Root growth of cluster-root-forming and mycorrhizal legumes in soils with heterogeneous P distribution

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Root growth of cluster-root-forming and mycorrhizal legumes in soils with heterogeneous P distribution

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Summary

Plant growth on soil in the beginning stages of pedogenesis is often limited by low availability of nutrients. Among the plants adapted to colonize such soils are many legumes. Legumes have the advantage that they do not depend on soil nitrogen. Instead, phosphorus (P) is usually the most limiting nutrient element for their growth. Like other nutrients and water, also P is in general distributed quite unevenly in soil. Many plants respond to this heterogeneity by preferential proliferation of roots into soil patches that are enriched in soluble P. Little is known how the presence of mycorrhizae affects this response, although it is known that they can be vital for the acquisition of P by plants in low-P soils. Other plants acquire tightly bound P from nutrient-poor and usually dry soils through special root structures called ‘cluster roots’ because of their bottle-brush like appearance. While some authors reported responses of cluster root growth to locally increased P exposure, it is not known how this response is affected by the distribution of soil moisture, which has been suspected to be an important factor in stimulating cluster root production.

The objective of this study was i) to test the propensity of Lotus corniculatus, a legume spontaneously colonizing soils at early stages of vegetation development, for preferential root allocation into P-enriched soil patches, ii) to investigate the effect of mycorrhizae on preferential root allocation in Lotus, and iii) to study cluster root allocation in soil with heterogeneous water and P distribution using Lupinus albus as test plant. The study was part of a multi-disciplinary research project investigating structures and processes governing geomorphological and vegetation pattern formation in the initial stages of ecosystem development.

To test the limitation of P for the growth of L. corniculatus and its propensity to preferentially grow into P-enriched soil patches we conducted an ingrowth core field experiment and a
climate chamber experiment with constructed soil P heterogeneity. In addition we conducted high-density samplings on undisturbed soil plots colonized by *L. corniculatus* on two recently restored sites: the artificial water catchment “Chicken Creek” (CCC) and a nearby experimental site (ES). In the field experiment, roots showed preferential growth into the P-fertilized ingrowth cores. Preferential root allocation was also found in the climate chamber experiment, where single *L. corniculatus* plants were grown in containers filled with ES soil and where a lateral portion of the containers was additionally supplied with a range of different P concentrations. In the high-density samplings, we excavated soil-cubes of 10x10x10 cm size from the topsoil of 3 mini-plot areas (50 x 50 cm) each on the ES and the CCC on which *L. corniculatus* had been planted (ES) or occurred spontaneously (CCC) and for each cube separated the soil attached to the roots (root-adjacent soil) from the remaining soil (root-distant soil). Root length density was negatively correlated with labile P (resin-extractable P) in the root-distant soil of the CCC plots and with water-soluble P in the root-distant soil of the ES plots. The results suggest that P depletion by root uptake during plant growth soon overrode the effect of preferential root allocation in the relationship between root density and plant-available soil P.

In the next study we used *Lotus japonicus* because of the greater genetic homogeneity of the available material instead of *L. corniculatus* to investigate the effect of mycorrhiza on root allocation patterns in response to heterogeneous soil P distribution. In a climate chamber experiment the following four P treatments were applied to gamma-sterilized low-P sandy soil: no additional P (control), homogeneous application of 28 mg P/pot, or application of either 9 mg or 28 mg P/pot applied to only one lateral third of the respective container. Each P treatment was combined with one of the following three AMF treatments: no mycorrhizae, *Glomus intraradices* (G. intra), indigenous mycorrhizal fungi (ind. AMF). Quantitative real-time PCR was used to assess the abundance of *G. intraradices* in the G. intra treatment and of
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G. mosseae and G. claroideum in the ind. AMF treatment. Both, mycorrhization and P fertilization strongly increased plant growth. Preferential root allocation into P-rich soil was found in absence but not in presence of mycorrhizal fungi. The two mycorrhizal treatments resulted in similar growth enhancement in the homogenous P treatments, while the indigenous mycorrhizal fungi had a stronger effect on growth than G. intraradices alone in the heterogeneous treatments. No clear evidence was found for preferential allocation of intraradical and extraradical hyphae in response to heterogeneous soil P distribution.

To address the third objective we performed another climate chamber experiment with constructed soil heterogeneities, in which we investigated the combined influence of spatial heterogeneity in soil water and P distribution on cluster root allocation by Lupinus albus. Single plants were grown for 35 days at a low or a high rate of water supply in containers filled with a P-poor sand to which either no P was added or which was fertilized homogeneously or heterogeneously (lateral section only) with the same total amount of P. In addition, heterogeneous distribution of water availability was established in half of the containers by using fine-grained instead of coarse-grained sand as substrate in a lateral third of the containers (opposite to the P-fertilized lateral section in the case of the treatments with heterogenous P). Water availability was limiting plant growth at the low rate of water supply, while the rate of P supply had no influence on plant growth. There was also no response in the allocation of total and clustered root length to heterogeneous P application. However, cluster roots were preferentially allocated in the sections with coarse sand in the treatments with low water supply, where soil water availability was lower in the coarse than in the fine sand. Total cluster root production was the same as in the treatments with homogeneous water distribution though, and only affected by initial P exposure of the developing root system. The results suggest that resource allocation to cluster root growth was a systemic response to initial plant P status, while the spatial allocation of cluster root growth was governed by the
distribution of available water, being stimulated in drier patches of soil when roots had access
to water at the opposite side of the containers.

In conclusion, the expected response of preferential root and cluster root allocation by the test
plants to P-enriched patches occurred under some conditions, but was not found at all under
other conditions. This unexpectedly high degree of plasticity in root growth response to soil
nutrient availability is likely to have important implications in the competition between plant
species for soil resources and must be considered when vegetation succession is investigated.
Zusammenfassung


In einer weiteren Studie verwendeten wir *L. japonicus* als Experimentalpflanze, weil das Saatgut von *L. corniculatus* zu heterogen war. Im Klimakammerversuch untersuchten wir die Auswirkung der Kolonisierung mit AMF auf die Wurzelallokationsstrategie von *L. japonicus*, die als Einzelpflanzen im Topf gezogen wurden. Zu diesem Zweck verwendeten


Zusammenfassend können wir sagen, dass die bevorzugte Allokation von Wurzeln in mit P angereicherten Bodenbereichen unter bestimmten Bedingungen stattfand, unter anderen Bedingungen aber nicht. Diese unerwartet hohe Plastizität der Wurzelallokation als Antwort auf unterschiedliche Nährstoffbedingungen hat wahrscheinlich wichtige Auswirkungen auf den Wettbewerb um Wasser und Nährstoffe im Boden zwischen Pflanzenarten und beeinflusst damit auch die Vegetationsstruktur.
1. Introduction

1.1. Background

1.1.1. Initial phase ecosystem development

Understanding the mechanisms of ecosystem development is crucial to assess the effects of changing environmental conditions, resulting from climate change, atmospheric deposition, land use change and other factors (Arndt, 2006; Emmerson et al., 2005; Gerwin et al., 2009; van der Putten et al., 2004). In this context, the Transregional Collaborative Research Centre (SFB/TRR 38) “Structures and processes of the initial ecosystem development phase in an artificial water catchment” (http://www.tu-cottbus.de/sfb_trr) addressed the question which mechanisms and processes govern the initial stages of soil and ecosystem development when the original soil and vegetation are completely removed due to natural (e.g. glaciation), or human (e.g. open-cast mining) impacts (Gerwin et al., 2009). The central hypothesis of the SFB was that initial patterns define and shape the development and later stages of an ecosystem. As a joint research site the artificial water catchment “Chicken Creek” was established in the Lusatian mining area near Cottbus.

The Chicken Creek Catchment (CCC) was constructed with quarternary calcareous sand from Saale-time Pleistocene deposits on a refilled open cast lignite mine about 30 km south of the city of Cottbus in the State of Brandenburg, Germany. A detailed description can be found in Gerwin et al. (2009). After construction was finished in September 2005, the site was left to re-vegetate spontaneously. In order to enable also manipulative and invasive field experiments with soil and plants under comparable conditions an “Experimental Site” (ES) was established in 2009 in the vicinity of the CCC using substrate of the same origin.
1.1.2. *Interactions between root allocation patterns and soil heterogeneity*

Plant roots interact in many ways with processes and structures shaping the development of an ecosystem. Roots absorb nutrients and water from the soil, introduce organic carbon and nitrogen, promote weathering and contribute in various ways to soil structure formation, thus strongly influencing soil properties and related biotic and abiotic processes (Huetsch *et al.*, 2002). On the other hand, biomass production and fitness of plants depend on resource availability (nutrient and water) and resource distribution in the soil. The distribution of resources is often quite heterogeneous in soils, not only at large scales but also at very small scales within the domain of a single root system (Farley and Fitter, 1999b; Gallardo and Parama, 2007; Gross *et al.*, 1995; Jackson and Caldwell, 1993). Many plants were found to show a preference to allocate their roots into soil patches with increased availability of growth-limiting resources such as water and nutrients compared to the surrounding soil (Hodge, 2010; Robinson, 1994).

1.1.3. *Initial ecosystems, P availability and N fixing legumes*

Limitations in the availability of soil nitrogen (N) and phosphorus (P) are frequent conditions during the early phases of ecosystem development (Vitousek *et al.*, 2010). In particular soil N concentrations are usually very low in undeveloped soil. Before soil organic matter has accumulated the main sources of N are biological N fixation and atmospheric deposition. Limitations in P supply are often not due to low total soil P concentrations but to low availability because of strong binding in solid phases of low solubility, particularly in unweathered and alkaline sediments. In the absence of fertilization, mineral weathering usually is the only relevant source of P at this stage, as long as there is no major release of soluble P from the decomposition of organic matter.
Pioneering legumes overcome the N limitation of young soils by symbiosis with N fixing rhizobia and are therefore often among the first plant species occurring on such soils. *Lotus corniculatus* for example, is spontaneously colonizing many of the restored sites on the open cast mining areas in Lusatia. In the same region other legumes (*Lupinus* species) are frequently used for soil remediation. The P requirements of legumes are in many cases higher than for other plants species and P is therefore often the most limiting nutrient for plant growth in unweathered soils (Sprent *et al.*, 1988)

**1.2. P nutrition of plants**

Phosphorus requirements of plants are high and besides nitrogen it is the most important nutritional element in plant tissue. It functions as a structural element in DNA (nucleic acids) and membranes (phospholipids), plays an important role as an energy carrier (ATP) and is involved in all major biosynthetic pathways as well in carbon partitioning between the vacuole and the cytosol (Marschner, 2012).

Dissolved inorganic P (specifically $\text{H}_2\text{PO}_4^-$) in soil solution is the only form of P directly available for uptake by roots (Hinsinger, 2001). It represents only about 0.1 % of total soil P (Blume *et al.*, 2012). Most P is present as orthophosphate in inorganic or organic compounds. In humic horizons organic P fractions can substantially contribute to dissolved inorganic P fractions through decomposition by microorganisms. In soils with low organic substance most of the P occurs as in form of minerals associated with Al, Fe and Ca, such as Al-phosphate, Ca-phosphate or Fe-phosphate. The majority of these minerals are highly insoluble, and only a small fraction of plant available P is bound in form of readily soluble precipitates or adsorbed by Fe, Al or Mn oxides. At moderately low pH Fe, Mn and Al phosphates control P concentrations in soil solution, while at higher pH Ca-phosphates control P concentrations in soil solution.
The uptake of P ions from soil solution is usually higher than the re-supply from the bulk soil to the roots with the water stream driven by transpiration (mass flow) (Hinsinger et al., 2011). As a consequence a few mm large P depletion zone arises around the roots. The resulting P gradients drive diffusive fluxes towards the roots, and these fluxes are considered the dominant process of P transfer to the roots.

1.3. Root adaptations for P uptake

Plants can overcome the limitation of P supply by increasing their absorptive surface or the solubility of P. Indeed, under P deficient conditions plants increase their root surface by allocating more resources to the roots and enhancing the root shoot ratio or by growing more and longer root hairs (Lambers et al., 2006). The solubility of P is often increased by enhanced exudation of organic acids and protons especially at the root tips (Hinsinger, 2001). Two important root adaptations have brought these strategies to its extreme, namely arbuscular mycorrhizal fungi (AMF) and cluster roots. While AMF increase the surface area for P absorption, cluster roots are specialized for soil P solubilization.

1.3.1. Arbuscular mycorrhizal fungi

Eighty percent of plant species form associations AMF (Smith, 2008). The association consists of the fungal partner transferring P taken up from soil via extraradical hyphae to the plant roots and in turn receiving assimilates from the plant. Assimilates from the plant are the only carbon sources for AMF, while P from soil can be uptaken by the roots or the fungus. From mycorrhizal roots extraradical hyphae grow up to 10 cm away from the root surface and allow the plants to acquire P from areas beyond the depletion zone in the rhizosphere (Jansa et al., 2005). AMF increase the absorptive area of roots and therefore improve the P supply of plants (Smith, 2008). The diameters of mycorrhizal hyphae are much lower than of roots. This
allows AMF to enter soil pores not accessible to roots and improve the P uptake rate of plants. Similar to plant roots, extraradical hyphae are only able to take up P from soil solution and therefore have access to the same P fractions in the soil as roots.

Although mycorrhizal associations are regarded as mutualistic in most cases, they may sometimes even be parasitic. Thus, the responses of plants to mycorrhization range from positive to negative (Johnson et al., 1997; Smith et al., 2009). In fact, it is still subject to discussion when associations may be considered mutualistic and when not. This certainly depends on many factors like the plant-fungus combination, stage of plant growth and P supply rate (Johnson et al., 1997; Smith et al., 2009). The P supply plays an important role for the plants response to mycorrhization. With increasing P supply the marginal benefit of biomass production per uptaken P decreases; therefore investments in P acquiring structures like AMF become less beneficial and should be reduced, otherwise resource allocation is not optimal for the plant (Johnson et al 1997). But although enhanced P supply often decreases mycorrhizal colonization of roots and production of extraradical hyphae, this is not always the case. It is evident that AMF symbiosis cannot be regarded as an optional strategy that is “implemented” by plants when P is low (Smith et. al. 2011).

Most plant-fungus combinations do not seem to be very specific, as most plant species form associations with different AMF species. However, preferences for individual AMF species seem to exist, as colonization density differs between plant-AMF species combination (Smith et al., 2011). Furthermore, neither do different AMF species induce the same response in a specific plant species, nor does a specific AMF species necessarily induce the same growth response in different plant species (Klironomos, 2003; Munkvold et al., 2004; Smith et al., 2004).
Most of the cited studies have been conducted with single mycorrhizal species. But in nature, it is normal that roots are colonized by many AMF species (Smith, 2008). Different AMF species vary in their growth rate and architecture of extraradical mycorrhizal hyphae (Jansa et al., 2005). Some authors suggest that the effect of multiple colonization on P uptake might be larger than of root colonization by a single species, because of spatial and temporal separation of P uptake by different AMF species (Koide, 2000).

1.3.2. Cluster roots

Cluster root formation is prominent in the Proteaceae family, which includes in particular the genera Banksia and Hakea, but there are also many cluster root forming species in other families like Casuarina, Acacia, Lupinus, Kennedia, Viminaria and Myria (Lamont, 2003; Shane and Lambers, 2005). Many of them can live in symbiosis with N₂ fixing bacteria of the genera Rhizobium and Frankia. Cluster roots are bottlebrush-like structures of densely haired rootlets clustered in specific sections along the axis of growing roots. They release large amounts of organic acids, protons and enzymes into the rhizosphere to increase solubilization of P. The organic acids build complexes with cations (Fe, Al, Ca, Al) associated with phosphate or exchange P from adsorption sites, while protons acidify the rhizosphere and enhance the solubility of phosphates. With these mechanisms cluster roots are able to mobilize P from non-labile P fractions that are un-available to non-cluster roots (Braum and Helmke, 1995; Lamont, 2003). As also metals such as Zn, Cu and Fe are solubilized, cluster roots may also be beneficial for the acquisition of metal micronutrients. The large absorptive surface of the cluster roots decrease the diffusive path for the uptake of solubilized P or other nutrients.

Cluster roots have a short life span, and their development follows a well predictable sequence of functional phases. In L. albus cluster rootlets release organic acids for
approximately one week after emergence, with an exudative burst between 2 and 4 days after the rootlets emergence (Hagstrom et al., 2001). A few days after a burst rootlets lose their function. The predictability of sequential phases makes them well suited to investigate physiological and ecophysiological processes that are relevant also for non-cluster roots (Skene, 2001).

Cluster roots are very expensive structures in terms of carbon costs and therefore their production usually decreases with increasing P availability in soil (Lambers et al., 2006; Lamont, 2003; Neumann and Martinoia, 2002; Shane et al., 2003; Shen et al., 2005; Shu et al., 2005; Shu et al., 2007).

