were recognised with the naked eye, were removed. This removes sources of auxin production and reduces auxin export from the shoot; it would also enhance accumulation of photosynthates in the shoot, and possibly enhance transport of assimilates to both root halves. The remaining six plants were transferred to the control treatment split-root pots, separating the roots into each side of the pot as described for the treatment plants. For both plants that had their shoot apex removed and control plants, individual plants were placed in split-root pots, with one root half deprived of P (-P) and the other half supplied with 75 µM P (+P).

**Experiment 2- Auxin (NAA) treatment**

Six plants were transferred into the treatment split-root pots by dividing the root mass into two groups and placing each group into one side of the split pots. Stock solution (0.1 mM) of 1-naphthylphthalamic acid (NAA) was prepared in ethanol and a diluted NAA solution was added to both sides of the split pots at a final NAA concentration of 1*10⁻⁹ M in 0.1% (v/v) of ethanol from day 31 until the harvest. The remaining six plants were control plants with roots separated into each side of the pot in split-root pots as described for the treatment plants. The control plants received 0.1% of ethanol from the same day that the NAA treatment started. For both of the NAA treatment and the control, plants were placed in the split-root pots, with one root half deprived of P and the other half supplied with 75 µM P.
For both Experiment 1 and Experiment 2, the entire nutrient solution (including P) for each root half was replaced daily in the split-root pots to maintain the P concentration in the root medium and also the pH of the nutrient solution. The plants were grown in a temperature-controlled glasshouse, with containers or pots half-immersed in a root-cooling tank maintained at 18–22°C until harvest. During these experiments, the average day/night temperatures were 24/16°C and 27/20°C, the average day-lengths were 11 h and 13.5 h, the average mid-day ambient light levels were approximately 870 and 1480 µmol photons m⁻² s⁻¹, and the average relative humidity was 57% and 48%, for Experiment 1 and Experiment 2, respectively. There were only four replicates for Experiment 1 as the two plants from the total six intended replicates were either about 38% smaller or around 25% larger than the mean plant size; there were only three replicates for the Experiment 2, because for the rest replicates, splitting of the root system into equal root halves was not successful when plants getting older, thus the two root halves were not evenly distributed in each pot of the split-root pots; those plants, therefore, had to be discarded.

Sugar analysis

Leaf and root material (either non-cluster or cluster roots) from Experiment 1 was harvested, weighed (fresh weight recorded) and frozen in liquid nitrogen. Samples were then transferred to a -80 °C freezer and then freeze-dried at -40 °C for 72 h. Freeze-dried samples were weighed and then ground to a powder using a stainless-steel ball-mill grinder. About 0.0500 g of the ground samples was extracted using 80 % (v/v) ethanol with gentle shaking in a hot water (80 °C) bath.

The samples of extracted supernatant were then stored at -80 °C until analysis using HPLC. The supernatants in 15–25 µL injections (depending on the expected
concentration of sugars in the samples) of the extracts were analysed by HPLC (600E pump, 717 plus auto-injector, Waters, Milford MA, USA) with an Alltech Evaporative Light Scattering Detector (ELSD, Grace Materials Technologies, Deerfield, IL, USA) as adapted from Slimestad and Vågen (2006).

Separation was achieved at 30 ± 0.5°C on a Prevail ES Carbohydrate column (250 x 4.6 mm i.d. with 5 µm packing; Grace Materials Technologies) using an isocratic mobile phase consisting of 25% Milli-Q water and 75% acetonitrile at the rate of 1 mL min⁻¹. Samples in the auto-injector were held at 10°C and the ELSD settings were; drift tube held at 80°C and high purity nitrogen flow rate 2.5 L min⁻¹ for nebulisation.

Calibration curves for each sugar were generated from peak area versus the mass of standard sugar injected, and a standard analysed every 10 samples to check for any instrument/detector drift. Data acquisition and processing was with Empower™ 2 (Waters) software. Retention times of sugar standards were used to identify sugars in the supernatants of samples mentioned above. Sugar concentrations were expressed per unit plant dry mass. Some examples of chromatograms of sugars (Fig. A2) from the HPLC performed can be found in the Appendix to this chapter.

Collection of root exudates

Root exudates were collected from individual cluster roots on the day of harvest in the glasshouse. Prior to root-exudate collection, 1 to up to 4 mature cluster roots from each root half of plants were carefully excised and washed briefly in fresh nutrient solution without P (root-exudate collecting solution). A known volume of nutrient solution without P just mentioned was added to the 5 mL tubes with screw lids; the nutrient solution was just covering the clusters. After a 1 h (for one half of all
replicates) or 1.5 h (for the other half of the replicates) collection (from 11:00 am) with gentle shaking at ambient temperature, sub-samples of the collecting solution from tube with cluster roots were taken and filtered through a 0.2 µm syringe filter [13-mm syringe filter with 0.2 µm Supor (PES) membrane] into a 1 mL vial. The solids were re-dissolved in a 1 mL vial containing 90% 25 mM KH$_2$PO$_4$ pH 2.5 with 10% (v/v) methanol. The vial was stored in a -20°C freezer until the carboxylates were analysed using HPLC.

