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# Towards a more efficient and sustainable fertilization through recycling phosphorus as struvite

**Dissertation**

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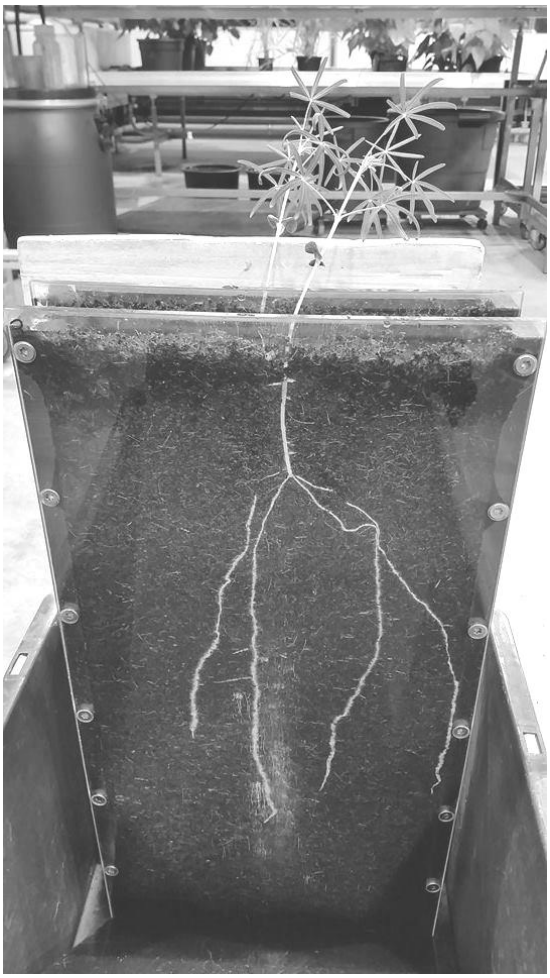
“Stay true and don’t forget your roots„



Abstract

Food and water security are dependent on the sustainable use of phosphorus (P). However, there is no single solution for achieving a phosphorus-secure future. P recovered and recycled from current waste streams (like manure) is an important approach to developing environmentally sustainable and biologically efficient fertilizers. In this framework, a promising example of a P recovery product from waste streams is struvite ($MgNH_4PO4\ 6H_2O$), a crystal precipitated after the anaerobic digestion of different biological waste streams. Struvite has reported positive results regarding yields and P uptake for different crop species; however, there are still some limitations. P availability from struvite is highly influenced by the soil pH, which can be further modified by nutrients added to the soil or by plant and microbial activy in the rhizosphere. The main challenges are i) to understand the essential aspects that have a major effect on struvite availability, and ii) to focus on those traits that will increase struvite availability and therefore improve fertilizer use efficiency. With this apporoach, struvite value will increase, making it more competitive against mineral fertilizers.

To understand which aspects modify struvite availability, the response of different plant species with contrasting strategies to mobilize nutrients (i.e. maize, lupine, and tomato) to various P applications was analyzed at different time points and plant growth stages. Likewise, the effect of soil pH, and method and duration of application were studied. Plant species have different morphological and physiological adaptations to increase the efficiency of P acquisition. Under this premise, a particular focus was put on root traits that would have an effect on phosphorus bioavailability and spatial availability.

It was concluded that struvite has the same P fertilizer efficiency as mineral sources regarding biomass production, P uptake efficiency, and allometric studies of root–shoot relations. Moreover, it was validated that the following traits contributed to increase struvite use efficiency: i) the results from the automatic shoot phenotyping analyses support the idea of struvite being a slow-release; compared to triple-superphosphate (TSP), struvite-fertilized plants had lower initial leaf area, but later higher biomass ii) plant responses were conditoned by the nutrients applied with the struvite. It was confirmed that nitrate increased root biomass due to a higher number of primary roots, while ammonium increased the phosphorus uptake efficiency from struvite due to rhizosphere acidification iii) it was observed that lupine plants acidified the soil due to a high release of carboxylates by the roots. In contrast with the readily available P source $K_2PO_4$, the carobxylate exudation increased when struvite was applied, mobilizing the struvite-P at neutral conditions; iv) the microbial community analyzed did not shift between fertilizers used, as much as between plant species.