1.4. Preferential allocation of non-cluster roots, cluster roots and mycorrhizal hyphae

In many studies it has been demonstrated that plants preferentially allocate roots into P-enriched patches (Ma et al., 2007; Ma et al., 2011; Robinson, 1994). The extent to which the spatial distribution of roots responds to the uneven distribution of P depends on plant species and plant nutrient status (Ma and Rengel, 2008; Robinson, 1994), degree of heterogeneity and patch sizes (Farley and Fitter, 1999a; Robinson, 1994; Wijesinghe and Hutchings, 1999). As P is of very low mobility in soil among the nutritional elements it is likely that under P limiting conditions the root density optimal for P acquisition might be higher than the optimal root density for the uptake of more mobile solutes such as NO$_3^-$ and water. More mobile nutrients could be depleted in P-enriched patches as a consequence of root proliferation for P. The costs of extraradical hyphae in terms of water, nitrogen and/or other resources per absorbing surface area might be lower than for roots because of the small diameter of hyphae (Hodge, 2005). Consequently, preferential foraging of roots for P could be (partially) replaced by preferential proliferation of extraradical hyphae, allowing the root system to forage for other resources. At least for some AMF species it has been shown that preferential growth of
extraradical hyphae into P-enriched patches occurs, when P was supplied in a compartment not accessible to roots (Cavagnaro et al., 2005; Shi et al., 2011).

While mutualistic interactions play an important role for the allocation of resources to AMF, cluster root production is mostly controlled by the plant itself. In many experiments cluster root production was increased in soil areas with increased nutrient or P availability, but there are also studies in which cluster root allocation did not respond to local P-enrichments or in which cluster root production was even reduced in parts of the root system where P supply rate was increased (Shane et al., 2003; Shane and Lambers, 2005; Shen et al., 2005; Shu et al., 2007). The spatial distribution of cluster roots strongly depends on the allocation of non-cluster roots parental to them. Hence the distribution of other resources determining the spatial allocation pattern of non-cluster roots strongly influence cluster root distribution. Water is certainly an important soil resource by itself but it is also the medium through which other soil resources are transported to the roots. The promoting effect of locally increased water availability on the root growth of plants that do not form cluster roots is well known (Hodge, 2010), but its effect on cluster root production has not been investigated yet.

1.5. Objectives

In this PhD-project we investigated the root allocation pattern of mycorrhizal Lotus corniculatus and cluster root forming Lupinus albus in soil with heterogeneous P distribution. These following questions are addressed in the three main chapters:

1. Does L. corniculatus preferentially allocate roots in P-enriched soil patches on the Chicken Creek Catchment?
The root allocation pattern of *L. corniculatus* was investigated in two experiments with constructed heterogeneity, one in the field and the other in a climate chamber. In addition a high-density root and soil sampling was conducted on undisturbed soil plots colonized by *L. corniculatus* on the Chicken Creek Catchment and on a second recently restored experimental site.

2. How do arbuscular mycorrhizal fungi influence preferential root growth?

To address this question a climate-chamber experiment was performed using *L. japonicus* as experimental plant instead of *L. corniculatus*, as the seeds we had obtained for the latter species produced plants of too much variability. The root allocation pattern of single mycorrhizal or non-mycorrhizal plants exposed to soil with no P or either heterogeneous or homogenous P fertilization was investigated. Mycorrhizal and non-mycorrhizal treatments were established using gamma-sterilized soil that remained un-inoculated or was inoculated with either *G. intraradices* or a naturally occurring mycorrhizal mixture of unknown composition. Abundance of *G. intraradices*, *G. mosseae* and *G. claroideum* in roots and soil were investigated using real time PCR.

3. How does heterogeneous water and P distribution in soil influence spatial root allocation of cluster-root forming *L. albus*?

Growth of cluster roots and non-cluster roots of *L. albus* was investigated in a climate chamber experiment, were water was supplied at high and low rates in containers filled with a P-poor sand to which either no P was added or which was fertilized homogeneously or heterogeneously in a lateral section of the plant container. In addition, heterogeneous distribution of water availability was established in half of the containers by using fine-grained instead of coarse-grained sand as substrate in a lateral third of the containers. Water
distribution and root growth in the containers was monitored using neutron radiography – a non-invasive technique to visualize roots and water in soil.

1.6. References


Introduction


2. Root growth of *Lotus corniculatus* interacts with P distribution in young sandy soil
Summary

Large areas of land are restored with unweathered soil substrates following mining activities in eastern Germany and elsewhere. In the initial stages of colonization of such land by vegetation, plant roots may become key agents in generating soil formation patterns by introducing gradients in chemical and physical soil properties. On the other hand, such patterns may be influenced by root growth responses to pre-existing substrate heterogeneities. In particular, the roots of many plants were found to preferentially proliferate into nutrient-rich patches. Phosphorus (P) is of primary interest in this respect because its availability is often low in unweathered soils, limiting especially the growth of leguminous plants. However, leguminous plants occur frequently among the pioneer plant species on such soils, as they only depend on atmospheric nitrogen (N) fixation as N source. In this study we investigated the relationship between root growth allocation of the legume *Lotus corniculatus* and soil P distribution on recently restored land. As test sites, the experimental Chicken Creek Catchment (CCC) in eastern Germany and a nearby experimental site (ES) with the same soil substrate were used. We established two experiments with constructed heterogeneity, one in the field on the experimental site and the other in a climate chamber. In addition, we conducted high-density samplings on undisturbed soil plots colonized by *L. corniculatus* on the ES and on the CCC. In the field experiment, we installed cylindrical ingrowth soil cores (4.5 x 10 cm) with and without P fertilization around single two-month-old *L. corniculatus* plants. Roots showed preferential growth into the P-fertilized ingrowth-cores. Preferential root allocation was also found in the climate chamber experiment, where single *L. corniculatus* plants were grown in containers filled with ES soil and where a lateral portion of the containers was additionally supplied with a range of different P concentrations. In the high-density samplings, we excavated soil-cubes of 10x10x10 cm size from the topsoil of 3 mini-plot areas (50 x 50 cm) each on the ES and the CCC on which *L. corniculatus* had been
planted (ES) or occurred spontaneously (CCC) and for each cube separated the soil attached to the roots (root-adjacent soil) from the remaining soil (root-distant soil). Root length density was negatively correlated with labile P (resin-extractable P) in the root-distant soil of the CCC plots and with water-soluble P in the root-distant soil of the ES plots. The results suggest that P depletion by root uptake during plant growth soon overrode the effect of preferential root allocation in the relationship between root density and plant-available soil P heterogeneity.
2.1. Introduction

Large areas of land are denuded of the original soil cover in the course of construction or mining projects and later restored, often using un-weathered soil substrates. The formation of spatial patterns in the physical and chemical properties of the developing soil during the initial stages of colonization by vegetation is an important aspect in the restoration of such land. The development of root systems plays a particular role in these processes. Roots form pathways for water flow and solute transport and are a primary source of organic matter input into soil (Huetsch et al., 2002). Processes such as the release of organic compounds, protons and carbon dioxide, consumption of oxygen, and uptake of nutrients and water can lead to steep gradients in chemical conditions and biological activities around roots, a phenomenon well known as “rhizosphere effect” (Hinsinger et al., 2005). Such gradients can have a strong influence on the patterns of mineral weathering and transformation, formation of humus, and the development of physical soil structure. Equally strong influences may also occur in the opposite direction, as the pre-existing heterogeneities in soil properties can also shape the patterns of root system development. For example, many plant species are known to respond to patchiness in the spatial distribution of growth-limiting nutrients by root proliferation in patches where these nutrients are enriched (Robinson, 1994).

Limitations in the availability of soil nitrogen (N) and phosphorus (P) are a particularly frequent condition during the early phases of ecosystem development (Vitousek et al., 2010). In the absence of fertilization, mineral weathering usually the only relevant source of P in this stage, as long as there is no major supply of P deriving from the decomposition of organic matter. Many pioneer plants are legumes, which do not depend on soil N, as they live in symbiosis with N-fixing rhizobia in their roots. Most of them, however, have high requirements for P (Sprent et al., 1988).
Phosphorus is often distributed quite heterogeneously in soil on the scale of a root system (Farley and Fitter, 1999; Gallardo and Parama, 2007; Gross et al., 1995; Jackson and Caldwell, 1993). Laboratory and greenhouse experiments with constructed heterogeneities and/or split root systems have shown that localized P supply can induce preferential root proliferation in many plant species (Denton et al., 2006; Kume et al., 2006; Ma and Rengel, 2008; Ma et al., 2007; Robinson, 1994; Weligama et al., 2007). Some authors also studied preferential root growth in response to localized P fertilization in the field (Buman et al., 1994; Caldwell et al., 1996; Eissenstat and Caldwell, 1988). In studies with artificially created heterogeneity the contrast in P concentrations between fertilized and non-fertilized soil patches was usually high. Little is known about the extent and relevance of preferential root growth in response to P patchiness under normal field conditions. (Mou et al., 1995) analyzed three-dimensional root distributions in monocultural Liquidambar styraciflua and Pinus taeda plantations in relation to available soil P, K and N concentrations and found that the fine root densities of both tree species increased with P and K but not with N concentrations in the topsoil. These stands were already in a later stage of ecosystem development, at which root, shoot and leaf litter decomposition already may have played a major role for the spatial distribution of soil nutrients. We are not aware of studies that investigated the effect of heterogeneous soil nutrient distribution on root allocation patterns in soils in the initial stage of the development of an ecosystem and which compared the response of roots to nutrient enriched soil patches under experimental conditions with the relationship between root allocation and soil nutrient distribution under undisturbed conditions in the field.

In this study we had the opportunity to investigate the root allocation strategy of the legume Lotus corniculatus in the man-made 6-ha Chicken Creek Catchment (CCC), which was established in 2005 in a Lusatian (in German: Lausitz) post-mining landscape in Eastern Germany to study initial ecosystem development on freshly deposited non-weathered
substrate on a catchment scale (Gerwin et al., 2009). *Lotus corniculatus* L. (bird’s Foot trefoil) is a perennial herbaceous early-succession plant, pioneering the colonization of post-mining landscapes in Lusatia.

On the catchment we sampled roots and soil at high-density on 3 mini-plot areas where *L. corniculatus* occurred spontaneously. Because disturbances in general and erosion risks in particular had to be kept at a minimum on the CCC, an experimental site (ES) with similar soil properties was established in the vicinity of the CCC, where soil and vegetation could also be experimentally manipulated. On this site we carried out the same mini-plot high-density sampling as on the CCC, but after growing *L. corniculatus* in monoculture. In addition, we performed a factorial plot experiment on this site and a climate chamber experiment with constructed heterogeneities to test the response of *L. corniculatus* to P-enriched soil also under more controlled conditions. We expected that soil patches with elevated concentrations in P would induce preferential root allocation and that we would therefore find a positive correlation between root length density and soil P in the high-density samplings.

### 2.2. Materials and Methods

#### 2.2.1. Site description

The Chicken Creek Catchment (CCC) was constructed on a refilled opencast lignite mine about 30 km south of the city of Cottbus in the State of Brandenburg, Germany. After construction was finished in September 2005, the site was left to re-vegetate spontaneously. A detailed description of the establishment and initial development of the catchment was given by Gerwin et al. (2009). In order to enable also manipulative and invasive field experiments
with soil and plants under comparable conditions, the before-mentioned “Experimental Site” (ES) was established in 2009 in the vicinity of the CCC using substrate of the same origin.

The substrate deposited on the CCC and the ES as soil parent material was quaternary calcareous sand from Saale-time Pleistocene deposits of the Lusatian ridge (in German: Lausitzer Höhenrücken). The soil parameters of the substrate on the ES and the CCC are illustrated in Table 1. Soil parameters for the CCC derive from a soil sampling campaign conducted in 2005 (Gerwin et al., 2009) and are averaged values of sampling points proximate to the investigated plots, while soil parameters for the ES represent values taken from soil sampled at the investigated plots.

The climate is temperate and slightly continental with high summer temperatures and pronounced drought periods during the growing season. The long-term average precipitation was given as 595 mm per year, and the mean air temperature as 9.3 °C (Gerwin et al., 2009). The main difference between the two sites was that the CCC was built as a large lysimeter with an impermeable clay liner at 2-3 m depth in order to collect all water at the catchment outflow, while there was free drainage from the ES soil. Consequently, a water table developed in the subsurface of the CCC in contrast to the ES, and as the hydraulic conductivity of the deposited substrate was lower than predicted, the water table rose to higher levels than planned, and at times some water even influenced the lower parts of the root zone.

2.2.2. Climate chamber experiment

The climate chamber experiment was performed at ETH Zürich. Single *L. corniculatus* plantlets were grown in Aluminium (Al)-containers of 27x27x1.2 cm internal volume
Table 1. Soil parameters of the Experimental Site (ES) and the Chicken Creek Catchment (CCC)

<table>
<thead>
<tr>
<th></th>
<th>Sand</th>
<th>Silt</th>
<th>Clay</th>
<th>Organic carbon (%)</th>
<th>Calcium carbonate (%)</th>
<th>pH (H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>96.3</td>
<td>1.6</td>
<td>2</td>
<td>&lt;0.01</td>
<td>0.82</td>
<td>8.50</td>
</tr>
<tr>
<td>CCC</td>
<td>86.0</td>
<td>8.0</td>
<td>6</td>
<td>0.14</td>
<td>0.65</td>
<td>8.07</td>
</tr>
</tbody>
</table>

filled with soil from the experimental site. We established 6 homogeneous soil treatments adding 5.7, 17, 34, 52, 85 or 102 mg P per pot (4, 12, 24, 37, 60 72 mg P kg⁻¹ soil) and 8 heterogeneous soil treatments. In the latter we added 5.7, 11.3, 17, 34, 51, 68, 85 or 102 mg P pot⁻¹, but only to a lateral third of the soil in each container (12, 24, 36, 72, 109, 145, 182, 218 mg P kg⁻¹ soil in the P-enriched soil area). Additionally, we established a control treatment with no P addition. All treatments were replicated three times, except for the highest heterogeneous treatment, which was replicated only twice. Mono-calcium-phosphate (Ca(H₂PO₄)₂.H₂O) was used as fertilizer.

To fill the soil into the containers, we laid them down on one side and removed the upward looking lateral wall of the other side. Then the soil, which had been thoroughly mixed with respective amounts of fertilizer before, was filled in three vertical bands of equal width (9 x 27 cm) into the containers. In the heterogeneous treatments, we always filled the P fertilized soil into the third on the right-hand side looking into the opened container. After filling, we closed the lateral wall and put the container into the upright position again. Care was taken to avoid pressing of the soil, and to achieve a dry soil bulk density of approximately 1.6 g cm⁻³ in all containers.
We planted a single pre-germinated seedling in the middle of each container, so that the distances to the left and the right compartment were the same. Plants were grown for 60 days in a climate chamber with a humidity of 60%, a 16:8 h day:night cycle and a respective 21/16 °C temperature cycle. During the day the photon flux was 250 µmol m⁻² s⁻¹. We watered the container on a weight basis to 50% water holding capacity (approx. 100 hPa water suction).

At harvest, we cut the shoots close to the soil surface and dried them to constant weight at 60°C. The roots were sampled separately from each third of the containers. After thoroughly washing the soil from the roots, they were placed into a water bath and scanned with an Epson scanner (Perfection V700, 400 dpi resolution). The scans were then analyzed for root length by means of WinRHIZO (Regent Instruments, Inc. Quebec Canada, version 2009a).

2.2.3. Ingrowth core experiment

For the factorial plot experiment on the ES we employed the ingrowth core method. Single *L. corniculatus* plantlets were grown on 18 plots of 50 x 50 cm size, on which fertilized (as described below) and non-fertilized cylindrical soil cores were installed vertically on a regular grid at distances of 10, 22 and 30 cm from the plant stem in the center of each plot (Fig. 1). The total rate P applied was 55 mg per plot. Thus, this treatment is denoted here as HET P 55 mg. Additionally, we established plots with homogeneous P fertilization of the entire topsoil (0-10 cm depth) and non-fertilized control plots in order to assess the potential P responsiveness of *L. corniculatus* on the experimental site. The rate of P application in the homogeneous fertilization treatment was the same as for the fertilized cores in the HET P 55 mg treatment, resulting in a total rate of 1080 mg P applied per plot. This treatment thus is denoted here as HOM P 1080.
Figure 1. Positioning of P-fertilized (grey circles) and unfertilized soil cores (unfilled circles) around single *L. corniculatus* plants (cross) on plots (50 x 50 cm) with heterogeneous P supply of the ingrowth core experiment. Soil cores were arranged on a 14 x 14 cm grid resulting in 4, 8 and 4 soil cores at 10, 22 and 30 cm distance from the plant, respectively.

To prepare the plots for planting, we excavated and bulked the entire topsoil (0-10 cm) of all plots, homogenized it thoroughly and divided it into two fractions. One fraction was mixed with 27 mg P kg⁻¹ soil (as monocalcium phosphate), while the other fraction remained unfertilized. At first, the ingrowth cores were established using steel cylinders of 10 cm height and 4.5 cm diameter placed in upright position on a 14 x 14 cm square grid. As the center of the plot was aligned with the center of the central square, this scheme resulted in 4, 8 and 4 ingrowth cores at 10, 22 and 30 cm distance from the center of the plot, respectively. Alternatively, half of the cylinders were filled with fertilized and unfertilized soil. After refilling the space around the cylinders with unfertilized soil, the cylinders were carefully removed. Similarly, just without previous ingrowth core installation, homogenized soil with or without fertilization was filled back into the plots of the respective homogeneous treatments. Each treatment was replicated six times.
Two months before the experiment started, we sowed *L. corniculatus* seeds on the ES to establish a pool of candidate plantlets. From this pool we selected plantlets of similar size and habitus and transplanted them on the 15th of April 2009 to the experimental plots. All plots were weeded once weekly. On the 1st of October 2009, we harvested the shoots and sampled all ingrowth cores. After transfer to the laboratory, the roots were processed and analyzed in the same way as in the climate chamber experiment.