**Carboxylate analysis**

The carboxylates in 100 µL injections of the extracts were analysed by HPLC [600E pump, 717 plus autosampler and 996 photodiode array (PDA) detector, Waters, Milford MA, USA] using an Alltima C$_{18}$ reverse-phase column as described by Cawthray (2003). The mobile phase was 93% 25 mM KH$_2$PO$_4$, adjusted to pH 2.5 with concentrated H$_3$PO$_4$, and 7% (v/v) methanol with a flow rate of 1 mL min$^{-1}$. Carboxylate exudates were expressed per unit cluster roots dry mass. Some examples of chromatograms of carboxylates (Fig. A3) from the HPLC performed can be found in the Appendix to this chapter.

**Plant measurements**

Fresh mass of shoots and roots (clusters and non-clusters) were determined following the harvest. In Experiment 2, cluster roots used in the calculation were those that formed after the application of NAA; these were separated from the old cluster roots observed before the treatment started.

After being freeze-dried at -40°C for 72 h, the dry mass was then determined. The plant parts were ground with a steel ball mill, and approximately 0.0500 g of the
subsamples was digested in a mixture of hot concentrated HNO₃: HClO₄ (3: 1, v/v). The digests were then analysed for total P concentration using the malachite green colourimetric method (Motomizu et al., 1983) by a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

Data were analysed with one-way ANOVA followed by LSD test (α = 0.05) using Statistix 8.1 (Analytical Software, USA). Means are presented with standard errors to indicate the variation of each measurement. Significant differences among means were tested at a significance level of $P \leq 0.05$. 
RESULTS

Experiment 1- Effects of shoot apex removal

Plant biomass

Shoot apex removal of *L. albus* plants resulted a significant decrease in plant biomass compared to that of intact plants, when plants were grown in a split-root system, with each root half receiving either 0 or 75 µM P (Fig. 1). The total plant biomass decreased by 21% in the plants that had their shoot apex removed, compared with the plant biomass in the intact plants.

![Graph showing plant biomass comparison](image)

**Fig. 1.** Total plant dry biomass of *Lupinus albus* for the treatment in which the shoot apex were removed, compared with control plants, harvested at 43 days after sowing in Experiment 1. Plants were harvested at 43 days after sowing, having been grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH₂PO₄). Error bars represent standard errors (n = 4 replicates). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA followed by LSD at $P \leq 0.05$.

For plants without their shoot apex removed, there were no significant effects of P supply on root mass (data not shown). However, biomass allocation to cluster roots in the root half receiving no P of the *L. albus* plants that had their shoot apex removed was
the same as that of the intact plants grown in a split-root system (Fig. 2). The percentage biomass invested in cluster roots of the root half receiving 75 µM P was not significantly different between the intact *L. albus* plants and the plants that had their shoot apex removed (Fig. 2).

**Fig. 2.** Percentage of cluster roots of *Lupinus albus* following shoot apex removal, compared with that in control plants, harvested at 43 days after sowing in Experiment 1. Plants were grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH$_2$PO$_4$). The percentages were calculated as dry mass of cluster roots divided by total root dry mass times 100. Error bars represent standard errors (*n* = 4). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA followed by LSD at *P* ≤ 0.05.

**Sugar concentrations**

The removal of shoot apex significantly and approximately doubled leaf sucrose concentration, compared with that in the intact plants (Fig. 3). By contrast, the leaf fructose and glucose concentrations were similar for both of *L. albus* plants that had their shoot apex removed and intact plants (Fig. 3).

However, the treatment in which the shoot apex was removed did not result in a significant increase in root sucrose concentration, compared with that for the intact
plants (Fig. 3). On the other hand, the plants that had their apex removed had a lower root fructose at 3.7, 2.9 mg g\(^{-1}\), and glucose concentration at 0, 0.2 mg g\(^{-1}\) in both –P and +P root halves, compared with that for the intact plants (Fig. 3).

**Fig. 3.** Sugar concentrations in mature leaves (A) and both root halves (B) of intact *Lupinus albus* plants and of plants with their shoot apex removed, harvested at 43 days after sowing in Experiment 1. Plants were grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH\(_2\)PO\(_4\)). Error bars represent standard errors (\(n = 4\)). Treatment means marked with the same letter are not significantly different within each organ group using a one-way ANOVA followed by LSD at \(P \leq 0.05\).