Throughout this thesis, the use of invasive and non-invasive techniques, revealed different plant responses at various growth stages above and below ground, depending on the P fertilizer applied. It was shown that struvite solubility will not only depend on the soil pH but also will be modulated by the plant species and the way in which it is applied (e.g combined with other nutrients). In addition to yield analyses, studies of root morphological and physiological adaptations to P application provided a more detailed report of traits that would increase struvite use efficiency. It was shown that struvite, with a slower release, has the potential to be a more efficient method of fertilizing plants than the application of conventional, highly soluble P fertilizers. Likewise, the use of plants that can actively acidify the soil, combined with the application of the

struvite with ammonium-N, will increase the P use efficiency. For future applications, those traits can be used to select candidate plants that will increase the use effiiciency of struvite, underlying mechanisms that will also ensure high yields. Those studies have the potential to be applied for other recovered products, increasing the efficiency and promoting the recycling of nutrients.

Zusammenfassung

Die Rückgewinnung von pflanzenverfügbarem Phosphor (P) aus Gülle und Abwasser in Form von Struvit ($MgNH_4PO_4$ $6H_2O$) ist ein wichtiger Ansatz zur nachhaltigen und effizienten Düngerherstellung. Struvit kann die Biomasseproduktion verschiedener Pflanzenarten fördern aber es hat sich gezeigt, dass dies und die P-Aufnahme in hohem Maße von der Pflanzenart, dem pH Wert des Bodens und der Art der Ausbringung, abhängt**.** Das bessere Verständnis der Faktoren, die die Verfügbarkeit und damit die Effizienz von Struvit als Düngemittel, beeinflussen, stehen im Mittelpunkt der vorliegenden Arbeit.

Die Pflanzen Mais, Lupine und Tomate (Spezies, die sich in ihrer P-Aufnahmestrategie unterschieden) bezüglich ihrer Antwort auf verschiedene P-Quellen, pH Wertes des Bodens und die Methode und Dauer der Ausbringung analysiert. Die Verwendung neuartiger Phänotypisierungtechnologie erlaubte hierbei die Vsualiserung des Pflanzenwachstum zu verschiedenen Zeitpunkten und Entwicklungsstadien. Struvit wurde gemeinsam mit Ammonium bzw. Nitrat ausgebracht um die Auswirkung der Stickstoffform auf den pH Wert der Rhizosphäre und somit auf die Löslichkeit von Struvit, die Nährstoffmobilisierung und –aufnahme zu untersuchen. Vergleichbar hierzu wurde die Bedeutung der Carboxylatexsudation der Wurzeln auf die Verwertbarkeit von Struvit getestet. Des Weiteren wurde der Einfluss von Struvit auf die mikrobielle Gemeinschaft im Substrat untersucht. Die Untersuchungen wurden in Bodensubstraten mit unterschiedlichen physikalischen und chemischen Eigenschaften durchgeführt.

Es konnte gezeigt werden, dass Struvit bezüglich der Biomasseproduktion, der Wurzel-Spross-Verhältnisse und der P-Aufnahmeeffizienz über die gleiche Effizienz als P-Dünger verfügt wie standardmäßig verwendete mineralische P-Dünger. Die Phänotypisierung des Sprosswachstums zeigte, dass Struvit über Eigenschaften eines Langzeitdüngers verfügt: verglichen mit Tripelsuperphosphat (TSP) zeigen die mit Struvit gedüngten Pflanzen zu Beginn der Untersuchung eine geringere Blattfläche, zum Ende des Experimentes jedoch die größere Biomasse. Das Pflanzenwachstum wurde auch durch die Nährstoffe, die zusammen mit Struvit ausgebracht wurden, beeinflusst. In Anwesenheit von Nitrat bildeten sich vermehrt Primärwurzeln, während Ammonium die Anzahl der Feinwurzeln erhöhte, was eine Erhöhung der spezifischen Wurzellänge zur Folge hatte. Lupinen sind in der Lage durch Wurzelexsudation von organischen Säuren das Bodensubstrat anzusäuern was in Anwesenheit von Struvit unter neutralen Bedingungen zur besseren Verfügbarkeit von P führte. Schließlich zeigte sich, dass durch Stuvit die Pflanzenart-spezifische Bakteriengemeinschaft der Rhizosphäre nicht verändert wurde.