2.2.4. **High-density sampling on the Chicken Creek Catchment and the Experimental Site**

After manual removal of existing plants, three otherwise undisturbed 50 x 50 cm mini-plots were seeded with *L. corniculatus* in spring 2008 at low, medium and high density, as specified in Table 2. Keeping the plots clean from other plants was the only manipulation of the plots during the growth of the *L. corniculatus* seedling. In spring 2009, the plants were harvested and the soil collected in 10x10x10 cm cubes. The same type of sampling was performed on three mini-plots of the same size in May 2010 on the CCC, with the difference that in contrast to the ES plots, *L. corniculatus* was present on these plots spontaneously. While plots were selected which were predominantly but sparsely populated with *L. corniculatus*, it was unavoidable that also other plants – exclusively grass species - were present as well.

The soil cubes were collected by means of metal boxes, which were driven side by side into the soil (25 cubes per plot). The samples (containing soil and roots) were transferred into plastic bags and immediately transported in thermo boxes into the laboratory, where they were stored in a refrigerator at 4 °C, until they were further processed and analyzed within the following 1-4 weeks. Roots with adherent field-moist soil were separated from the remaining soil, in the following referred to as *root-distant soil*, by means of a 4 mm sieve. Grass roots in the CCC samples were easily distinguished and separated from *L. corniculatus* roots. Grass
roots and the soil attached to these roots was excluded from soil or root analysis. The soil adhering to the roots, in the following referred to as *root-adjacent soil*, was left to air-dry for 5 minutes and then gently removed using a brush. Root-adjacent and root-distant soil samples were stored separately in small parchment paper bags for subsequent chemical analyses. After thorough washing, the roots were analyzed in the same way as in the experiments described before.

**Table 2.** Number of *L. corniculatus* plants and coverage (%) per plot (50 x 50 cm) at the experimental site (ES) and Chicken Creek Catchment (CCC) for low (plot 1), intermediate (plot 2) and high (plot 3) vegetation density. For the coverage of plot 2 and plot 3 on the CCC numbers in brackets refer to the coverage of *L. corniculatus* plus the co-occurring grass species.

<table>
<thead>
<tr>
<th></th>
<th>Plot 1 ES</th>
<th>Plot 2 ES</th>
<th>Plot 3 ES</th>
<th>Plot 1 CCC</th>
<th>Plot 2 CCC</th>
<th>Plot 3 CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plants</td>
<td>6</td>
<td>7</td>
<td>9</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>per plot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coverage (%)</td>
<td>16</td>
<td>36</td>
<td>48</td>
<td>16</td>
<td>44 (90)</td>
<td>48 (100)</td>
</tr>
</tbody>
</table>

Water-soluble P and calcium (Ca) concentrations and the pH of root-distant and root adjacent soil samples were analyzed in 1:2.5 soil-to-solution extracts, using bi-distilled water for extraction (Meiwes *et al.*, 1984). After adding the water, the slurries were shaken for 1 h and then left to settle for 16 h at room temperature, centrifuged for 5 min at 3000 rpm and filtered (512 ½ folding filter, Whatman; Dassel, Germany) following the method of (Schlichting *et al.*, 1995). The filtrates were analyzed for Ca and P by means of inductive coupled plasma optical emission spectroscopy (iCAP 6000 series, Thermo Scientific, Germany). The CCC samples were also analyzed for anion- and cation-resin extractable P using the method of (Saggar *et al.*, 1990) Phosphorus concentrations in solution were
determined photometrically (Van Veldhoven and Mannaerts, 1987). In the following we refer to the resin-extractable P as labile P.

2.2.5. Statistical analysis and calculations

We used normal quantile–quantile plots to check for deviations from normal distribution of random effects and residual errors. The labile P, water-soluble P and root length data from the CCC samples were log-transformed to achieve normality. In all other cases no transformation was necessary.

In the climate chamber experiment, we calculated root allocation as the difference of root length in the right third of the container (fertilized in the heterogeneous treatments) and the left third (unfertilized in the heterogeneous treatments). We used the protected Fisher LSD test for multiple comparisons. If the lower boundary of the 95% confidence interval was greater than zero, root allocation was considered preferential.

The datasets of the high-density samplings were analyzed separately for the two sites. We standardized root length and soil parameters by plots to achieve mean values of 0 and variances of 1 for all parameters on each plot. Then we pooled the standardized data of the three plots of each site and calculated Pearson correlation coefficients of the soil parameters, distance from the stem and root length. Distance from the stem of a sampled cube was calculated as the distance from the center of the cube containing the nearest plant and the center of the cube in question. Rhizosphere effects for labile P, water-soluble P, Ca and pH were determined as the difference between concentrations of the root-adjacent and root-distant soil in a cube sample.
2.3. Results

2.3.1. Climate chamber experiment

The growth habitus of the experimental plants showed considerable variation in the climate chamber experiment, indicating substantial genotypic variability among the seeds. As a result, neither heterogeneous nor homogeneous P fertilization showed a significant influence on shoot dry weight production (ANOVA, p<0.05). At low P supply shoot biomass tended to increase with increasing level of fertilization (Fig. 2 a).

In the heterogeneous treatments, root length was always significantly higher in the P-fertilized part of the containers than in the unfertilized part (Fig. 2, b). In the homogeneous treatments, root length, as to be expected, did not significantly differ between the two sides of the containers. Despite the large variability in plant growth, the experiment thus revealed a clear preferential root growth response to increased P concentration.

2.3.2. Ingrowth core experiment

As we selected the plants according to their size, growth habitus and leaf shape for the ingrowth core field experiment, it can be assumed that they were genetically much more homogeneous than in the climate chamber experiment. Fresh-weight production of the shoot biomass was 2.5 times higher in the homogeneous P fertilization (HOM P 1080) treatment than in the ingrowth core (HET P 55 mg) and control treatments (No P addition, Fig. 3, a). Root length was larger in the P fertilized ingrowth cores than in the unfertilized cores (2-way ANOVA, p<0.05) and decreased with increasing distance from the stems of the plants (2-way ANOVA, p<0.05). As Fig. 3 b shows, the effect of P on root length production was strongest close to the plant stems and decreased with distance. The gradient of decrease appeared to be larger for the P fertilized cores than the control cores, but the interaction between P fertilization and distance from the stem was not significant.
Figure 2. (a) Shoot dry weight production of L. corniculatus grown in containers filled heterogeneously (stippled bars) or homogeneously (grey bars) with soil. (b) Preferential root allocation was calculated as the difference of root length in the right third of the container (P-fertilized in the heterogeneous treatments) and the left third of the container (unfertilized). Error bars refer to the standard error of the mean. Preferential root allocation was significant in all heterogeneous treatments.
Figure 3. (a) Shoot fresh weight production of single field-grown *L. corniculatus* plants grown on plots with heterogeneous P fertilization (HET P 55), no P addition (No P addition) or homogeneous P supply (HOM P 1080). (b) Root length density in fertilized and unfertilized ingrowth cores of HET P 55 at 10, 22 and 30 cm from the stem of the plants. Error bars refer to the standard error of the mean. P fertilization and distance had a significant effect on root length density in the ingrowth cores (2-way-ANOVA, p<0.05)
2.3.3. High-density samplings

Root length density did not significantly change with distance from the stems on the CCC plots (Table 4), while it decreased with increasing distance from the stems on the ES plots (Table 3, Fig. 4). Root length density also decreased with increasing water-soluble P on the ES plots, while no correlation between these two variables was found on the CCC plots. A similar negative relationship as between water-soluble P and root length density on the ES was found between labile P and root length density on the CCC (Fig. 5). In contrast to the finding that the relationships of root length density with stem distance and water-soluble P were both negative, water-soluble P was not affected by stem distance on the ES plots. But distance from the stem had a positive effect on labile P on the CCC, while it showed no influence on water-soluble P. Soil Ca concentration increased with root length density on the ES plots and decreased with distance from the stem. Soil pH and Ca were negatively

<table>
<thead>
<tr>
<th></th>
<th>Root length</th>
<th>Distance</th>
<th>Water-sol. Ca</th>
<th>pH</th>
<th>Water-sol. P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length</td>
<td>0.0034</td>
<td>0.0000</td>
<td>0.2437</td>
<td>0.0207</td>
<td></td>
</tr>
<tr>
<td>Distance</td>
<td>-0.35</td>
<td>0.0009</td>
<td>0.2607</td>
<td>0.6949</td>
<td></td>
</tr>
<tr>
<td>Water-sol. Ca</td>
<td>0.6</td>
<td>-0.44</td>
<td>0.0206</td>
<td>0.6859</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>-0.14</td>
<td>0.14</td>
<td>-0.32</td>
<td>0.0283</td>
<td></td>
</tr>
<tr>
<td>Water-sol. P</td>
<td>-0.28</td>
<td>-0.05</td>
<td>-0.06</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>
correlated on both sites. Furthermore, pH increased also with distance on the CCC, but not on the ES.

Water-soluble Ca was on average 28 mg/kg higher in root-adjacent soil than in root-distant soil on the ES (Fig. 6), while the pH of root adjacent soil was on average 0.4 units lower than the pH of root-distant soil. A similar but weaker rhizosphere effect on water-soluble Ca as in the ES soil was also found in the CCC samples, whereas no consistent effect on pH was detected. While we found no significant rhizosphere effect on water-soluble P in ES soil, it tended to be higher in root-adjacent than in root-distant soil for all three plots. In contrast to this trend, water-soluble P concentrations tended to be slightly lower in root-adjacent than in root-distant Chicken Creek soil. On the other hand, labile P was higher in root-adjacent than in root-distant soil of the Chicken Creek plots, similar to the rhizosphere effect on water-soluble P of the ES soil.

**Table 4.** Pearson correlation coefficients (lower part of the table) and p-values (upper part of the table) for root length, distance from the stem, water-soluble Ca, pH and water-soluble P on the Chicken Creek Catchment (CCC). The numbers in italic indicate significant correlations between the respective variables (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Root length</th>
<th>Distance</th>
<th>Water-sol. Ca</th>
<th>pH</th>
<th>Water-sol. P</th>
<th>Labile P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length</td>
<td>0.1512</td>
<td>0.7251</td>
<td>0.4737</td>
<td>0.3016</td>
<td>0.0038</td>
<td></td>
</tr>
<tr>
<td>Distance</td>
<td>-0.17</td>
<td>0.0372</td>
<td>0.0256</td>
<td>0.1762</td>
<td>0.0066</td>
<td></td>
</tr>
<tr>
<td>Water-sol. Ca</td>
<td>-0.04</td>
<td>-0.24</td>
<td>0.0004</td>
<td>0.0000</td>
<td>0.0584</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>0.08</td>
<td>0.26</td>
<td>-0.40</td>
<td>0.0783</td>
<td>0.7651</td>
<td></td>
</tr>
<tr>
<td>Water-sol. P</td>
<td>-0.12</td>
<td>0.16</td>
<td>-0.46</td>
<td>0.21</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>Labile P</td>
<td>-0.33</td>
<td>0.31</td>
<td>-0.22</td>
<td>0.03</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>


2.4. Discussion

The results of the fertilization experiment on the ES area clearly show that low soil P was limiting the growth of *L. corniculatus* in the unfertilized soil and that *L. corniculatus* responds with root proliferation into P-enriched soil under these conditions. The climate chamber experiment, where all other heterogeneities had been evened out by soil homogenization, confirmed that preferential allocation of root growth is indeed a response of *L. corniculatus* that can be induced by heterogeneous P distribution. The ability to respond to locally increased P availability with enhanced root proliferation has been shown also for many other plant species (Denton *et al.*, 2006; Kume *et al.*, 2006; Ma and Rengel, 2008; Ma *et al.*, 2007; Robinson, 1994; Weligama *et al.*, 2007) in climate chamber experiments, but seldom in the field (Buman *et al.*, 1994; Eissenstat and Caldwell, 1988).

The negative correlations of root length density with labile P and water-soluble P in the root-distant soil on the high-density sampling plots of the two field sites is in direct contrast to the results of the experiments with constructed heterogeneity. They suggest that plant-available soil P was quite rapidly depleted by root uptake and that this depletion had a stronger influence than preferential root proliferation into P-rich soil on the relationship between root length density and soil P at the time of sampling. Furthermore, it indicates that the influence of the roots extended into zones around the roots beyond our operationally defined root-adjacent soil. The negative correlation between root length and labile and water-soluble P is also in contrast to the results of Mou *et al.* (1995), who found a positive correlation between root length growth and soil P in *Liquidambar styraciflua* and *Pinus taeda* monocultures. However, in contrast to our sites, these stands were already in a stage of ecosystem development at which P recycling with root and shoot litter decomposition was probably a major process determining P distribution in soil. Recycling of P by litter decomposition could result in high contrasts between P-rich and P-poor patches, as P is
Figure 4. The relationship between root length density and water-soluble P (first row) or water-soluble Ca (second row) as well as the relationship between the distance from the stem and root length density (third row) or water-soluble Ca (fourth row) investigated in the high-density sampling on the Experimental Site (ES) in the top-soil (0-10 cm) of the plots with low (plot 1), intermediate (plot 2) and high vegetation density (plot 3).
Figure 5. The relationship between root length density and labile P (first row) as well as the relationship between the distance from the stem and labile P (second row), water-soluble Ca (third row) or pH (fourth row) investigated in the high-density sampling on the Chicken Creek Catchment (CCC) in the topsoil (0-10 cm) of the plots with low (plot 1), intermediate (plot 2) and high vegetation density (plot 3).
Figure 6. The rhizosphere effect for pH, water-soluble Ca, water-soluble P and labile P on the plots with low (plot 1), intermediate (plot 2), high (plot 3) vegetation density and the pooled data for the three plots (plot 1+2+3) on the Chicken Creek Catchment (CCC) and the Experimental Site (ES) is calculated as the difference between the value for the respective parameter in the soil attached to the root and the value in the remaining soil of cubic samples taken from the top 10 cm of the soil profile. Boxplots illustrate the median (horizontal line), the interquartile range (box), 1.5 times the interquartile range (whiskers) and outliers (dots).
Root growth of *L. corniculatus* interacts with P distribution in young sandy soil

extracted from the entire volume of soil colonized by roots, while P release via necromass decomposition would be spatially much more concentrated as it would occur in close relationship with the allocation of the mass of decaying roots.

Comparing the results of the high-density samplings with those of the experiments with constructed heterogeneities, it must be considered that the variation in plant-available soil P was in average much smaller in the undisturbed field soil than the contrasts between fertilized and non-fertilized soil in the latter experiments. Furthermore, the plants sampled on the CCC had much more time to develop their root systems and extract soil P than in the ingrowth core and the climate chamber experiment. Thus, it is quite plausible that preferential root allocation into initially P-rich soil occurred, but was subsequently masked by the opposite effect of P depletion. It is also conceivable that P heterogeneity in the undisturbed field soils was too small to trigger preferential root growth allocation in P-enriched soil zones. Several authors investigating root distributions in relation to soil nutrient distributions suggested that P heterogeneity in their study soils was too low to become relevant for root allocation in herbaceous plants, but not for trees (Farley and Fitter, 1999; Gallardo and Parama, 2007; Gross et al., 1995).

In apparent contrast to the notion that P becomes depleted with time in the rhizosphere (Hendriks et al., 1981; Hinsinger et al., 2011b; Wang et al., 2005), we observed elevated concentrations of labile P fractions in soil adjacent to roots as compared to soil farther away from the roots in the high density samplings. Likely reasons for this effect are P solubilization by root exudation overriding P uptake by roots (Hinsinger et al., 2011b). Given that P is a rather immobile nutrient element in soil, the direct influence of roots on P concentrations only extends a few mm at most into the adjacent rhizosphere soil (Hinsinger et al., 2011b). By exudation of organic acids such as citric acid, which diffuse into the surrounding rhizosphere
soil and mobilize phosphate from solid phases, plants can substantially increase the flux of soil P to their roots. Such solubilization can result in higher average concentrations of labile or water-soluble P in the rhizosphere than in the bulk soil, even when the total P concentration is reduced and despite a concentration gradient in dissolved P towards the root surface. Support for this interpretation comes from findings of P depletion in the rhizosphere immediately adjacent to the root surface and P enrichment above bulk soil level in the outer zone of the rhizosphere just a few mm farther (Hinsinger et al., 2011b; Hinsinger and Gilkes, 1996; Hubel and Beck, 1993).

Whether accumulation or depletion of P is found in the rhizosphere, thus, may also depend on the extent to which soil adjacent to the root surface is included in “rhizosphere” soil samples and explain why some authors found depletion of P and others accumulation of P in the rhizosphere. (Hinsinger et al., 2011a) suggest that the interaction of P uptake rate and P solubilization through exudates are responsible for P concentration pattern.