*Phosphorus concentrations*
There was no significant difference in leaf P concentration between plants with their shoot apex removed and intact *L. albus* plants (Fig. 4A).

![Phosphorus (P) concentration in (A) mature leaves and (B) root halves of intact *Lupinus albus* plants and of plants with their shoot apex removed, harvested at 43 days after sowing in Experiment 1. Plants were grown hydroponically in split-root pots, with one root half receiving no P and the other half 75 µM P (in the form of KH$_2$PO$_4$). Error bars represent standard errors (*n* = 4). Treatment means marked with the same lower-case letter are not significantly different within each organ group using a one-way ANOVA followed by LSD at *P* ≤ 0.05.]

Phosphorus concentration of the P-supplied root halves were approximately four-fold higher than that of the P-deprived root halves of plants that had their shoot apex removed which is similar to that for the intact plants (Fig. 4B). However, root P concentrations in the plants that had their shoot apex removed were not significantly
higher compared with that of the intact plants in either the root half receiving no P or 75 µM P (Fig. 4B).

_Carboxylate exudation_

Rates of citrate exudation by cluster roots from individual root halves of plants that had their shoot apex removed were similar to those of intact plants, irrespective of P supply to the root halves (Fig. 5).

**Fig. 5.** Carboxylate exudation from mature cluster roots of *Lupinus albus* for the treatment, in which plants with their shoot apex removed, compared with the control – intact plants, harvested at 43 days after sowing in Experiment 1. Plants were grown hydroponically in split-root pots, with one root half receiving no P and the other half 75 µM P (in the form of KH₂PO₄). Malate exudation is in white, citrate is in black, and fumarate is in grey; no other carboxylate detectable in the HPLC assay. Error bars represent standard errors (n = 4 replicates). Treatment means marked with the same letter are not significantly different within each organ group using a one-way ANOVA followed by LSD at ⁰P ≤ 0.05.

In the P-deprived root halves, there was no significant difference in the malate exudation rate from cluster roots between the plants that had their shoot apex removed and intact plants (Fig. 5). However, malate exudation by cluster roots from +P root half of plants without shoot apex was significantly less, compared with that from –P root
half and also that from both root halves of intact plants (Fig. 5). There were only very little fumarate released from the cluster roots of each root halves, ranging from approximately 0.1-0.2 µmol g$^{-1}$ h$^{-1}$ (Fig. 5).

**Experiment 2- Effects of auxin (NAA) application**

*Plant biomass*

The synthetic auxin, NAA (1 * 10$^{-9}$ M), supplied to both root halves had no significant effect on plant biomass of *L. albus* plants, compared with that of the control plants (Fig. 6). The total root dry mass was slightly higher in +P root halves than that in –P root halves for both plants with root halves supplied with exogenous NAA and control plants, but that difference was not significant (data not shown).

![Graph showing plant biomass comparison](image)

**Fig. 6.** Total plant dry biomass of *Lupinus albus* for the treatment, in which exogenous auxin was supplied to the roots, compared with control plants in Experiment 2. Plants were harvested at 42 days after sowing, after having been grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH$_2$PO$_4$). Error bars represent standard errors ($n = 3$ replicates). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA followed by LSD at $P \leq 0.05$. 
The exogenous NAA treatment resulted in a significant increase in the percentage of cluster-root formation for both root halves, compared with that of the control plants. The percentage of cluster roots for the –P root halves in the exogenous NAA treatment was 25%, which was 1.8 times higher than that of the control plants (Fig. 7), and the percentage of cluster roots for the +P halves was 9.7%, which was 1.5 times higher than that of the control plants (Fig. 7).

Fig. 7. Percentage of cluster roots of *Lupinus albus* as affected by exogenous supply of NAA to the roots, compared with that in control plants in Experiment 2. The cluster roots used in the calculation were those formed after application of NAA. Plants were harvested at 42 days after sowing. Plants were grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH$_2$PO$_4$). The percentages were calculated as dry mass of cluster roots divided by total root dry mass times 100. Error bars represent standard errors ($n = 3$ replicates). Treatment means marked with the same lower-case letter are not significantly different within each group using a one-way ANOVA followed by LSD at $P \leq 0.05$.

*Phosphorus concentrations*

The exogenous NAA supplied to both root halves of *L. albus* did not result in increases of P concentrations in mature leaves or root halves, compared with control plants (Fig. 8A).
Phosphorus concentrations of the P-supplied root halves was approximately five-fold and six-fold higher than that of the P-deprived root halves in the control plants and the plants with both root halves supplied with NAA (Fig. 8B). However, P concentrations in plants with root halves treated with NAA were not significantly higher than that of control plants in either –P or +P root half (Fig. 8B).

**Fig. 8.** Phosphorus (P) concentration in (A) mature leaves and (B) root halves of *Lupinus albus*, in which exogenous auxin was supplied to the roots, compared with control plants in Experiment 2. Plants were grown hydroponically in split-root pots, with one root half receiving no P and the other half 75 µM P (in the form of KH₂PO₄). Error bars represent standard errors (n = 3 replicates). Treatment means marked with the same lower-case letter are not significantly different within each organ group using a one-way ANOVA followed by LSD at $P \leq 0.05$. 


Carboxylate exudation

Citrate exudation rates by the most recently matured cluster roots from an individual root half of plants with roots supplied with NAA were not significantly different from that of the control plants, irrespective of P supply to the root halves (Fig. 9). However, the rate of malate exudation from cluster roots in the P-deprived root halves of the plants with root halves exposed to NAA was significantly higher than that of the control plants (Fig. 9). There was no difference in malate exudation from the +P root halves between treated and control plants. In terms of fumarate, the exudation rates were very low, ranging from 0.3-0.7 µmol g⁻¹ h⁻¹, compared with those of citrate and malate for both the treatment that had their root halves supplied with NAA and the control plants (Fig. 9).

Fig. 9. Carboxylate exudation from mature cluster roots of *Lupinus albus* as affected by an exogenous supply of NAA to the roots, compared with that in control plants in Experiment 2. Plants were grown hydroponically in split-root pots, with one root half receiving no P and the other half 75 µM P (in the form of KH₂PO₄). Error bars represent standard errors (n = 3 replicates). Treatment means marked with the same letter are not significantly different within each organ group using a one-way ANOVA followed by LSD at $P \leq 0.05$. 

![Graph showing carboxylate exudation](image-url)
DISCUSSION

The objective of the present study was to investigate how cluster-root formation and carboxylate release in *L. albus* are regulated by sugars, auxin and the local internal P concentration. The results indicate that the activity of cluster roots, *i.e.* carboxylate exudation, is specifically dependent on a low P concentration in the roots, rather than being systemically controlled.

*Combined effect of a local low P signal and an auxin signal on cluster-root formation and malate exudation*

Exogenous application of 1-naphthylphthalamic acid (NAA) to the both root halves of *L. albus* plants, which were grown hydroponically in split-root pots, with one root half receiving no P and the other half sufficient P, had significant and positive effects on the percentage of cluster roots in both root halves (Fig. 7). However, the activity of cluster roots, *i.e.* the exudation of malate was enhanced only when the root half was exposed to no P together with exogenous NAA (Fig. 9). This finding demonstrates that *L. albus* produced more cluster roots and then exuded more malate in the presence of both an auxin signal and a local low-P signal in the root growth medium, given no significant differences in leaf P concentrations between treatments and controls for both two experiments (Fig. 8).

*Effect of shoot apex removal on cluster-root formation*

Shoot apex removal enhanced sugar concentration in leaves but not in the root halves, irrespective of different P supply in different root halves (Fig. 3). Previously, exogenous sucrose has been found to induce cluster-root formation and also enhance the expression of genes encoding a phosphate transporter (*LaPT1*) and an acid phosphatases
(LaSAPI) (Liu et al., 2005, Müller et al., 2007, Zhou et al., 2008); as a result, sucrose has been considered as a systemic signal in long-distance signalling under P starvation (Hammond and White, 2008, Yang and Finnegan, 2010, Hammond and White, 2011). However, shoot apex removal resulted in a decrease in investment of root mass in cluster roots, because there was a significant decrease in the percentage of cluster roots for the +P root halves of the plants that had their apex removed, compared to that of other root halves (Fig. 2). Since there was no increase in sugar concentration in the roots upon removal of the shoot apex, no increase in cluster roots was to be expected (Fig. 2); the observed decrease was likely due to less auxin exported from the shoot to the roots. Below, we explore a possible role of auxin in the induction of cluster-root formation in \textit{L. albus} grown in a split-root system.

\textit{Effect of root auxin application on cluster-root formation}

Exogenous NAA application to both root halves resulted in a significant increase in the percentage of cluster roots in both root halves, especially in the –P half (Fig. 7). The present results agree with the findings that auxin application leads to the initiation and induction of cluster roots at P-limited conditions (Gilbert et al., 2000, Skene and James, 2000). In addition, the results of the present study confirm the findings that the percentage of cluster roots of the control \textit{L. albus} plants with the root half deprived of P was significantly higher than that of the other half supplied with 75 μM P (Shane et al., 2003).
Effect of shoot apex removal and root auxin application on the functioning of cluster roots

The removal of the shoot apex did not result in enhanced carboxylate release irrespective of local P supply; instead, it caused lower carboxylate-exudation rates, compared with those of intact plants for +P root half (Fig. 5).