While lower water-soluble or labile P concentrations in root-distant than in the root-adjacent soil can be explained by P solubilization through root exudates, the rhizosphere effect does not explain the negative correlation observed between root density and labile or water-soluble P in the root-distant soil. A likely candidate would be soil P extraction via arbuscular mycorrhizal fungi (AMF). Extraradical mycorrhizal hyphae can grow far beyond the zone directly influenced by the roots and extract P from soil up to 10 cm away from the root surface (Jansa et al., 2005). Mycorrhizal fungi can contribute much more than direct root uptake to the P nutrition of plants. (Smith et al., 2004) for example showed that 50 to 100% of the P accumulated in the shoots of three plant species was taken up via mycorrhizal fungi. If the density of extraradical mycorrhizal hyphae was positively correlated with root length density and root age, then this could explain why P depletion increased with root length
density in the root-distant soil. Indeed we found that the roots of three randomly selected \textit{L. corniculatus} plantlets were colonized with AMF in the climate chamber experiment.

While the positive rhizosphere effect on labile P in the CCC plots was in line with the corresponding effect on water-soluble P in the ES plots, it was surprising that the rhizosphere at the same time had the opposite effect on water-soluble P, a fraction closely related to labile P, in the CCC soil. This puzzling result may be explained by the different water regimes of the two sites and their effect on soil carbonate dynamics. The CCC was under the influence of a fluctuating groundwater table in the subsoil, in contrast to the ES. At times, the water table was high enough that through the capillary fringe above the water table even topsoil roots could probably tap into this source during some periods. Thus, the vegetation on the CCC plots could consume much more water than on the ES, and this transpirational water stream could result in a substantial upward flow of calcium carbonate saturated solution from the groundwater table to the roots at certain times. Calcium supplied in excess of plant uptake (Hinsinger \textit{et al.}, 2005) would have accumulated in the rhizosphere and eventually precipitated as CaCO$_3$, in particular when the partial pressure of CO$_2$ decreased during drying phases. Thus, the pH buffer capacity provided by CaCO$_3$ was periodically replenished in the rhizosphere of the CCC plots, maintaining pH at similar or even higher levels as in the bulk soil and keeping water-soluble P at correspondingly low levels. In contrast, as the buffer was gradually depleted, pH values decreased and water-soluble P concentrations increased in the rhizosphere of the ES plots (Fig. 5). The fact that, unlike the concentration of water-soluble P, the concentration of resin-extractable P was higher in the rhizosphere than in the bulk soil of the CCC plots suggests that a comparatively large fraction of this P had been mobilized from less available P pools by root exudates and bound in labile, but not water-soluble form, e.g. on ion-exchanging sites.
The negative correlation between stem distance and root length density was more pronounced in the one-year-old plants on the ES than in the plants of the CCC plots, which were on average older than one year. This indicates that with plant age new root growth is increasingly allocated at greater distances from the stem. This could be a response to nutrient depletion around older parts of the root system as long as zones farther away are still more abundant in nutrients and water. Indeed, we found an increase of labile P in the root-distant soil with distance from stems on the CCC. Most authors investigating herbaceous plants or grasses found that root length density decreased with distance from stem after one growing season (Buman et al., 1994; Majdi et al., 1992; Milchunas et al., 1992). But they did not study perennial growth. For trees, some authors found that within the sampled range the distance from the stem had no influence on root length density (Millikin and Bledsoe, 1999). In line with our observations, these findings suggest that an initial dependence of root density on stem distance disappears with plant age.

The results suggest that patches with spontaneous P enrichment in the CCC and ES field soils that was due to natural spatial variability (i.e. heterogeneity that was not experimentally constructed) only persisted for less than a year, before they were depleted by root P uptake into patches. This would mean that also the pattern of new root growth allocation would shift accordingly during that time frame and with it the spatial pattern of root influences on the surrounding soil, including weathering, organic matter deposition and other processes affecting soil formation. Our study shows that the responsiveness of plant root allocation to nutrient enriched soil, as obviously found under well-controlled experimentally manipulated conditions, may not necessarily translate into a corresponding, easily interpretable relationship between root and nutrient distribution under undisturbed field conditions even at the early stage of the development of an ecosystem.
The experiments with heterogeneous and homogeneous P fertilization showed that P was a limiting factor for the growth of *L. corniculatus* on the experimental soil and that the plants preferentially allocated roots into P-enriched zones in this soil. The results of the high-density samplings, on the other hand, indicate that P depletion by roots (and probably also mycorrhizal fungi) had a more dominating influence on the spatial relationship between root length density and soil P concentrations in the field soil without artificially enhanced P heterogeneity within the first year after plant establishment. Assuming that *L. corniculatus* plants responded with preferential root allocation also to local P enrichment that was present in the undisturbed and untreated field soils due to natural spatial variability, this means that depletion of these patches by root P uptake subsequently turned them into their opposite, i.e. patches with decreased P availability. While the combined effect of preferential root growth and soil P depletion by roots is expected to reduce contrasts between soil patches of higher and lower P-availability during the initial stages of soil development, other processes may oppose this trend by the generation of new heterogeneities, in particular locally concentrated P inputs with leaf and root litter.

2.5. Acknowledgements

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2.6. References


Root growth of *L. corniculatus* interacts with P distribution in young sandy soil


3. Interaction between root growth allocation and mycorrhizal fungi in soil with patchy P distribution

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Submitted to *Plant and Soil*
Summary

Many plants preferentially grow roots into P-enriched soil patches, but little is known about how the presence of arbuscular mycorrhizal fungi (AMF) affects this response. *Lotus japonicus* (L.) was grown in a low-P soil with (a) no additional P, (b) homogeneous P (28 mg/pot), (c) low heterogeneous P (9.3 mg/pot), and (d) high heterogeneous P (28 mg/pot). Each P treatment was combined with one of three mycorrhiza treatments: no mycorrhizae, *Glomus intraradices*, indigenous AMF. Real-time PCR was used to assess the abundance of *G. intraradices* and the indigenous AMF *G. mosseae* and *G. claroideum*. Mycorrhization and P fertilization strongly increased plant growth. Homogeneous P supply enhanced growth in both mycorrhizal treatments, while heterogeneous P fertilization increased biomass production only in treatments with indigenous AMF inoculation. Preferential root allocation into P-enriched soil was significant only in absence of AMF. The abundance of AMF species was similar in P enriched and unfertilized soil patches. Mycorrhizae overrode proliferation of roots into P-enriched patches. Root colonization by a mixture of fungal species was more advantageous for the plants to cope with heterogeneous soil P availability than forming an association with *G intraradices* alone.
3.1. Introduction

Phosphorus (P) is an essential macronutrient element that is limiting plant growth in many natural and cultivated soils. Many soils are deficient in plant available P, because the availability of dissolved P is often limited by the low solubility of Ca, Fe and Al phosphates or strong binding to specific sorption sites (Hinsinger, 2001). When roots take up P from the rhizosphere solution, re-supply from the bulk soil is limited by solubilization of P from the solid matrix and transport of the dissolved P to the roots. Diffusion is the main transport process, and diffusivity of P is a critical factor that often co-limits the P uptake rate of roots in combination with the low solubility of solid P phases.

The P nutrition of around 80% of all plant species benefits from a mutualistic association with arbuscular mycorrhizal fungi (Smith and Read, 2008). Arbuscular mycorrhizal fungi (AMF) overcome the problem of low P diffusivity by growing their hyphae into soil zones not yet depleted in P and consequently exploiting a much larger soil volume than roots. While these associations are usually beneficial for both organisms, AMF can also negatively affect plant growth, depending on environmental conditions and the type of AMF-plant combination (Johnson et al., 1997; Smith et al., 2009). With increasing concentrations of soil P that is directly available to the roots, there is less benefit of the mycorrhizal association for the plant and thus, mycorrhizal root colonization and production of extraradical mycorrhizal hyphae generally decrease (Smith et al., 2011).

Phosphorus is usually distributed heterogeneously in soil, also at the scale of a single root system (Jackson and Caldwell, 1993). This can be an important factor for plant P acquisition. A given amount of soluble P tends to be more easily available for uptake if it is locally concentrated than if it is homogeneously distributed within the volume of soil accessible to the roots (Kume et al., 2006). This beneficial effect of heterogeneous P
distribution is attributed to the following reasons: (a) Sorption strength generally decreases with increasing concentration, and higher P concentration gradients result in locally larger diffusive fluxes of P in the soil solution (Kovar and Barber, 1989). (b) Local enrichment allows to reduce the average length of diffusive pathways for soil-root transfer of P by preferential root allocation (morphological plasticity) in P-enriched soil patches (Robinson, 1994). (c) It also allows for increased P uptake efficiency by concentrating P membrane transporter activity in zones of high soil P availability (physiological plasticity) within less root mass than in case of homogeneous soil P distribution (Jackson et al., 1990). (d) Similarly, the efficiency of P acquisition by plants can be increased by preferential growth of extraradical mycorrhizal hyphae (Hodge, 2005). Preferential allocation of roots into nutrient-enriched patches is the phenomenon that is best investigated among the strategies of plants to adapt to heterogeneous distribution of resources in soil. The degree to which the spatial distribution of roots responds to the uneven distribution of a nutrient in soil can be quantified by the so-called ‘precision of root allocation’, defined as the difference in root mass density between a P enriched patch of soil and an unfertilized control patch in relation to the overall root mass density (Einsmann et al., 1999). It depends on plant species and plant nutrient status (Ma and Rengel, 2008; Robinson, 1994), as well as on nutrient type, degree of heterogeneity and patch sizes (Farley and Fitter, 1999; Robinson, 1994; Wijesinghe and Hutchings, 1999).

Preferential allocation of mycorrhizal hyphae and effects of AMF on plant growth in soil with heterogeneous P distribution has been investigated much less than preferential root growth. Several AMF species were found to grow extraradical hyphae preferentially into P-enriched soil compartments (Cavagnaro et al., 2005; Shi et al., 2011). However, roots were excluded from the P-enriched soil by screens, as the objective was to study the potential contribution of AMF to plant P acquisition under conditions of exclusive P availability to AMF and not hyphal allocation in unrestrained competition with plants roots, which may have
led to a different outcome of the experiment (Smith et al., 2009). Hodge (2005) hypothesized that AMF may be more flexible in responding to temporary patches of increased nutrient availability than roots, as the metabolic cost of hyphal length growth is much less because of their smaller diameter. Providing assimilates for preferential allocation of AMF hyphae could therefore be more economical for plants associated with AMF than investing into own root growth to explore and exploit P-enriched patches (Tibbett, 2000), given that ‘delegating’ soil P mining to AMF would have the additional advantage that the roots would have more freedom to forage for other nutrients and water in soil zones with low P availability. Investigating effects of AMF on preferential root growth in P-enriched patches accessible to roots and mycorrhizal hyphae, Cui and Caldwell (1996) in fact found that preferential allocation of Agropyron desertorum roots into N and P enriched patches was decreased in mycorrhizal compared to non-mycorrhizal plants and that mycorrhization increased P uptake from soil with heterogeneous, but not with homogeneous P distribution. The authors suggested that extraradical mycorrhizal hyphae pre-empted the P-enriched soil and eventually improved plant P uptake, but did not investigate the abundance of extraradical or intraradical hyphae. On the other hand, there may also be conditions in which locally increased P supply makes direct P uptake by the roots more efficient for a plant than P acquisition through AMF hyphae, as the strength of P adsorption to mineral or organic soil phases decreases with concentration.

Using Lotus japonicus (L.) as a model plant, the aim of this study was to investigate interactions between plant growth, spatial allocation of roots, root colonization by AMF and development of extraradical hyphae in soil with heterogeneous P distribution. The research questions were: (a) Does plant growth enhancement by AMF depend on the spatial distribution of soil P? (b) In case of preferential root allocation into P-enriched soil, how is it influenced by AMF? We addressed these questions by performing a factorial container
experiment with constructed soil P distributions, comparing treatment combinations with heterogeneous and homogeneous P distribution at different levels of total P application and three mycorrhizal treatments of the sterilized test soil: no AMF inoculation, inoculation with *Glomus intraradices*, and reinoculation with indigenous mycorrhizal fungi. No screens were used to separate P-enriched from untreated P-poor soil, so all soil was accessible for both the roots and AMF hyphae.

3.2. Materials and Methods

3.2.1. Soil and AMF treatments

The experimental soil was a pleistocene sediment extracted from the forefield of an open cast mine near Cottbus (Welzow Süd, Germany). The soil consisted of 88.9 % sand, 8.8% silt and 2.3% clay, the organic carbon content was 0.17% and the calcium carbonate content 1.34 %. Bicarbonate-extractable (Olsen *et al.*, 1954) P was low with 1.9 mg kg\(^{-1}\) soil, the pH was 8.8. The soil was sterilized by gamma-irradiation (25-75 kGy) and inoculated with AMF-free solution extracted from non-sterilized soil by filtering a soil-water suspension with a 45 μm mesh. The aim of this inoculation was to re-introduce the non-fungal indigenous microbial soil flora. After this inoculation, the soil was partitioned into three batches, one for the following three rates of P application in form of Ca-monophosphate (Ca(H\(_2\)PO\(_4\))\(_2\).H\(_2\)O) solution: 0, 20 or 60 mg P per kg soil. Each soil batch was further partitioned into three sub-batches for inoculation with indigenous AMF (‘ind. AMF’ treatment), *G. intraradices* (‘G. intra’ treatment) and non-viable AMF (‘no AMF’ treatment) In all three AMF treatments, inoculum consisting of spores, colonized root fragments (chopped to less than 2 cm in length) and soil attached to the roots as well as extraradical mycelium fragments was thoroughly mixed into the sterilized soil (w/w) at a ratio of 5 g inoculum per 100 g mixture. The inoculum of *G. intraradices* had been produced under
glasshouse conditions in open-pot cultures of *G. intraradices* Schenck & Smith isolate BEG 158 using potting mix planted with leek (*Allium porrum L.*). The inoculum of indigenous AMF was obtained from a previous pot culture of *L. corniculatus* using the experimental soil. For the no AMF treatment, indigenous AMF inoculum obtained from this culture was applied after gamma sterilization.

### 3.2.2. Filling of containers

Teflon-coated aluminium containers of 27x27x1.4 cm size (L x H x B) were used for the experiments. To fill them with the experimental soils, the containers were layed down on one side and removed the upward looking lateral wall of the other side. Then, P- and AMF-treated soil (see above) was taken from the respective (sub)batches and filled into the containers in three vertical bands or sections of equal width (9 x 27 cm), according to the scheme of the selected treatment (see below). Corresponding to the position of the sections, when looking from above at the open container, they will be referred to as ‘left’ (LS), ‘middle’ (MS) and ‘right’ (RS) sections. The mycorrhizal inoculant was always the same in the three sections of a given container and differed only between treatments. Heterogeneous conditions were established only with regard to P distribution in the respective treatments. In the 2 homogeneous P treatments (see next section) all soil filled into the three sections of a container had the same P concentration. In the 2 heterogeneous P treatments (see next section) soil fertilized with either 20 or 60 mg P kg⁻¹ was filled into the right container sections and soil with no added P into the left and middle sections. No barriers or screens were installed to separate the three soil sections physically. After filling, the lateral wall was mounted and the container put into upright position again. The total mass of soil filled into each container was determined by weighing. It varied between 1.33 and 1.45 kg.
3.2.3. Experimental design

Three AMF treatments were applied in combination with 4 P treatments in a fully randomized factorial design. For each of the mycorrhizal treatments (no AMF, G. intra, ind. AMF), 4 P treatments were established: 0 P HOM (homogeneous soil with no P addition); 28 P HOM (homogeneous soil with addition of 20 mg P kg⁻¹, equivalent to 28 mg P per container); 9.3 P HET (heterogeneous filling using soil with 20 mg P kg⁻¹ for the right container section, equivalent to 9.3 mg P per container); and 28 P HET (heterogeneous filling using soil with 60 mg P kg⁻¹ for the right container section, equivalent to 28 mg P per container. Thus, one of the heterogeneous treatments (9.3 P HET) had the same soil P concentration in the fertilized soil section as in the homogeneous fertilization treatment (28 P HOM), while in the other (28 P HET) the same amount of P was applied per container, but concentrated in one third of the soil packing. Each of the 12 treatment combinations was replicated 4 times.

3.2.4. Plant establishment and growth conditions

Seeds of *L. japonicus* ecotype GIFU (Department of Molecular Biology, University of Aarhus, Denmark) were sterilized with 1 % of hypochlorite solution (diluted commercial bleach) and germinated on filter paper. A single seedling was planted in the middle of each aluminium container, equidistant to the two lateral sections LS and RS. Plants were grown in a climate chamber with relative aerial humidity of 60 % with a 16 h : 8 h day/night cycle with 21/16 °C temperature, respectively. During the day the photon flux was 250 µmol m⁻² s⁻¹. Plants were watered to 50 % waterholding capacity (approx. 100 hPa water suction). All plants in the experiment developed functioning nodules for N-fixation.
3.2.5. Sampling and chemical soil analysis

For initial characterization of the experimental soil, soil texture was determined using the hydrometer method after wet oxidation of the organic matter using hydrogen peroxide (FAL, 1996b). Organic matter content was determined using the dichromate method (FAL, 1996c) and carbonate content was measured by volumetric analysis of the CO₂ that evolved after addition of 4 M HCl to the soil (FAL, 1996a). Soil pH was measured in a 1:2.5 soil water suspension (FAL, 1996d). At the end of the experiment after 104 days of growth, shoots were harvested by clipping them at the soil surface, dried to constant weight at 60°C, weighed and stored in an exsiccator until they were analyzed for P and other nutrient concentrations. After removal of the shoots, soil was collected from two adjacent points in each section at 4, 8, 12 and 16 cm depth using a thin-walled metal tube. These 8 samples of each section were pooled to one composite sample per section and frozen at -23°C. The soil packings were then divided with a knife into right, middle and left sections according to the original filling design, and the roots were sampled by thoroughly washing the soil from the roots. The roots were imaged using a scanner (EPSON, Expression 10000XL) and frozen at -23°C. Three days later, the root samples were lyophilized to constant weighed, cut into small pieces and stored in an exsiccator until they were analyzed for AMF, dry weight, root length, and nutrient concentrations. Root samples were digested in 15 ml 69 % HNO₃ in a heating block at 120°C. Phosphorus was analyzed in the experimental solutions by means of ICP-OES (Vista-MPX, Varian). Nitrogen was analyzed using a CN analyzer (Flash EA, Thermo Electron Corporation). In the “0 P HOM non-myc” treatment no N analysis was possible because all root material was consumed by the other analyses. Root length was determined in the scanned root images using Win-Rhizo software (Regent Instruments Canada Inc., Ottawa, Canada). Except for imaging and AMF analysis, shoot samples were analyzed in the same way as root
samples. Like the plant samples, the soil samples were lyophilized and stored in an exsiccator until analysis for dry weight as well as P and N concentrations.

3.2.6. AMF analysis of root and soil samples

The abundance of individual AMF taxa in root and soil samples was assessed by means of quantitative real-time PCR (qPCR). Molecular quantification is now firmly established in microbial ecology and also frequently applied to quantify AMF (Gryndler et al., 2012; Jansa et al., 2008; Kiers et al., 2011; Thonar et al., 2012). While there are disparities between staining-microscopy and qPCR methods (Gamper et al., 2008; Jansa et al., 2008; Krak et al., 2012; Lendenmann et al., 2011), it is tedious to discuss which method gives ‘better’ results, as they measure different biological entities and have both their methodological problems, which need to be controlled and accounted for in the interpretation of results. Here we were interested in the assessment of treatment effects on AMF abundance and not in comparing AMF abundances in our experimental systems with results of other experiments, plant species or soil types. Thus, we used molecular quantification as method of choice, following Thonar et al. (2012). Briefly, subsamples of the lyophilized soil and root samples were milled in a ball mill MM 200 (Retsch). Then, DNA was extracted from aliquots of 15-20 mg milled root sample and 500 mg milled soil sample using the DNeasy Plant Mini Kit (Qiagen) and the PowerSoil DNA Isolation Kit (MoBio), respectively. All samples were spiked with 2x10^{10} copies of internal DNA standard (linearized plasmid carrying a fragment of the Cassava Mosaic Virus, GenBank accession AJ427910) before DNA extraction. The analyses were carried out using Roche chemistry (Lightcycler TaqMan Master) and Lightcycler 2.0 (Roche). Preliminary analyses showed that Glomus mosseae and G. claroideum were detectable using the existing qPCR markers in root samples from the soil re-inoculated with indigenous AMF. The markers used were targeting the nuclear large ribosomal subunit (LSU) of Glomus intraradices, G. claroideum, and G. mosseae. The marker system for quantification of internal
standard recovery was described by Thonar et al. (2012), and the mitochondrial LSU marker for *G. intraradices* (mt5) was described by Couillerot et al. (2012). The results of the qPCR (Cq values) were converted to copy numbers of individual AMF taxa per unit weight of roots and corrected for extraction efficiency using the internal standard recovery as described in Thonar et al. (2012).

3.2.7. Data analysis and statistics

The precision (Pr) of root and AMF allocation was determined by calculating the differences in root dry weight, root length, extraradical and intraradical LSU copy numbers between RS and LS and dividing them by the total of the respective root or AMF parameter for the entire container. In addition, we estimated the precision of extraradical and intraradical hyphae allocation relative to root mass by taking the respective differences in LSU copy numbers per unit root mass between the two lateral sections RS and LS and dividing them by the average density of the respective LSU copies in the entire container (i.e. total numbers of LSU copies in the container divided by total root mass). Using regression analysis, p-values were calculated to assess whether the precision of root and AMF allocation calculated in terms of these parameters was significantly different from 0 or not, taking p < 0.05 as criterion for non-random, i.e. preferential allocation.

Differences between treatments were assessed by Analysis of Variance (ANOVA) using the statistical software package R (R developmental Core Team, 2008). For shoot and root dry weights the analysis was performed on square root transformed data in order to fulfill the normal distribution assumption of the test. For the same reason, the analysis of LSU copy numbers was performed on log-transformed data. Pairwise comparison of differences between treatment means were determined by means of the Least Significant Differences (LSD)
method, if main effects and interactions between the main effects were significant (ANOVA); otherwise a Bonferroni correction was applied.

![Figure 1](image)

**Figure 1.** Shoot and root dry weights of *L. japonicus* grown in soil without AMF (no AMF), soil inoculated with *G. intraradices* (*G. intra*) and soil reinoculated with indigenous AMF (ind. AMF) after sterilization for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). Error bars give the standard error of the means. Different letters indicate significantly different means according to least significant difference (LSD) multiple range test following significant ANOVA (p<0.05).

### 3.3. Results

#### 3.3.1. Biomass production

Treatment effects on shoot and root biomass were similar. Mycorrhization led to a strong increase in biomass production (Fig. 1), except for the treatment with *G. intraradices* in
combination with heterogeneous P application, where biomass did not differ from the same P treatment without AMF inoculation. Compared to the respective treatments without P fertilization, P application always increased biomass, except for the heterogeneous P applications to soil inoculated with *G. intraradices*. There was no significant difference in biomass production between the treatments with high heterogeneous and homogeneous P applications.

**Figure 2.** Phosphorus concentrations of shoots and roots of *L. japonicus* grown in soil without AMF (no AMF), soil inoculated with *G. intraradices* (G. intra) and soil reinoculated with indigenous AMF (ind. AMF) after sterilization for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). Error bars give the standard error of the means. Different letters indicate significantly different means as determined by Bonferroni corrected multiple comparison (p<0.05) for shoot P concentrations and by least significant difference (LSD) multiple range tests following ANOVA (p<0.05) for root P concentrations.
supply (i.e. when the same total amount of P was applied) in combination with indigenous or no AMF, while biomass production was lower in the heterogeneous than in the homogeneously fertilized treatments with G. intraradices.

3.3.2. P concentration

Treatment effects on root and shoot P concentrations were in many respects similar to the described pattern of biomass responses, although the effects on P concentrations were generally less distinct than those on biomass. Overall, mycorrhization and P application increased plant P concentrations. For the root P concentrations also the interaction of AMF and P treatment had a significant effect (p<0.05, ANOVA, Fig. 2). Pairwise comparison (Bonferroni corrected for the shoots and LSD corrected for the roots, p<0.05) revealed a significant effect of mycorrhization only in the case of the unfertilized control, where inoculation with G. intraradices increased shoot and root P concentration compared to the non-mycorrhizal plants. Application of P had a significant effect on shoot and root P concentration only in the non-mycorrhizal plants when P was applied heterogeneously at the higher of the two experimental levels. No P treatment effect on shoot or root P concentrations was found in the mycorrhizal plants.

3.3.3. Precision of root allocation

Treatment effects on the precision of root allocation were similar for root length and root dry weight (Fig. 3, Table 1). Both, root biomass and root length were significantly increased in the P-enriched soil sections in the heterogeneous P treatments when no viable AMF had been added to the soil. The precision of root biomass allocation was also increased in the treatments with low-level heterogeneous P application, when the soil was inoculated with indigenous AMF. This effect was only rather weak, however, and similar in magnitude as the slightly negative precision of root length allocation in the homogeneously fertilized
treatment in the absence of mycorrhizae. As soil conditions were homogeneous in the latter case, we consider these effects as false positives.

*Figure 3.* Root biomass (a) and root length (b) in the three sections of the containers (LS left, MS middle, RS right) for *L. japonicus* grown in soil without AMF (no AMF), soil inoculated with *G. intraradices* (*G. intra*) and soil reinoculated with indigenous AMF (ind. AMF) after sterilization for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). In the heterogeneous P treatments P was added only to the right sections (RS).
3.3.4. Abundance of mycorrhizal gene copies

In the treatments inoculated with *G. intraradices* alone, P fertilization level and distribution did neither affect the average densities of mitochondrial or nuclear intraradical LSU copies per unit root mass, nor the total numbers of mitochondrial or nuclear extraradical LSU copies per container (Table 2). The only significant P treatment effect on LSU copy numbers of *G. intraradices* was that the density of extraradical mitochondrial LSU copies per

**Table 1.** Root biomass and root length allocation in *L. japonicus* grown in soil without AMF (no AMF), soil inoculated with *G. intraradices* (*G. intra*) and soil reinoculated with indigenous AMF (ind. AMF) for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). The precision values give the differences in root length or root biomass between the right and the left section of a container divided by the total root length or biomass in all three sections (i.e. including middle section) of the container. Positive values indicate preferential root allocation to the P-enriched soil sections in the heterogeneous P treatments. Regression analysis was used to determine if precision was significantly different from zero. *p < 0.05, **p < 0.01. Different letters indicate significant differences between means according to least significant difference (LSD) multiple range post-hoc test following ANOVA (p<0.05).

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<td>0.07</td>
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</table>
unit root mass was lower in the treatment with homogeneous P fertilization than in the treatments with heterogeneous or no P application. The densities of intraradical LSU copies and of extraradical nuclear LSU copies per unit root mass showed a similar, though at p=0.05 statistically not significant effect (Table 2). In the treatments with indigenous AMF, P fertilization had some effect on the density of intraradical LSU copies per unit root mass and on the total number of extraradical LSU copies produced by G. mosseae, but not on the LSU

Table 2. Natural logarithms of the numbers of ribosomal subunit (LSU) copies of AMF hyphae in roots (intraradical) and soil (extraradical) in the 4 P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). LSU copy numbers for G. claroideum and G. mosseae were determined in the treatments inoculated with the indigenous AMF mixture. Mitochondrial and nuclear LSU copy numbers of G. intraradices were determined only for the treatments in which the soil was inoculated with G. intraradices alone. Different letters indicate significantly different means according to least significant difference (LSD) multiple range post-hoc test following significant ANOVA (p<0.05). Both root and soil samples from nonmycorrhizal treatments yielded undetectable LSU copy numbers for all tested AMF taxa and thus are not shown here.

<table>
<thead>
<tr>
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<th>Intraradical Hyphae</th>
<th>Extraradical Hyphae</th>
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<tbody>
<tr>
<td></td>
<td>LSU copy number</td>
<td>LSU copy number</td>
</tr>
<tr>
<td></td>
<td>mg⁻¹ root mass</td>
<td>mg⁻¹ soil</td>
</tr>
<tr>
<td><strong>G. claroideum</strong></td>
<td></td>
<td></td>
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<tr>
<td>0 P HOM</td>
<td>11.02 (±0.27)</td>
<td>6.46 (±0.44)</td>
</tr>
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</tr>
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<td>10.67 (±0.31)</td>
<td>6.94 (±0.51)</td>
</tr>
<tr>
<td>28 P HET</td>
<td>10.13 (±0.31)</td>
<td>7.20 (±0.44)</td>
</tr>
<tr>
<td><strong>G. mosseae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 P HOM</td>
<td>8.56 (±0.60)a</td>
<td>3.56 (±0.48)a</td>
</tr>
<tr>
<td>28 P HOM</td>
<td>11.34 (±0.60)</td>
<td>7.32 (±0.48)</td>
</tr>
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<td>11.71 (±0.70)</td>
<td>6.60 (±0.56)</td>
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<td>11.21 (±0.70)</td>
<td>6.36 (±0.48)</td>
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<td><strong>G. intraradices</strong></td>
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</tr>
<tr>
<td>(nuclear DNA)</td>
<td>0 P HOM</td>
<td>13.42 (±0.36)</td>
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<td></td>
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<td>13.50 (±0.36)</td>
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<td>6.60 (±0.31)</td>
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<tr>
<td>28 P HET</td>
<td>13.15 (±0.38)</td>
<td>6.67 (±0.31)</td>
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of *G. claroideum* (Table 2). The densities of intraradical and extraradical *G. mosseae* LSU copies per unit root mass as well as the density of extraradical *G. mosseae* LSU copies per unit soil mass were lower in the unfertilized treatment than in the fertilized treatments.

3.3.5. *Precision of mycorrhizal gene copy numbers and root-related densities*

The P treatments had generally no influence on the precision of LSU copy allocation, neither for the nuclear nor the mitochondrial genes, (Table 3, Fig. 4, Fig. 5). Only the precision of extraradical LSU copies produced by *G. claroideum* per unit root mass showed a significant P fertilization effect, as relatively more copies were allocated in the lateral soil section without than in the section with P application in the heterogeneous treatments. This effect was not strong, however, and of similar magnitude as also observed in the homogeneous P treatments with *G. intraradices*, where extraradical nuclear LSU copies were more abundant without detectable reason in the left than in the right section.
Table 3. Precision of intraradical and extraradical hyphae allocation, analyzed in terms of intraradical and extraradical LSU copy numbers per unit root mass, as well as extraradical LSU copy numbers per unit soil mass for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). LSU copy numbers for G. claroideum and G. mossea were determined in the treatments inoculated with the indigenous AMF mixture. Mitochondrial and nuclear LSU copy numbers of G. intraradices were determined only for the treatments in which the soil was inoculated with G. intraradices alone. The precision values give the differences in respective copy numbers between right and left lateral soil sections divided by the respective total LSU copy number in the entire container. Positive values indicate preferential hyphae allocation in the P-enriched soil sections in the heterogeneous P treatments. Regression analysis was used to assess if precision was significantly different from zero: * p < 0.05, ** p < 0.01. Different letters indicate significant differences between means according to least significant difference (LSD) multiple range post-hoc test following ANOVA (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>LSU copy number</th>
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<tr>
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<td>Prec.</td>
<td>Std.e.</td>
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<tr>
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Figure 4. Abundances of *G. intraradices* hyphae, given in terms of intraradical mitochondrial (a) and extraradical mitochondrial (c), as well as intraradical nuclear (b) and extraradical nuclear (d) LSU copy numbers, in soil inoculated with *G. intraradices* after sterilization in the three sections of the containers (LS left, MS middle, RS right) for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). In the heterogeneous P treatments P was added only to the right sections (RS).
Figure 5. Abundances of $G$. claroideum (a,c) and $G$. mosseae (b,d) hyphae, given in terms of Intra-radical (a,b) and extra-radical (c,d) LSU copy numbers, in soil reinoculated with indigenous AMF after sterilization in the three sections of the containers (LS left, MS middle, RS right) for the four P treatments: no fertilization (0 P HOM), homogeneous P supply (28 P HOM), low heterogeneous P supply (9.3 P HET), high heterogeneous P supply (28 P HET). In the heterogeneous P treatments P was added only to the right sections (RS).

3.4. Discussion

3.4.1. AMF and P fertilization enhanced growth of Lotus japonicus

The results show that the growth of $L$. japonicus was severely inhibited by lack of P in the unfertilized soil and in absence of viable AMF. Given the low level of available P in the
experimental soil, this was in agreement with expectation. Inoculation with AMF without additional P supply reduced this limitation to a similar extent as the high rate of total P application in our experiment. The fact that P fertilization in combination with the indigenous AMF treatment resulted in still larger biomass than without AMF treatment suggests that none of the experimental treatments with P applications alone was sufficient to fully alleviate the P limitation of plant growth. In fact, an average soil P fertilization rate of 20 mg kg\(^{-1}\) is still rather low compared to rates required for maximum growth that have been reported for other legumes (Abbott et al., 1984; Demiranda et al., 1989; Schweiger et al., 1995).

While P was clearly limiting the growth of mycorrhizal plants grown in unfertilized soil, it cannot be deduced from our results whether P was still growth-limiting in the heterogeneous P treatments when AMF were present. Root P concentrations were higher and shoot P concentrations not lower in the heterogeneous P treatments with \textit{G. intraradices} than with indigenous AMF, while more biomass was produced in the latter. This could mean that a factor other than P became limiting with increasing P supply in the heterogeneous treatments and that the indigenous AMF was more efficient to remove this limitation than \textit{G. intraradices}. Another possible explanation, further discussed below, is that \textit{G. intraradices} itself limited P acquisition by the plants and that the high P concentrations in the root samples were due to P stored in intraradical fungal tissue not available to the plants (Kiers et al., 2011). The partitioning of P between fungus and root cells depends on P supply and differs widely among AMF species and could explain differences in total root P concentrations between the mycorrhizal treatments in our experiment.
3.4.2. Preferential allocation of roots into P-enriched patches was inhibited by mycorrhizal fungi.