The present results show that exogenous NAA induced cluster-root formation (Fig. 6), as expected (Gilbert et al., 2000, Skene and James, 2000, Meng et al., 2012). Interestingly, in the present study, rates of carboxylate exudation from mature cluster roots were only enhanced for the –P root halves exposed to NAA (Fig. 9). The percentage of cluster roots increased with application of NAA in both root halves, compared with that of control plants (Fig. 7). However, the rate of carboxylate exudation by cluster roots was only enhanced when both root halves were treated with NAA where the local P supply was low (Fig. 9).

In conclusion, the results of this study indicate that local low-P signalling was stronger than systemic auxin signalling for controlling malate exudation from cluster roots when plants were grown in a split-root system.

Concluding remarks

When *L. albus* was grown in split-root pots, cluster-root investment in the root half receiving no P was not significantly different between intact control plants and the plants that had their shoot apex removed; however, the percentage of cluster roots of the root half receiving 75 µM P in intact plants was slightly but significantly higher than that of the plants that had their shoot apex removed. The plants had higher leaf sugar
concentration following removal of the shoot apex and any buds that were produced subsequently. After both root halves were supplied with the synthetic auxin, naphthalene acetic acid, cluster-root production increased with the application of auxin in both root halves, irrespective of P supply and P concentration. The two experiments indicate that cluster-root formation in P-deprived *Lupinus albus* is controlled by a systemic factor. However, the functioning of cluster roots, *i.e.* carboxylate release, was dependent on a local low-P status of the roots. Without auxin, there were no significant effects of P supply on cluster-root formation and carboxylate release. Future research is warranted on the effect of exogenous feeding of sucrose to leaf petioles, and also adding auxin to the leaf on the formation and functioning of cluster roots.

ACKNOWLEDGMENTS

X.W. was supported by the University of Western Australia and the China Scholarship Council for UWA China scholarships and by the School of Plant Biology for project funding. We are grateful to Jon C. Clements for kindly providing the *Lupinus* seeds, to David Strack for being a great help with the experiments, to Ray Scott at the former UWA Combined Workshop and Rob Creasy and Bill Piasini at the School of Plant Biology’s Plant Growth Facility, to Patrick Hayes for kind help with sugar extraction, to Greg Cawthray for analyses of sugars and carboxylates, and also to many friends for their support and encouragement.

LITERATURE CITED

**Beveridge CA, Symons GM, Turnbull CGN. 2000.** Auxin inhibition of decapitation-induced branching is dependent on graft-transmissible signals regulated by genes *rms1* and *rms2*. *Plant Physiology, 123*: 689-697.


Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. *Plant and Soil*, **349**: 89-120.


FIG. A1. Two photographs of *Lupinus albus* in the split-root culture in Experiment 1. Plants were grown hydroponically in split-root pots, with one root half receiving no phosphorus (P) and the other half 75 µM P (in the form of KH$_2$PO$_4$).
Fig. A2. Sugar chromatograms performed by reversed-phase (RP)-high-performance liquid chromatography (HPLC) with (A) 1000 mg L$^{-1}$ mixed standard, (B) mature leaf of intact *Lupinus albus* and (C) mature leaf *L. albus* plants, which had their apex removed (Experiment 1). Separation of sugars performed by HPLC was achieved at 30 ± 0.5°C on a Prevail ES Carbohydrate column using an isocratic mobile phase with 25% Milli-Q water and 75% acetonitrile, 1 mL min$^{-1}$; an Alltech Evaporative Light Scattering Detector (ELSD) detection at 210 nm.
Fig. A3. A chromatogram of carboxylates exuded from mature cluster roots of *Lupinus albus*, where one root half receiving 75 µM P (in the form of KH$_2$PO$_4$) and both root halves receiving an exogenous supply of 1-naphthylphthalamic acid - NAA (Experiment 2). Separation of carboxylates performed by reversed-phase (RP)-high-performance liquid chromatography (HPLC) with 93% 25 mM KH$_2$PO$_4$ pH 2.5 with 7% methanol, 1 mL min$^{-1}$; PDA detection at 210 nm.
CHAPTER 5