In the absence of AMF, root growth was preferentially allocated to P-enriched soil sections in the heterogeneous P treatments. With AMF inoculation this response disappeared. This effect may be attributed to the enhanced availability of soil P also in the unfertilized sections of the containers for roots associated with AMF. In mycorrhizal roots the contrast in P availability between P fertilized and unfertilized soil would have been considerably reduced in comparison to the treatments without AMF, as judged from the magnitude of the AMF effects on plant growth and P uptake in the treatments without fertilization. Ma and Rengel (2008) found in *Triticum aestivum* that the precision of root allocation decreased with enhanced P status. Also Cui and Caldwell (1996) who studied root length growth in *Agropyron desertorum* found that preferential root allocation into P-enriched soil patches decreased. In the presence of AMF. But in the study of Cui and Caldwell (1996) this AMF effect was rather moderate in comparison to our experiment, where preferential root allocation nearly or even completely disappeared in presence of AMF. This strong AMF effect on preferential root allocation is particularly remarkable in the case of the *G. intraradices* treatments. As the P fertilization effects show, P was still limiting plant growth even in the presence of AMF when no P fertilizer was supplied, and thus some preferential root allocation, as observed in the heterogeneous low P treatment with indigenous AMF, could still have been beneficial for the plants by enhancing P acquisition.

An explanation for the particularly strong inhibition effect of *G. intraradices* on preferential root allocation may be related to its apparent capacity to downregulate or even inhibit P transporters in root cell membranes (Smith *et al*., 2004). As a result, the sensitivity of roots for the detection of soil P gradients and thus the plant’s capacity to respond to them with preferential growth could be strongly reduced. Smith *et al.* (2004) showed that in
association with *G. intraradices*, *Linum usitatissimum* L., *Medicago trucatula* L. and *Lycopersicon esculentum* Mil. acquired 80-100 % of their P via the fungus, while fungal contribution to P acquisition was much less in association with *G. caledonium* and *Gigaspora rosea*. The sensitivity of plants to detect P gradients in soil might also be reduced due to P depletion in the rhizosphere as a result of hyphal P uptake. Li *et al.* (1991) found that P concentrations around mycorrhizal white clover roots were much lower than around non-mycorrhizal roots for white clover.

3.4.3. **Plants associated with indigenous AMF mixture can cope better with heterogeneous P distribution in soil than in association with *G. intraradices* alone**

In soil with homogeneous P distribution, the treatments with *G. intraradices* or the mycorrhizal mixture did not significantly differ in their effects on plant biomass and P accumulation, suggesting that both mycorrhizal treatments had similar effects on plant P acquisition under these conditions. In contrast to the homogeneous treatments, heterogeneous P fertilization increased biomass production only in the treatments with indigenous AMF mixture, but not in the *G. intraradices* treatments. The difference suggests that there were AMF species in the mycorrhizal mixture that were more beneficial for the experimental plants than *G. intraradices* in soil with heterogeneous P distribution. This would be consistent with the hypothesis that *G. intraradices* had an inhibiting effect on the direct uptake of soil P by the roots and that P absorbed by the fungus was partially retained in the fungal tissue. The plants growing in the homogeneously fertilized treatment had the advantage that they had access to P-enriched soil right from the beginning of the experiment and thus could have developed a stronger root system than in the other P treatments before being colonized by the fungus.
The difference between *G. intraradices* and indigenous AMF treatments may have also been due to AMF species in the mixture of indigenous AMF that were able to develop faster and explore the P-enriched soil at an earlier stage in the experiment than *G. intraradices*. Jakobsen *et al.* (1992) demonstrated that *Acaulospora laevis* hyphae spread faster and further away from *Trifolium subterraneum* roots than hyphae of *Glomus* species and acquired more P at greater distances from the root surface. Preferential allocation of hyphae may have been another factor contributing to the difference in AMF effects on plant growth in the heterogeneous P treatments. Although no preferential hyphal growth was detected in the analyzed AMF species, there may have been other species in the inoculum of indigenous AMF that responded by preferential hyphal allocation in the P-enriched soil sections.

3.4.4. *The effect of P fertilization on mycorrhizal abundance differed between AMF species*

If mitochondrial LSU copy numbers are taken as a proxy for fungal biomass, then homogeneous P fertilization did not stimulate extraradical hyphal growth of *G. intraradices*, although it promoted root and shoot biomass production. This is consistent with the findings of previous studies and also with optimal resource utilization theory, which predicts that plants should devote less assimilates to the fungus and more to the roots when metabolic costs decrease for direct P acquisition by the roots (Smith 2011). It is not possible to determine if there was a similar trend with P application in the relationship between root and fungal biomass in the indigenous AMF treatments, because only two AMF species out of an unknown number of AMF species colonizing *L. corniculatus* were analyzed; but the results show that P effects on hyphal growth can differ between AMF species. While there was no significant influence of P fertilization on the abundance of *G. claroideum*, the abundance of *G. mosseae* was strongly increased by P fertilization. Our results are in line with previous findings that P fertilization effects on fungal growth can vary considerably between AMF species (Graham, 2000; Cavagnaro, 2005) and that in soils extremely poor in available P low
rates of P fertilization can even increase extraradical and intraradical hyphal production in a mycorrhizal fungus (Abbott et al., 1984). When roots are colonized by a mixture of AMF species plants can selectively reward those fungi with assimilates that deliver most P per unit of carbon, while withholding assimilates from AMF species providing P at higher C costs (Kiers et al., 2011). As plant assimilate allocation to P acquisition from AMF depends on plant P status and direct soil P uptake by roots, it can be expected that the relative competitive strength of AMF species will change with soil P availability and accessibility.

The lack of clear treatment effects on the spatial distribution of LSU copies between fertilized and unfertilized soil indicates that the analyzed AMF species did not respond to soil P heterogeneity with preferential hyphal growth. This was not to be expected under conditions where plant growth was still P-limited. Few experiments have investigated preferential hyphal growth in soil. Their results indicate that hyphal growth patterns vary considerably with experimental conditions, host plant and AMF species. Contrary to our results, Gavito and Olsen (2003) found increased hyphal length density in soil patches enriched in P in G. intraradices and Scutellospora calospora associated with Trifolium subterraneum. Also Shi et al. (2011) showed that G. intraradices, G. etunicatum and G. mosseae preferentially grew hyphae into P-enriched soil patches, while Cavagnaro et al. (2005) found preferential hyphal growth in G. intraradices, but not in G. mosseae or Gigaspora rosea. In contrast to our study, however, plant roots were kept out of the P-enriched soil patches by means of screens that only allowed the fungal hyphae to pass. Thus, there was no direct competition between roots and hyphae for P in these patches, which might have favoured preferential hyphal growth into them to exploit this ‘monopole position’.

The main finding of this study is that interactions with mycorrhizal fungi may completely override preferential root growth responses of plants to P patchiness in soil. It appears that this effect was primarily due to the fact that the plants could acquire sufficient P
via the AMF to satisfy their demand for growth. The advantage of such a ‘delegation’ of nutrient acquisition is that it gives roots more freedom to explore a larger soil volume and forage for other resources in demand for growth. Our results furthermore show that hosting a larger variety of mycorrhizal fungi was most beneficial for the plants under conditions of heterogeneous P distribution, while there was no added advantage compared to the colonization with one species alone in the homogeneous P treatments, indicating that the main benefit for plants of association with multiple-species AMF in comparison to monocolonization is an enhanced capacity of the plants to adapt to variable soil conditions.

3.5. Acknowledgements

This study was part of the Transregional Collaborative Research Centre 38 (SFB/TRR 38). We gratefully acknowledge financial support by the German Research Foundation (DFG) and the Ministry of Science, Research and Culture of Brandenburg (MWFK, Potsdam).

3.6. References


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FAL, RAC, FAW. (1996d) Ph in water suspension (1:2.5) and ph in CaCl₂ suspension (1:2.5). Swiss reference methods of the Federal Agricultural Research stations, Swiss Federal Research Station FAL, RAC, FAW, Zurich, Switzerland


4. Cluster root allocation of *Lupinus albus* in soil with heterogeneous P and water distribution

Bernd Felderer, Peter Vontobel, Rainer Schulin

Submitted to *Journal of Experimental Botany*
Summary

Cluster roots are structures formed by many plants adapted to soils low in available phosphorus (P). Low soil P availability is often aggravated by dry soil conditions. In this study we investigated the combined influence of spatial heterogeneity in soil water and P distribution on the allocation of cluster root formation in *Lupinus albus*. Single plants were grown for 35 days at a low or a high rate of water supply in containers filled with a P-poor sand to which either no P was added or which was fertilized homogeneously or heterogeneously. In addition, heterogeneous distribution of water availability was established in half of the containers by using fine-grained instead of coarse-grained sand as substrate in a lateral third of the containers. Plant growth increased with water supply rate, but P fertilization had no influence on shoot and root biomass production. While there was no response in cluster root allocation to heterogeneous P application, cluster roots were preferentially allocated in the soil sections with lower water availability, when overall water supply rate was low. Total cluster root production was the same as in the treatments with homogeneous water distribution though, and only affected by initial P exposure of the developing root system. This suggests that resource allocation to cluster root growth was a systemic response to initial plant P status, while cluster root growth was stimulated in drier patches of soil when roots had access to water at the opposite side of the containers.
4.1. Introduction

Phosphorus (P) is the most limiting nutrient for plant growth in many agricultural and natural ecosystems (Hu and Schmidhalter, 2005; Vance et al., 2003). The solubility and mobility of P is generally low in soil as compared to nitrogen (Hinsinger et al., 2011). Phosphorus in soil solution is the only source of P directly available to the roots and is rapidly absorbed by the roots. Resupply of P from the bulk soil to the rhizosphere is determined primarily by diffusive but also mass flow. Limited availability of water is a further constraint of plant growth in many ecosystems (Hu and Schmidhalter, 2005; Vance et al., 2003). As not only mass flow but also diffusion coefficients of solutes decrease with decreasing soil water content, dry soil conditions can also cause or aggravate P limitation of plant growth (Bhadoria et al., 1991).

To increase P uptake from soil solution, roots often enhance P solubility in the rhizosphere by exuding organic acids such as citric acid (Gardner et al., 1983; Lamont, 2003; Shane and Lambers, 2005). Many plant species, particularly of the families Proteaceae and Fabaceae, but also others, have specialized in this strategy by developing so-called cluster roots for the acquisition of soil P fractions that are not available to “normal” roots (Lamont, 2003). Cluster roots are bottlebrush-like structures of densely haired rootlets clustered in specific sections along the axis of growing roots (Lambers et al., 2006; Purnell, 1960; Shane and Lambers, 2005). As not only P, but also metals are solubilized by organic acids, cluster roots may also be beneficial for the acquisition of metal micronutrients such as Zn and Cu. On the other hand, cluster roots are very expensive structures in terms of carbon costs, and therefore reduced production is usually found in soils with increased P availability (Lambers et al., 2006; Lamont, 2003; Neumann and Martinoia, 2002; Shane et al., 2003; Shen et al., 2005; Shu et al., 2005; Shu et al., 2007).
As for other soil resources, there is often remarkable heterogeneity in the spatial distribution of P and water in soil, even within the domain of a single root system (Farley and Fitter, 1999; Jackson and Caldwell, 1993). Preferential allocation of roots in soil patches or zones with increased P and water availability has been reported for many plant species (Kume et al., 2006; Ma and Rengel, 2008; Ma et al., 2007; Weligama et al., 2007). Little is known on such root growth responses to heterogeneous soil water distribution for plants developing cluster roots, while contrasting findings have been reported for responses of cluster root formation to P heterogeneity. Some authors found that localized P supply stimulated cluster root production (Shen et al., 2005; Shu et al., 2007) in *Lupinus albus*, while others found no stimulation (Shane et al., 2003; Shane and Lambers, 2005). Shane et al. (2003) even found that cluster root production was inhibited by locally increased P supply in *L. albus*. If the availability of soil P increases with water content and cluster root growth responds to locally increased P supply, it can be expected that also localized water supply affects cluster root production. In two studies it was found that cluster and non-cluster root growth occurred during the wet season in *Hakea* and *Banksia* species, anticipating shoot growth (Lamont, 1976; Lamont, 2003). In the *Hakea* species cluster root growth was induced by irrigation during the dry season (Lamont, 1976).

These results suggest that localized supply of water has a stimulating effect on cluster root growth. However, effects of heterogeneities in soil water distribution on spatial cluster root allocation patterns apparently have not been studied so far. This is surprising as water is often considered the main driver of root allocation (Hodge, 2010). Difficulties in establishing and maintaining sufficiently well-defined heterogeneities in soil water distribution over an adequate period of root growth may be a major reason for this lack of experimental data. In comparison to P, water is highly mobile in soil and will quite rapidly redistribute if it is added locally. In addition, the storage capacity of soil for water is generally small in comparison to
Cluster root allocation of *Lupinus albus* in soil with heterogeneous P and water distribution

the consumption of water by growing plants making frequent replenishment necessary during a root growth experiment. Recently, neutron radiography (NR) was shown to provide an elegant way to cope with these difficulties in climate chamber experiments (Carminati *et al.*, 2010; Menon *et al.*, 2007; Moradi *et al.*, 2009; Oswald *et al.*, 2008). This imaging technique is based on the absorption and scattering of neutrons by hydrogen as the main attenuation agent in soil. It is non-invasive and allows simultaneous imaging of roots and soil water distributions, provided that there is sufficient contrast in water content between soil and roots. A plant species highly suitable to investigate interactions between roots and soil water by means of NR is *Lupinus albus* (Menon *et al.*, 2007). It is also a model plant species for cluster root studies, having the advantage that it forms no mycorrhizal associations that could confound cluster root effects on P uptake (Lambers *et al.*, 2006; Shane and Lambers, 2005). Being a legume it can form an association with nitrogen fixing rhizobia and then does not depend on soil nitrogen status.

Taking advantage of the potential of NR imaging, the objective of this study was to investigate the effects of spatially heterogeneous soil water and P availability on the initiation of cluster root formation in *Lupinus albus* under different water supply conditions. Plants grown in sand-filled containers with constructed heterogeneity in soil P and/or water availability were subjected to different combinations of the following experimental treatments: (i) heterogeneous versus homogeneous P application and no P addition, (ii) heterogeneous versus homogeneous soil water distribution; (iii) low versus high rate of irrigation. Root growth and soil water distribution were monitored over time by repeated NR imaging. At the end of the experiment, plants were harvested and the partitioning of normal roots and cluster roots between soil sections of different water and P availability was determined.
4.2. Materials and Methods

4.2.1. Experimental substrates

The two experimental substrates used in this study both contained 85 % (w/w) P-poor pleistocene sediment extracted from the forefield of an open cast mine near Cottbus (Welzow Süd, Germany) and 15 % (w/w) of either a coarse grained (300-900 µm) or of a fine grained silica sand (40-70 µm). The particle size distributions of the two substrates, briefly referred to as fine sand and coarse sand in the following, are given in Figure 1, and chemical properties are given in Table 1. The water retention curves of the two experimental substrates (Fig. 2) show that water storage capacity (the change in water content per unit change in soil water potential) was higher in the coarse sand than in the fine sand between 0 and 80 hPa and

![Particle size distributions](image)

**Figure 1:** Particle size distributions of the two experimental substrates, determined by means of laser diffraction particle size analysis using an LS 13 320 Beckman Coulter Counter.
4.2.2. Experimental setup

between 160 and 220 hPa soil water tension, but lower between 80 and 160 hPa and between 220 and 690 hPa. To establish the P treatments 3 levels of P fertilization were applied as Ca-monophosphate (Ca(H2PO4)2,H2O) to the coarse sand (0, 10 and 30 mg P per kg sand) and 2 P levels to the fine sand (0 and 10 P per kg sand).

Table 1. Chemical properties of the two experimental substrates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coarse sand</th>
<th>Fine sand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± Standard Deviation</td>
<td>Mean ± Standard Deviation</td>
</tr>
<tr>
<td>pHCaCl2</td>
<td>7.94 ± 0.02</td>
<td>8.51 ± 0.02</td>
</tr>
<tr>
<td>EC (dS m⁻¹)</td>
<td>0.068 ± 0.004</td>
<td>0.081 ± 0.003</td>
</tr>
<tr>
<td>POlsen(mg/kg)</td>
<td>1.89 ± 0.12</td>
<td>1.87± 0.70</td>
</tr>
<tr>
<td>CaWater (mg/kg)</td>
<td>49.33 ± 0.46</td>
<td>37.03 ± 0.21</td>
</tr>
<tr>
<td>MgWater (mg/kg)</td>
<td>2.23 ± 0.44</td>
<td>1.40 ± 0.12</td>
</tr>
<tr>
<td>KWater (mg/kg)</td>
<td>10.03 ± 0.82</td>
<td>8.90 ± 0.26</td>
</tr>
<tr>
<td>NaWater (mg/kg)</td>
<td>6.53 ± 1.44</td>
<td>42.13 ± 0.84</td>
</tr>
<tr>
<td>FeDTPA (mg/kg)</td>
<td>4.44 ± 0.18</td>
<td>5.00 ± 0.07</td>
</tr>
<tr>
<td>MnDTPA (mg/kg)</td>
<td>2.78 ± 0.05</td>
<td>2.97 ± 0.09</td>
</tr>
<tr>
<td>ZnDTPA (mg/kg)</td>
<td>0.17 ± 0.017</td>
<td>0.18 ± 0.017</td>
</tr>
</tbody>
</table>

pHCaCl2: soil ph (FAL, 1996b); EC: Electrical conductivity in 1:2.5 soil water suspension; POlsen: NaHCO3 extractable P (Olsen et al., 1954); CaWater,MgWater, KWater,NaWater refer to water-extractable Ca, Mg, K and Na concentrations (FAL, 1996a); FeDTPA, MnDTPA, ZnDTPA refer to DTPA-extractable Fe, Zn and Zn concentrations (Reed and Martens, 1996);

4.2.3. Container filling

The aluminium (Al) containers had an internal size of 27 x 27 x 1.2 cm (L x H x B). The inner sides of the walls were coated with Teflon to prevent Al toxicity. During filling the containers were layed down onto one side, while the wall of the opposite, upward looking lateral side was removed. Then the substrate was filled in in three vertical sections of 9 cm
Figure 2. Water retention curves of the two experimental substrates determined by means of the pressure membrane method (Klute, 1986). The vertical lines represent calculated water suctions at medium container depth in the treatments with heterogeneous substrates (as described in the Materials and Methods section). The vertically aligned numbers give the corresponding water contents. The grey areas represent the calculated water suction ranges between maximum and minimum water contents in the high (dark grey) and low (after DAG 12, light grey) water supply treatments.
width each, using for each section the sand mixture assigned to it according to the respective treatment. No barriers separated the sections. Depending on their position when looking from above at the open container during filling, the sections will be referred to as left (LS), middle (MS) and right (RS) in the following. After filling was completed, the lateral wall was replaced, and the container was put back into upright position. Care was taken to avoid pressing of the soil and to achieve a dry soil bulk density of approximately 1.7 g cm$^{-3}$ in all containers. The net dry weight of the fillings varied between 1.33 and 1.45 kg.

4.2.4. Experimental setup

Three P treatments were established in combination with homogeneous and heterogeneous water distribution as well as high and low overall water supply rate. In the homogeneous P treatments, sand to which 10 mg P per kg sand (P hom) or no P (P no) had been added was used for all three sections. In the heterogeneous P treatments (P het), sand fertilized with 30 mg P per kg was filled into the right section and sand with no P fertilization into the other two sections, resulting in a total of 14 mg fertilized P per container, which is the same total as in the treatment with homogeneous P fertilization. For the treatments with heterogeneous water (W het) distribution, fine sand was filled into the left section and coarse sand into the other two sections. Only coarse sand was used for treatments with (horizontally) homogeneous water distribution (W hom). Treatments with high (+) and low water (-) supply were established as described below. The plant containers were arranged in 4 blocks in the climate chamber, with all treatments represented at randomly assigned positions in each block. A few plants showed atypical rooting patterns, e.g. due to tap root injury during transplanting, in the NR images. These plants were excluded from analysis.
4.2.5. Plant growth conditions

Seeds of *L. albus* (var. Amiga) were germinated on filter paper. Single seedlings were transplanted to the middle of each Al-container (equidistant to the LS and RS sections) 2 days after germination (DAG), when the tap-root was approx. 3 cm long. Except for the times, when the containers were moved to PSI for NR imaging, the plants were kept in a climate chamber at 60% humidity, a 16/8 hours day/night cycle, and 21/16 °C day/night temperature, respectively. During the day, the photon flux was 450 µmol m⁻² s⁻¹. Plants were watered from above with high or low water supply rates. In the treatments with low water supply, an initial water content of 16.3-17.6% was established on DAG 2 and subsequently no water was applied until DAG 12. From DAG 12 on, equal irrigation rates were supplied to each plant container with low water supply 2 to 3 times a week. The water supply rate between the irrigation dates ranged from 5-30 ml. In the treatments with high water supply, plants were irrigated 2-3 times a week with equal water supply rates ranging from 17-60 ml depending on irrigation date. The total amount of water supplied over 5 weeks of growth was 148 ± 5 mm in the treatments with high water supply and 84 ± 4 mm in the treatments with low water supply.

4.2.6. NR imaging

All containers were NR imaged 12, 19 and 25 days after germination. For this purpose, the containers were transported to PSI at Villigen, Switzerland, and carried back to ETH at Zürich on the following day after imaging. In the treatments with high water supply, the removable walls on the side through which the containers had been filled were temporarily taken off on the day before NR imaging to increase NR contrast between roots and soil by enhancing evaporation. Neutron radiography imaging was performed at the thermal neutron facility NEUTRA at PSI in Villigen (Switzerland). The NR set up has been described in detail.
Cluster root allocation of *Lupinus albus* in soil with heterogeneous P and water distribution by Moradi *et al.* (2009). A Li$_6$ scintillator was used as a neutron detector and a CCD camera with an array of 1024 x 1024 pixel. The nominal resolution was 0.01765 cm per pixel.

### 4.2.7. Plant harvest and analysis NR imaging

After 35 days of growth, shoots were harvested by clipping them at the soil surface. After weighing, drying and milling, the shoot samples were digested in 15 ml of 69 % HNO$_3$ in a heating block at 120°C. Phosphorus was analyzed in the experimental solutions by means of ICP-OES (Vista-MPX, Varian).

For the analysis of root parameters, the following root washing technique was applied: The front wall of the container was removed and a rectangular metal grid (27x 27 cm, mesh size: 4 mm) was placed onto the soil on the upwards looking open side of the Al container. The Al container was carefully flipped over, so that the open front site was facing downwards with the soil laying on the metal grid, loosened from the soil and removed. Then the soil was thoroughly washed through the metal grid with a shower. The roots were transferred onto a Plexiglas screen from the grid with minimal displacement from their original position in the container. Finally, roots were scanned with a conventional scanner.

### 4.2.8. Image analysis

Neutron radiography images were processed using the method of Menon *et al.* (2007) to correct for beam variation and root segmentation and to produce a bi-color image of the root system. Both the corrected NR images and the root scans were analyzed for total root length. In addition, we determined the length of root sections carrying cluster roots in the bi-color images after tracing these sections in the images by hand. All root length measurements were performed separately for the left (LS), the middle (MS) and right (RS) section, using WinRhizo. The length of root axes carrying cluster roots will be shortly referred to as cluster length in the following.
4.2.9. Assessment of water availability in heterogeneous water distribution treatments

For logistical reasons, the dynamics of short-term changes in soil moisture distribution were determined in a separate experiment. For this purpose, additional containers with heterogeneous water distribution, but no addition of P, were prepared as described before and treated with either high or low water supply. Plants were grown at ETH for 16 days under the same conditions as described before and then transferred to PSI, where they remained for the entire NR imaging period, which ended on DAG 28. At PSI, the plants were kept at room temperature in the hall of the NEUTRA facility with illumination provided through temporarily installed plant growth lamps (Nurturelite 125 W blue). In the treatments with low water supply, 10 ml of water were added on DAG 24 and 30 ml on DAG 25. In the treatments with high water supply 60 ml of water were supplied on DAG 21. The containers were NR imaged on DAG 17, 22 and 24 and 27 in the treatments with low water supply and on DAG 16, 21 and 22 and 24 in the treatments with high water supply. The NR images were processed as described before. Changes in soil moisture distribution were visualized by pixelwise subtraction of an NR image taken at the beginning of an interval from the respective image taken at the end of the interval.

Averaged water contents and matrix potentials of the fine and coarse sand sections in the heterogeneous treatments were calculated by partitioning the total mass of water in the container between the two sands according to their (interpolated) water retention curves under the assumption of hydraulic equilibrium and negligible non-linearity in the vertical water content gradients resulting from gravity, i.e. by solving the following equation for the matrix (or soil water) potential \( \psi \) at medium depth:

\[
W(\psi)/V = \frac{1}{3} \theta(\psi)_{\text{fine}} + \frac{2}{3} \theta(\psi)_{\text{coarse}} \tag{1}
\]
where $W(\psi)$ is the total mass of water in the container at matrix potential $\psi$, $V$ is the bulk volume of the soil, and $\theta(\psi)_{\text{fine}}$ and $\theta(\psi)_{\text{coarse}}$ are the water contents of the fine and the coarse sand at matrix potential $\psi$, respectively.

4.2.10. Data analysis

To quantify deviations from horizontal symmetry in root and cluster root allocation, the precision of root and cluster root allocation was calculated as the respective difference in root length or ‘cluster length’ (i.e. length of root sections carrying cluster roots) between RS and LS divided by the respective values of total root length or cluster length in the containers. In addition, we also calculated the (normalized) precision of cluster root allocation relative to root length as the difference between RS and LS in cluster length per root length divided by the average ratio between cluster length and total root length of the respective container. A value significantly different from zero indicates preferential root allocation in either RS ($>0$) or LS ($<0$). Significance of precision of root allocation was tested with a Bonferroni corrected 95% confidence interval for each treatment combination.

Treatment effects on shoot dry-weight, shoot P concentrations, root length and cluster length were analyzed using a linear mixed model. The fixed factors were P fertilization, water distribution and overall water supply rate as well as their interactions. The block was taken as random factor. Significance of effects was tested using variance analysis (ANOVA). The statistical software package R was used for all statistical analyses (R developmental Core Team, 2008).
4.3. Results

4.3.1. Water availability and distribution

The averaged soil water contents fluctuated between 15% and 25% in the treatments with high water supply, with a slight tendency to decrease over time (Fig. 3). In the treatments with low water supply, the soil water contents initially ranged between 15 and 17%; after irrigation was discontinued for 10 days after transplanting, they dropped to 8 – 10% within seven days and then gradually decreased to values between 4 and 6 % with the development of the plants.

The soil water potentials inferred from the experimental water contents varied between 60 (equivalent to field capacity) and 120 hPa at high water supply and between 160 and close to 500 hPa in the treatment with low water supply (Fig. 2), indicating that water availability was no limitation for plant growth in the former, but became an increasingly limiting factor towards the end of the experiment in the latter treatment.

The water retention curves show that always more than 10% of the bulk volume of both substrates was air-filled porosity, so that we can safely assume that aeration was sufficient at all times at both water supply rates, but in particular at the lower rate (Allmaras et al., 1988; Lipiec and Hakansson, 2000).

The NR images presented in Figure 4 show that the water content was generally higher in the fine sand than in the coarse sand sections in the heterogeneous soil water treatments. At low water supply it also fluctuated more strongly over irrigation cycles in the fine sand, in line with the higher specific water capacity of the fine sand in the dry range of the water retention curves. Conversely, water content fluctuations were larger in the sections with coarse than with fine sand over wetting-drying cycles in the treatments with high water supply.
Infiltrating water redistributed much more rapidly in the containers with high than with low water supply rate. Figure 4 shows that no wetting front was visible any more already within one day after watering in both substrates in the treatment with high water supply rate, while the wetting front was still clearly visible more than 2 days after water application in the treatment with low water supply and moved faster in the fine sand than in the coarse sand.

Given that horizontal redistribution was very slow, the larger water content fluctuations in fine sand in the treatments with low water supply indicate that water availability for plants was higher in the fine sand than in the coarse sand in these treatments, whereas the larger fluctuations in coarse sand in the treatments with high water supply do not necessarily mean that the opposite was the case in the treatments with high water supply. Water suctions did never reach levels known to limit water uptake by roots in the latter treatments, and due to a much higher hydraulic conductivity associated with the higher wetness horizontal fluxes could have much more easily balanced differences in available water between the sections in contrast to the treatments with low water supply.
Figure 3. Water content variation over time in containers with heterogeneous (dashed lines) and homogeneous (solid lines) substrates and no P addition (blue lines), homogeneous P fertilization (green lines) or heterogeneous P fertilization (red lines).
Figure 4. Neutron radiography (NR) images of soil water distribution at different points in time (grey-value images) and of the changes between these time points (color images) in treatments with heterogeneous substrate at low (a) and high water supply (b). The numbers above the grey value images refer to the volumetric water content (%) at the respective days (DAG: day after germination) given below the images. The colors in the colored images indicate no change (light blue), decrease (green-yellow-red-white) or increase (dark blue) in water content between two subsequent dates.
4.3.2. Shoot growth and shoot P concentrations

Water distribution and water supply rate had a significant influence on shoot biomass, whereas P had no effect (ANOVA, $p<0.05$). Shoot biomass was not only reduced by low water supply, but also smaller in the homogeneous than in the heterogeneous distribution treatments. The latter effect appeared to be stronger at low than at high water supply; but the interaction effect of these two factors was not significant (Fig. 5). The lack of a P effect on shoot growth was not related to a lack of plant response in P accumulation: P fertilization led to a significant increase in shoot P concentrations (ANOVA, $p<0.05$). Shoot P concentrations decreased inversely to shoot biomass with increasing water supply rate (ANOVA, $p<0.05$). It did not differ between treatments with heterogeneous or homogeneous P supply (Fig. 5). The P fertilizer effect on shoot P concentration was less pronounced at high than at low overall water supply rate (ANOVA, $p<0.05$).

4.3.3. Root growth

Figure 6 shows the typical herringbone root system of *L. albus*, consisting of a central tap root from which first-order laterals ‘with determined growth’ and first-order laterals ‘with undetermined growth’ are branching off. Laterals with determined growth ceased growth 12 days after germination. The lateral sections were colonized only by first-order laterals with undetermined growth (including cluster roots) and laterals of higher order. Only few first order laterals had reached the lateral sections by DAG 12 in the treatments with low water supply. At this time the soil water content was low enough to limit plant growth.
Cluster root allocation of *Lupinus albus* in soil with heterogeneous P and water distribution

**Figure 5.** Shoot dry weights (a) and P concentrations (b) of *L. albus* grown at low (-) or high water supply (+), in homogeneous (W hom) or heterogeneous (W het) substrate with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). Bars represent averages of all plants with herringbone root system. The numbers of replicates is given at the bottom of each bar in the lower graph. The error bars give the standard errors of the means.
Only the rate of water supply had a significant effect on root length growth (ANOVA, p<0.05). In the treatments with low water supply it was in average slightly larger than in the treatments with high water supply (Fig. 7). Also the vertical allocation of root length growth differed between these treatments. At high water supply more root length was produced at the top and the bottom of the containers than at intermediate depths, while the opposite was the case at low overall water supply (Fig. 6, A.1 in the appendix). The rate of root length growth was almost constant over time, while most of the cluster length was produced towards the end of the experiment between DAG 25 and 35 (A.2 in the appendix). While P fertilization had no effect on root length production, cluster length showed a strong negative influence of P fertilization (ANOVA, p<0.05), which was more pronounced at high than low water supply (Fig. 7) and stronger for homogeneous than heterogeneous P fertilization. At low water supply, the effect of heterogeneous P fertilization on cluster length (compared to no fertilization) was not significant. Heterogeneous water distribution had a slight negative influence on cluster length (ANOVA, p<0.05), while there was no significant influence of the water supply rate (Fig. 7).

4.3.4. Spatial allocation of root growth

None of the treatments led to a significant imbalance in root length allocation to one side of the containers (Fig. 8). In contrast, there was a strong preference of cluster roots to grow in the right section (RS) of the containers, i.e. opposite to the side of increased water availability, in the treatments with heterogeneous water distribution and low water supply (Fig. 8 and 9). This effect was associated with a reduced cluster length in the left section of the containers (LS), and it was independent of P application. A similar trend occurred at high water supply only in the treatment with heterogeneous P and water distribution.
Figure 6. Examples of root system development at low (a) and high (b) overall water supply. The colors indicate root growth from germination to 12 (red) days after germination (DAG), DAG 12 to 19 (blue) and DAG 19 to 25 (green). The two examples were not selected to represent average cases but to highlight the differences between the two water supply treatments. Quantitative analysis of root length distributions over depth is given in the appendix (A.1).

4.4. Discussion

4.4.1. Effects of water and P on shoot, root and cluster root growth

The results suggest that growth was not limited by P, even in the unfertilized soil at low water supply, whereas water availability was a limiting factor at the low supply rate. Limitation by water supply was not only reflected in the shoot biomass response to the treatments, but also in the increased allocation of growth to the root system with water shortage. Furthermore, the difference in root distribution profiles between treatments with
Figure 7: Cluster length (a) and root length (b) of L. albus grown at low (-) or high water supply (+) in homogeneous (W hom) or heterogeneous (W het) substrate with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). The numbers at the bottom of the bars in the lower graph give the number of replicates. The error bars give the standard errors of the means.
Figure 8. Cluster length (a) and root length (b) of *L. albus* in the left (LS, light grey) and right container section (RS, dark grey bar) at low (-) or high water supply (+) in homogeneous (W hom) or heterogeneous (W het) substrate with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). The error bars give the standard errors of the means.

High and low water supply showed a close correspondence to the different dynamics in water availability. As the soil started to dry out again from the surface in the treatments with low water supply while the wetting fronts had not yet reached the container bottoms, soil water was available for most of the time at intermediate depths in the treatments with low water supply. On the other hand, the much more rapid movement of the wetting fronts in the treatments with high water supply made it probably advantageous for roots to forage at the bottom of the containers for nutrients carried with the infiltrating water. The observed root
growth patterns can thus be understood as an adaptation to optimize the acquisition of a limiting soil resource.

The lack of a P fertilization effect on shoot biomass production was in contrast to P limited growth observed on the same soil in Lotus corniculatus (Felderer et al., 2013). It may have been due to a higher P acquisition efficiency of L. albus and/or to a larger P supply from the seeds. Until a functional root is formed plants must rely on P stored in the seed. With growth initial P stocks get progressively diluted, and plants become more and more dependent on uptake of soil-P. Such a dilution effect is indicated by the observed inverse relationship between shoot P concentration and shoot biomass. To find out whether seed P storage overrode a potential limitation due to low soil P availability, a longer duration of our experiment would have been required.