General Discussion and Conclusion
5.1 Key research findings and implications

5.1.1 Key research findings

Much research has been conducted on investigating mechanisms and strategies for plant adaptations to a limited phosphorus (P) availability (Gardner and Parbery, 1982, Vance et al., 2003, Zinn et al., 2009, Lambers et al., 2012, Lambers et al., 2013). Cluster-root formation combined with its functioning in carboxylate release is considered one of the most effective strategies to access poorly available soil P sources when P availability is extremely low (Lambers et al., 2008b). Researchers have explored factors regulating cluster-root formation in white lupin (Keerthisinghe et al., 1998, Watt and Evans, 1999, Gilbert et al., 2000, Shen et al., 2005, Zhou et al., 2008) and other cluster-root-bearing species (Shane et al., 2003a, Hocking and Jeffery, 2004, Zúñiga-Feest et al., 2010, Lambers et al., 2012, Delgado et al., 2013). However, whether the variation in biomass allocation to cluster roots among *Lupinus* species is an environmentally or genetically regulated process is unknown. An understanding of these special traits to increase P acquisition could also lead to enhanced long-term agricultural sustainability. This thesis focused on the potential benefits of cluster roots to sustainable agricultural production. The aim of this thesis was to investigate the relationship between cluster-root formation, plant P status, medium P concentration and plant relative growth rate.

The major research findings of this thesis are:

1. Phosphorus treatments induced species-specific variation in cluster-root formation in three *Lupinus* species. A significant but incomplete suppression of the percentage of cluster roots in *L. albus* and *L. pilosus* was observed when P supply exceeded 15 mg P kg\(^{-1}\) sand. Complete suppression was found in *L.
atlanticus at the highest P supply; this species never invested more than 20% of its root weight in cluster roots. Based on multiple regressions, relating cluster-root formation to both leaf P concentration and relative growth rate (RGR), it was concluded that cluster-root investment varied in different Lupinus species as dependent of leaf P concentration, irrespective of RGR.

2. Day-length treatments induced differences in RGR. Slower-growing plants grown at a shorter day-length showed reduced biomass allocation to cluster roots in all three Lupinus species. Multiple regression analysis showed that the percentage of cluster roots was strongly correlated with plant P status, and not with plant RGR for both L. albus and L. pilosus.

3. Variation in cluster-root formation among these Lupinus species cannot be explained by species-specific variation in RGR or in leaf P concentration.

4. Removal of the shoot apex of Lupinus albus grown with a split-root system led to a doubling in leaf sucrose concentration, without a change in root sugar concentration, and hence no increase in sugar-induced cluster-root formation was to be expected.

5. Exogenous supply of a synthetic auxin, 1-naphthalene acetic acid (NAA), to the roots resulted in a higher percentage of cluster-roots for both root halves; however, the exudation of carboxylates from cluster roots was only enhanced in the –P root halves.
6. Cluster-root formation in the investigated *L. albus* plants was predominantly regulated by an auxin signal, with no evidence for sugar signalling, as sugar concentrations in the roots were unaffected by the removal of the shoot apex; carboxylate release was independent of auxin, but depended on a local low-P signal.

5.1.2 Research implications

Variation among the present lupin species in cluster-root formation in my study might indicate that there are different strategies for cluster-root-forming *Lupinus* species. There are, indeed, different strategies among *Lupinus* species under P limitation: a higher investment in root biomass to cluster roots with a limited relatively slow rate of carboxylate release occurs in *L. albus*; conversely a limited investment in cluster roots but with abundant carboxylate exudation has been found in *L. cosentinii* (Pearse et al., 2007).

We have found that *L. albus* invested a considerable fraction of its root biomass in cluster roots, as long as the leaf P status was low. This is similar to the findings for the same species in the literature (Shane et al., 2003b, Shen et al., 2003, Pearse et al., 2006a, Abdolzadeh et al., 2010). This is in agreement with previous findings for other *Lupinus* species. For example, *L. cosentinii* produces fewer cluster roots than some other *Lupinus* species do; however, it can accumulate higher level of carboxylates in its rhizosphere (Pearse et al., 2006b, Pearse et al., 2007). For *L. angustifolius*, which produces no cluster roots, it is controversial, with Römer et al. (2000) reporting that it releases more carboxylates than *L. albus* when no P is supplied; however, other authors observed that it exudes less carboxylates (Bolland, 1997, Hocking and Jeffery, 2004).
Interestingly, *L. atlanticus*, *L. cosentinii* and *L. angustifolius* in the genus *Lupinus* (Leguminosae) showed similar results to the findings of Delgado et al. (2014) on *Embothrium coccineum*; under low-P conditions, this South American Proteaceae species, produces less cluster roots, but releases relatively greater amounts of carboxylates per unit cluster-root mass, compared with south-western Australian Proteaceae species, which produce far more cluster roots with relatively less release of carboxylates. *Embothrium coccineum*, naturally grows in young, P-rich volcanic soils, which have a low P availability. Its strategy of less investment of carbon in cluster-root growth and greater investment in cluster-root functioning would appear optimal to release P effectively when there is a large store of strongly sorbed P. Conversely, Proteaceae species that thrive on ancient, P-impoverished south-western Australian soils, produce more cluster roots but release less carboxylates (Shane et al., 2004), compared with *E. coccineum*. This strategy would appear optimal to release P effectively, when there is low total P that is only weakly sorbed. Therefore, this indicates that differences in cluster-root formation and functioning among Proteaceae species are related to soil P status in the contrasting environments. The same contrasting strategies may operate in different *Lupinus* species, referred to above, and this may also be related to their capacity to maximise P uptake from contrasting soils.