Along this line of reasoning, the fact that cluster length was increased in the treatments with no P addition in comparison to homogeneous P fertilization, may be understood as a preparation of the plants for later stages of development when they would become fully dependent on P acquisition from soil. Cluster roots are costly for a plant in terms of assimilates, and their production is reduced if they are not needed, as it was the case in the homogeneous P fertilization treatments. This raises the question why cluster root production was not reduced in the treatments with heterogeneous P fertilization at low overall water supply rate, although shoot P concentrations were increased to similar levels as in the homogeneous P fertilization treatment. This finding means that cluster root growth was neither determined by the actual soil P level to which the roots developing cluster roots were exposed, nor by the shoot P status at that time, while the soil P level nonetheless seemed to play some role. An explanation could come from the fact that no P was applied in the middle section of the containers with heterogeneous P fertilization. Thus, the plants were exposed to
Figure 9. Precision of cluster length allocation (relative to total root length) of L. albus at low (-) or high overall water supply (+) in homogeneous (W hom) or heterogeneous (W het) substrate with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). The precision of cluster root allocation relative to root length was calculated as the difference in cluster length per root length between the right (RS) and the left container section (LS) divided by the average ratio between clustered and total length of the roots in the respective container. A value significantly different from zero indicates preferential root allocation in either RS (>0) or LS (<0). The error bars represent the 95% confidence interval of the mean.

P poor soil during the initial stage of root system development in these containers as in the containers with no P application and in contrast to those with homogenous P fertilization. This suggests that cluster root growth at later stages responded to P supply during early root system development.
4.4.2. Effect of horizontal heterogeneities on root allocation patterns

The fact that neither the allocation of total root length nor the allocation of cluster root length was affected by heterogeneous P application provides further evidence that the P effect on root growth was a systemic response to plant P status and not the result of a local response to soil P availability. These results are in line with the hypothesis of Shane et al. (2003) that cluster root formation depends primarily on shoot P status and much less on the exposure of cluster-forming roots to local soil P concentrations. While some studies showed that localized P availability can influence cluster root allocation, the effects that have been reported are very diverse. In a split-root pot experiment Shu et al. (2007) found that *L. albus* allocated more cluster roots in P-enriched soil than in unfertilized P-poor soil. Apart from a higher level of P enrichment (60-80 mg P kg\(^{-1}\)) than in our experiment, the difference in responses may also be due to differences in the genotypes and experimental systems used. Studying cluster root responses of *L. albus* to heterogeneous P exposure in hydroponics supplying P exclusively to one half of a split root system and essentially no P at all to the other half, Shane et al. (2003) found no difference in cluster root production between the two root system halves at low to moderate contrasts in P supply and even a slight reduction in cluster root formation in root halves that were exposed to very high P levels compared to the roots exposed to nutrient solution without P. On the other hand, using a similar system, Shen et al. (2005) found that cluster root production of *L. albus* was enhanced in P-containing solution at P concentrations that were even higher than in the study of Shane et al. (2003).

The lack of a cluster root allocation effect in the heterogeneous P treatment was in striking contrast to the strong stimulation of cluster root growth on the sides with lower water availability in the containers with heterogeneous soil texture and low water supply. The fact that this stimulation was associated with a corresponding reduction in cluster length on the other side, so that the cluster length in total remained unaffected, provides further evidence
for a pre-determined systemic regulation of cluster root growth. But it seems that this systemic control only determines the total amount of resource allocation to cluster root growth, while its spatial allocation showed a strong response to local soil conditions.

As the effect of the heterogeneous soil texture on cluster root allocation was limited to the treatments with low water supply, it was probably directly or indirectly related to the resulting difference in soil moisture conditions. Otherwise, we should have expected some effect also in the treatments with high water supply, in which there was essentially no difference in soil water availability between the two substrates. If different water availability for root uptake was the main reason for the effect of the heterogeneous substrate treatment on cluster root allocation at low water supply, then the fact that cluster root growth was boosted on the side of lower (instead of higher) water availability under these conditions suggests that horizontal water redistribution via the roots was involved. Root-mediated transfer of moisture from wetter to drier soil zones allows roots to grow and mine for nutrients even in extremely dry patches of soil (Burgess et al., 2000; Lambers et al., 2006). In fact, cluster roots are probably most advantageous for a plant if they can operate under conditions of their own, local water supply. The diffusion of solutes is strongly reduced in dry soil (Bhadoria et al., 1991; Jungk and Claassen, 1997), and as exudates thus remain more concentrated for longer time around roots in dry than in moist soil, more P can be solubilized and less solubilized P is lost from the rhizosphere into the bulk soil.

Another factor that differed between the two substrates in the treatments with low water supply rate and appeared to be related primarily to the difference in moisture was mechanical resistance. When we sampled and cleaned the roots, we found that with the lower moisture content the coarse sand was also firmer than the fine sand in these treatments. It has been found in some plants that mechanical inhibition of lower order root growth can promote branching off of higher order laterals (Bingham and Bengough, 2003; Goss, 1977).
Considering that cluster roots are modified laterals, mechanical inhibition of first order lateral root growth may by analogy have promoted cluster root growth more in the coarse than in the fine sand in the treatment with low water supply.

In conclusion, the main finding of this study is that under conditions of water limitation cluster root growth of *L. albus* was stimulated in patches of reduced water availability resulting from textural heterogeneity, whereas it showed no response to heterogeneity in soil P. The only clear P effect was that total resource allocation to cluster root growth was related to the level of soil P to which the roots were exposed at the initial stages of growth. These results suggest that the control of cluster root growth depends both on systemic regulation and local soil conditions. Although P supply influenced the systemic response, water distribution – and not P distribution – was responsible for the spatial allocation pattern.

4.5. Acknowledgements

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4.6. References


Cluster root allocation of *Lupinus albus* in soil with heterogeneous P and water distribution

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**FAL, RAC, FAW.** 1996b. Ph in water suspension (1:2.5) and ph in CaCl₂ suspension (1:2.5). Swiss reference methods of the Federal Agricultural Research stations, Swiss Federal Research Station FAL, RAC, FAW, Zurich, Switzerland


5. Conclusion and outlook

5.1. P fertilizer effects on different experimental plant species

The results presented in the previous chapters show that soil P availability was a growth-limiting factor for *Lotus corniculatus* and *Lotus japonicus* on the soil of the experimental site nearby the Chicken Creek Catchment, but not for *Lupinus albus*. For *L. corniculatus* we found that P fertilization enhanced growth in the ingrowth core field experiment but no clear P fertilizer effect in the climate chamber experiment with constructed soil P heterogeneity was found (Chapter 2). We attributed the lack of a clear P effect in the climate chamber experiment primarily to the high genetic variability of the seeds. For the field experiment we then selected plants of similar size to reduce this variability. As we could not find a more homogeneous germplasm for the allogamous *L. corniculatus*, we decided to use homogeneous germplasm of the self-pollinating *L. japonicus* for the subsequent experiments with arbuscular mycorrhizal fungi (AMF) instead. Low shoot P concentrations and stunted growth of non-mycorrhizal *L. japonicus* grown in unfertilized soil show that soil P available to normal roots was extremely low in the unfertilized soil. By increasing the absorptive surface and the diffusive pathways of P through a network of external mycorrhizal hyphae P acquisition was strongly enhanced in the mycorrhizal plants (Smith, 2008), although AMF are not able to solubilize P fractions not available to plants (Smith, 2008). Nonetheless, P was still growth-limiting also for the mycorrhizal plants, when no P fertilizer was supplied. In contrast, no growth promoting effect of P fertilization was found on this soil for *L. albus* (Chapter 4). One explanation is that the seeds of *L. albus* are much bigger than those of *L. corniculatus* and *L. japonicus* and thus can store substantially larger amounts of P. In addition, we feel that also the higher P solubilization and acquisition capacity of cluster roots compared to normal roots played an important role. The P desorbed and solved from
Conclusion and Outlook

relatively strongly bound P fractions might have been abundant and could have overridden the P fertilizer application effect.

5.2. The role of AMF for plant growth of *L. japonicus* and its potential role for *L. corniculatus*

Arbuscular mycorrhizal fungi were found to play an important role for P nutrition on the experimental site (ES) and probably also on the Chicken Creek Catchment (CCC). Although we did not directly quantify the abundance of AMF spores in soil from the CCC, we could indirectly show that root colonization occurred in *L. corniculatus* grown on the soil taken from the experimental site nearby the CCC. We found that *L. japonicus* roots were colonized by *G. mossae, G. claroideum* and probably also other AMF species, when sterilized soil was inoculated with non-sterilized chopped rootlets of *L. corniculatus* that had been grown on this soil. In addition to the strong growth response of *L. japonicus* to mycorrhization, these results suggest a high abundance of AMF in the experimental soil. The AMF did not only enhance plant P nutrition but probably influenced also P distribution in the soil. The depletion of P in root distant soil, which was detected in the two intensive 3-D soil sampling campaigns on the CCC and on the experimental site nearby, thus could have been due to uptake by extraradical hyphae of the mycorrhizal fungi.

For *L. japonicus* the association with mycorrhizae was essential to survive in the unfertilized soil, and also in the treatments with P fertilizer addition mycorrhization still had a beneficial influence on plant growth. The genetical similarity of *L. corniculatus* and *L. japonicus* suggests that AMF would have had a similar positive influence on *L. corniculatus* (Handberg and Stougaard, 1992). Nevertheless, caution should be exercised in comparing responses between *L. corniculatus* and *L. japonicus*, as these species show marked differences in their growth rates. Plant growth rates can have a strong influence on mycorrhizal root
colonization. Plants with slower root growth might be more responsive to mycorrhization than slowly growing plants (Smith et al., 2011; Smith et al., 1992). If it is hypothesized that the rates of extraradical hyphal growth and mycorrhizal root colonization are not affected by root growth rate and, therefore, the density of colonization in relation to root length decreases with increasing root growth rate. With lower colonization and thus density of contact or transfer sites, the fraction of P taken up directly by the roots becomes larger relative to the fraction of P supplied through the mycorrhizal pathway. But despite the higher rates of root growth in *L. corniculatus* than in it closed relative *L. japonicus*, it seems likely that AMF would have played an important role in P acquisition also for *L. corniculatus*. Unfortunately, we have no data on the abundance of mycorrhizal inoculum in the untreated soil of the CCC or the ES. So there is no basis for speculations whether plant nutrition could have been improved by increasing the abundance of inoculum in the field. This knowledge would be important to assess the potential of soil amendments with mycorrhizal inoculum for remediation purposes of recently restored sites.

Our results also show that *L. japonicus* coped better with heterogeneous P distribution in soil when it was associated with indigenous AMF mixture than only with *G. intraradices*. In contrast to the homogeneous treatments, in which also inoculation with *G. intraradices* increased plant growth, heterogeneous P fertilization increased plant growth only in the treatments with indigenous AMF mixture, demonstrating that association with multiple-species AMF can enhance the capacity of plants to cope with heterogeneous soil conditions more than association with a single AMF species. As it is likely that this also holds for other plant species, it is important to monitor the development of AMF species composition with vegetation development on the field sites to investigate growth patterns and success of single plant species in a plant community. It becomes even more relevant if we consider that this
development is likely to be associated with changes in the spatial patterns of soil nutrient and water distribution.

5.3. Spatial allocation of roots, cluster roots and AMF.

Local enrichment of soil P had different effects on the root allocation patterns of *L. corniculatus*, *L. japonicus* and *L. albus*. While we detected root proliferation into P-enriched soil patches in *L. corniculatus* grown under field conditions, where we could not sterilize the soil, and in non-mycorrhizal *L. japonicus*, no such allocation pattern was found in mycorrhized *L. japonicus* and in *L. albus*. In the latter species, which does not form associations with AMF, neither the allocation of normal nor of cluster roots responded to heterogeneous P distribution.

If preferential root allocation into P-enriched soil patches also occurred in the soils of the two field sites, it was certainly much less pronounced than in the experiments with constructed heterogeneities, because the contrasts in P concentration were much weaker in the absence of experimental manipulation. In any case, the results obtained from the high density field soil samplings suggest that P depletion by roots had a more dominating influence on the spatial relationship between root length density and labile P concentrations in soil than preferential root proliferation into P-rich soil patches. But if our assertion is valid that these heterogeneities increase with progressing ecosystem development, then we should expect that preferential root growth will become more important with time on the two field sites.

The fact that mycorrhization overrode preferential root allocation in *L. japonicus* can not only be explained by the improved plant P status that resulted from mycorrhization, as plant growth was still not maximal in the treatments with *G. intraradices* and heterogeneous P application even at the higher rate of additional P application, which means that preferential
root allocation could have further enhanced P accumulation. This suggests that also other factors were important for the inhibition of preferential root allocation. The difference to *L. corniculatus* in root allocation responses might be attributed to the lower growth rate of *L. japonicus* leading to a higher density of AMF colonized roots. Root colonization by AMF has been found to result in downregulation of P transporter in the epidermis of roots (Smith *et al.*, 2004), which may reduce the sensitivity of roots to variations in soil P and thus suppress preferential root allocation. The lack of preferential root allocation in mycorrhizal *L. japonicus* was not compensated by preferential allocation of extraradical hyphae in the treatments with *G. intraradices*. As discussed in the introduction, the mycorrhizal association is not a state implemented or controlled by the plant and therefore not necessarily optimal for plants P uptake or shoot growth (Smith *et al.*, 2011). In search of assimilates, efficient P uptake might be less beneficial for the fungus than finding a maximum number of roots (Gavito and Olsson, 2008). Different fungal species will differ in their allocation strategies, depending also on the nature and specific conditions of plant-host interactions (Cavagnaro *et al.*, 2005; Gavito and Olsson, 2008; Shi *et al.*, 2011). Unfortunately, it was not possible for us to assess the spatial allocation patterns of extraradical hyphae in the treatments inoculated with the indigenous AMF mixture, but quantify only two out of the unknown number of AMF species.

In contrast to the two *Lotus* species, the allocation of resources to roots is not influenced by mycorrhizal fungi in *L. albus*. As mentioned before, we did not find preferential root or cluster root allocation to P-enriched soil patches in this species, only a systemic effect of initial root exposure to soil P on total cluster root production. Also other studies found no response of localized P fertilization on cluster root allocation (Shane *et al.*, 2003; Shane and Lambers, 2005). A possible explanation for the low sensitivity of cluster root production to locally increased P supply might be the different abilities of cluster and non-cluster roots –
which are parental to the cluster roots – to acquire P fractions of different availability. The P fraction available for non-cluster roots can in general be assumed to represent only a small fraction of soil P available for cluster roots. If spatial correlation of these P fractions is low, then preferential allocation of cluster roots in soil patches enriched in P available for non-cluster roots might not substantially enhance the acquisition of P by cluster roots and, therefore, will have little effect on cluster root allocation.

The lack of a cluster root allocation effect in the heterogeneous P treatment was in striking contrast to the strong stimulation of cluster root growth in soil patches with lower water availability and finer soil texture when overall water supply was limiting plant growth. It seems that cluster roots are more effective in solubilizing soil P in dry soil than in moist soil. To explain the observation that cluster root growth was boosted in the treatment with heterogeneous soil texture at low water supply on the side of the containers with lower (instead of higher) water availability we hypothesized that horizontal water redistribution via the roots occurs (Burgess et al., 2000; Lambers et al., 2006). Root-mediated transfer of moisture from wetter to drier soil zones allows roots to grow and mine for nutrients even in extremely dry patches of soil. It would be interesting to test this hypothesis by using neutron radiography in combination with the application of heavy water (D₂O) to monitor the postulated re-distribution of water through the root system Heavy water has a higher absorption coefficient for neutrons than water and could therefore be used as a tracer (Zarebanadkouki et al., 2012).

Interestingly, there was more shoot biomass in the treatments with heterogeneous than with homogeneous soil under water limiting conditions, although the irrigation rates and the averaged soil water contents were the same for treatments of both water distribution patterns, indicating a higher water use efficiency on the heterogeneous soil. This enhanced water use efficiency in heterogeneous treatments could be adopted to improve soil restoration
techniques in arid regions and should be tested in field experiments. Similarly to our climate
chamber experiment, heterogeneities in water distribution could intentionally be introduced
by using soil of different substrate distribution.

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6. Appendix: Supplementary material for chapter 4

Figure A.1: Vertical root length distribution 35 days after germination of L. albus at low (-) or high overall water supply (+) in homogeneous (W hom) or heterogeneous (W het) substrate and with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). The five bars given for each treatment combination represent the vertical distribution of root length from the top (lightest shading) to the bottom (black) of the containers. The error bars are the standard errors of the mean. At high water supply more root length was produced at the top and the bottom of the containers than at intermediate depths, while the opposite was the case in the treatments with low water supply.
Figure A.2: Root length and cluster length production of L. albus in containers with horizontally heterogeneous (dashed lines) and homogeneous (solid lines) soil water availability and no P addition (blue lines), homogeneous P fertilization (green lines) or heterogeneous P fertilization (red lines). While root length production was almost linear over the experimental growth period, most of the cluster roots were produced between 25 and 35 days (harvest) after germination.
Figure A.3: Shoot element concentrations of L. albus grown at low (-) or high water supply (+), in homogeneous (W hom) or heterogeneous (W het) substrate with no additional P supply (P no), homogeneous P fertilization (P hom) or heterogeneous P fertilization (P het). Bars represent averages of all plants with herringbone root system. The error bars give the standard errors of the means.
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