Taken together, there might be different strategies among cluster-root forming *Lupinus* species, similar to what has been discovered among Proteaceae species. One strategy is to produce more cluster roots but not release as many carboxylates; the other strategy is to produce less cluster roots but release more carboxylates per unit cluster mass. Carboxylate exudation from cluster roots is therefore not only important for plants naturally occurring on P-impoverished soils, but also when soils contain large amounts of P, of which most is poorly available for plants lacking specialised P-mining
roots (Lambers et al. 2012). There is probably a trade-off between carbon investment in cluster roots and carbon use for production of carboxylates, as dependent on soil P conditions. The optimal strategy is the one that mobilises the greatest amount of P for the smallest investment of carbon. That optimal strategy depends on soil P status. In P-impoverished soils, adapted plants invest more root biomass in cluster roots, in order to explore and take up the small amount of P from low-P soils, and conserve carbon by not releasing more carboxylates than required to mobilise the small amount of P from the soil in which they occur naturally.

5.2 Limitations of the Thesis and Future Research Directions

5.2.1 Key Limitations of the Thesis

1. Limited harvest times in Chapter 2: Growing plants over a relatively long period of time means that a plant’s relative growth rate is not constant, but declining over time (Poorter and Remkes, 1990, Lambers et al., 2008a). It would be better to have more harvests (5 harvests in total, i.e. day 14, 21, 28, 35, 42 and 49 after sowing) over 50 days in order to reveal ontogenetic drift of the plant’s relative growth rate, from faster-growing at an period to slower-growing at a later period.

2. Lack of sugar analysis for the day-length experiment in Chapter 3: I did not measure shoot and root sugar concentrations in this day-length experiment. It would be good to test root sugar concentrations under different day-length treatments, in order to explore possible sugar signalling at a transcriptional level leading to initiation of plant responses to P starvation.
3. No developmental or time-course study of cluster roots: I did not monitor the developmental stages of cluster roots during the growth of plants. As cluster roots are short-lived, the mass of cluster roots at harvests cannot fully represent changes between each harvest. It would be great to record the exact stages of cluster roots in a future study.

4. Some problems in plant sizes or unevenly distributed root halves of the split-root pot were encountered in Chapter 4, as splitting of the root system into equal root halves was not successful for all intended replicates, and some had to be discarded. It would be good to have more plants and also keep a closer eye on the roots during plant growth for better splitting or distributing of the roots.

5. Due to time constraints, I did not consider the role of all the potential signalling mechanisms that could be involved in cluster-root formation in lupins in response to P-deficiency. The thesis examined local P signals and auxin signals and some aspects of sugar signals on cluster-root formation and functioning, but not signals of strigolactones or microRNAs. I also did not include molecular aspects study of mechanisms involved in regulating cluster-root formation and functioning.

5.2.2 Future Research Directions

Lupins are of enormous potential to be developed or further enhanced as low-input crop and pasture species to improve the soil for later grain crops or planted trees (Lambers et al., 2013). Thus, they could have a much greater role in food and economic sustainability in low-input agroecosystem such as in Africa (Yeheyis et al., 2011, Yeheyis et al., 2012). Improved understanding of cluster-root traits in P acquisition can enhance new cultivar breeding programs in the future. These breeding programs should
utilise genotypic variation in traits of cluster roots and their functioning likely to improve P acquisition. Further investigations on the ability of some lupin species (such as *L. cosentinii*) to thrive on extremely nutrient-impoverished soils would be rewarding (Lambers et al., 2013).

It would be of interest to study in the future traits of cluster roots in *Lupinus* species to mine limited P and access also relatively unavailable organic P (Richardson et al., 2011, Lambers et al., 2013). *Lupinus* species have evolved on young volcanic soils with small reserves of P, e.g., Arenosols with low P availability, and also on the world’s most ancient and P-impoverished soils in south-western Australian. On young volcanic soils, with large reserves of P sorbed onto Fe$^{3+}$ and Al$^{3+}$ oxides and hydroxides, *Lupinus* species are likely ideal crops to target for liberating P in order to achieve a high yield, e.g. in Chile and West Africa (Baer et al., 2006, Gweyi-Onyango et al., 2010).

In addition, future research will be required to study the differences among the lupin species in cluster-root formation, in particular, the potential roles in carboxylate release in those *Lupinus* species that produce less cluster roots and also in those that do not produce cluster roots. The optimal strategy is the one that mobilises the greatest amount of P for the smallest investment of carbon. That optimal strategy depends on soil P status. Possible modelling studies are warranted in further investigations on this optimal strategy among *Lupinus* species as dependent on soil conditions.

The cause of variation in cluster-root formation among *Lupinus* species observed in the present study also warrants further investigation. More research on characterising the relationship between plant P status and signal compounds, such as auxins, sugars, strigolactones and also mRNA is needed. Auxins play a role in cluster-root formation
(Skene and James, 2000, Hocking and Jeffery, 2004, Donoso-Ñanculao et al., 2013), and hence there may be differences in the strength of this hormonal signal among the present *Lupinus* species. Shoot-derived sugar signals (sucrose, glucose and fructose) control plant P-starvation responses, including cluster-root formation (Liu et al., 2010, Müller et al., 2007, Zhou et al., 2008), hence the strength of this signal might vary among lupin species. Strigolactones, whose production increases under P deficiency (Yoneyama et al., 2007, López-Ráez et al., 2008, Kohlen et al., 2011), may play a role as well in differences in cluster-root formation among *Lupinus* species, given their role in lateral root formation and in the regulation of shoot architecture in response to P deficiency (Kapulnik et al., 2011). MicroRNAs act as a systemic low-P signal (Zhu et al., 2010), and this signal might also vary among *Lupinus* species. The balance or cross-talk between these signalling compounds need further intensive study in order to determine the exact mechanism and regulation on cluster-root formation and functioning. These also allow us to determine potential differences among lupin species. An understanding of the variation in cluster-root formation and carboxylate release among *Lupinus* species could be used in future selections of genotypes to better inform future breeding programs towards improved agricultural sustainability.

### 5.3 Conclusion

By combining insights from reports in the literature and the model presented in my published experiment, I have introduced a conceptual model of the impacts of genotype and environment on signalling pathways involved in cluster-root formation and function in lupins in low-P conditions which identifies targets for future breeding programs for enhanced P acquisition (Fig. 1).
FIG. 1. Conceptual model of the impacts of genotype and environment on signalling pathways involved in cluster-root formation and function in lupins. (A) Interaction between cluster-root formation, plant phosphorus (P)-uptake, shoot P status and relative growth rate (RGR). Positive effects are depicted as solid black lines, negative effects as solid light grey lines. Cluster roots increase P-uptake rate, which stimulates plant growth and increases plant P status. Phosphorus treatments caused a significant but incomplete suppression in *L. albus* and *L. pilosus* when P was supplied at a moderate level, but complete suppression was found in *L. atlanticus*. *Lupinus* species grown at a similar P availability in the root medium, but with a higher growth rate, as dependent on day-length showed a greater investment in cluster roots. Contrary to our expectation, cluster-root investment did not correlate with relative growth rate. (B) The combined positive effects of regulators such as a local low P signal and an auxin signal on cluster-root formation and malate exudation in cluster-rooted *L. albus*. Intact plants, plants with exogenous auxin supplied to both root halves, and plants that had their apex removed were grown hydroponically in split-root systems with one root half receiving no P and the other half 75 µM P (in the form of KH₂PO₄).
Cluster-root production is suppressed at high leaf P concentration, but only marginally depends on RGRs in *Lupinus* species. Species-specific variation in RGR or leaf P concentration cannot account for variation in cluster-root formation among *Lupinus* species. Split-root experiments demonstrated that cluster-root formation was predominantly regulated by an auxin signal and carboxylate release by the local P concentration. The combined positive effects of regulators such as a local low-P signal and an auxin signal on cluster-root formation and malate exudation in cluster-rooted *L. albus* provide new insight into further understanding of the ecology of lupins. In addition, these findings could be useful in future plant breeding for increased P acquisition, *i.e.* common non-cluster-root-forming plants may benefit from increased cluster roots and their root exudates. Our results demonstrate that in environments with a low P availability, exogenous auxin application would boost cluster-root formation; however, cluster-root functioning is dependent on a local P signal, rather than auxin. When supplies of quality rock phosphate for P-fertiliser production are dwindling, cluster-rooted species in combination with the functioning of cluster roots, *i.e.* carboxylate release, can increase acquisition of P and therefore improve future agricultural production and sustainability.
LITERATURE CITED


APPENDIX

The Alternative Respiratory Pathway Mediates Carboxylate Synthesis in White Lupin Cluster Roots under Phosphorus Deprivation

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