Zusammenfassend lässt sich feststellen, dass Struvit einen Langzeitdünger darstellt, der das Potenzial hat, präziser und effizienter zu wirken als konventioneller schnell-löslicher P-Dünger und dass Pflanzen, die das Substrat aktive ansäuern, bzw. die Kombination von Struvit mit Stickstoff in Form von Ammonium die P-Effizienz erhöhen.

Die vorliegende Studie kann darüber hinaus als Leitfaden zur Untersuchung weiterer rückgewonnener Nährstoffe dienen, um ihre Effizienz als Pflanzendünger und damit das Recyceln von Nährstoffen zu fördern.

Abbreviations

DAS Days after sowing

DCL Diameter class length

DW Dry weight

KP Potassium-phosphate

MD Measurement day

MFA Multi-factor analyses

PLA Projected leaf area

PUE Phosphorus uptake efficiency

rDCL Relative diameter class length

SRL Specific root length

TRL Total root length

TSP Triple-superphosphate

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# 1. Introduction

## 1.1 Towards a more sustainable fertilization: Nutrient removal, recovery, and recycling

In the coming years, a critical challenge will be to meet future food demands while reducing the negative impact on the environment produced by the agricultural systems, main forces of global environmental degradation (Herrero and Thornton 2013). The increasing human population is expected to require a 60% increase in food production by 2050 (Alexandratos and Bruinsma 2012). According to current trends, to meet this future demand while decreasing environmental impacts it is vital to improve resource use efficiency as well as plant yields (Ray et al., 2013; Garnett et al., 2013).

In recent decades, changes in consumption patterns (i.e., increasing demand for livestock products) have produced an increase of the global livestock sector (raising animals for food production and cultivation of feed crops). Today, it contributes to 70% of the global agricultural output (Thornton 2010), being the main cause of environmental degradation (FAO 2012). Feed production is the world's largest user of farmland (FAO 2012) and relies on the application of intensive and unsustainable use of fossil-fuel-based fertilizers. Besides that, another environmental impact that comes together with increasing livestock production is the increment of animal excreta (manure). Only in Europe, more than 1.27 billion tons of animal manure are produced per year, being the largest source of nitrate and phosphate pollution, and the primary cause of eutrophication of inland freshwater and marine environments (Withers and Haygarth, 2007).

There are different approaches to meet future food demands reducing environmental impacts. Precision agriculture, (i.e., promoting changes in fertilizer management according to site conditions), is seen as one possibility to improve farming practices. Traditionally, precision agriculture has been linked to the use of high technology such as remote sensing or GPS (Mulla 2013). At this thesis outlines, the concept of precision agriculture will mean moving towards a more "efficient fertilization" by analyzing ways in which to use resources to improve crop production and decrease environmental impacts (i.e. adapting fertilization to crop necessities).

Another strategy that aims to reduce negative environmental impacts is the improvement of animal waste treatment. In this framework, several EU projects have developed new technologies to process manure that are economically feasible and environmentally sustainable. One of these initiatives is ManureEcoMine, an EU-based PhD-candidate-funding project, aimed at producing green fertilizer upcycled from manure. Manure is therefore not considered as waste, but as a mining resource of organic carbon and nutrients, while decreasing the use of non-renewable rock-

derived fertilizers. The new technology developed is sustained on the following concepts: maximum recovery of nutrients, energy self-sufficiency, minimal greenhouse gas emission, and high-performance fertilizer production; thus contributing to have a more “sustainable fertilization”. The project was integrated by a consortium of eight partners including five research institutions; two farms; two engineering companies that designed the pilot plant; one center for contaminant analyses and one substrate company. Results are available at http://www.manureecomine.ugent.be. The goals are summarized in Fig. 1.

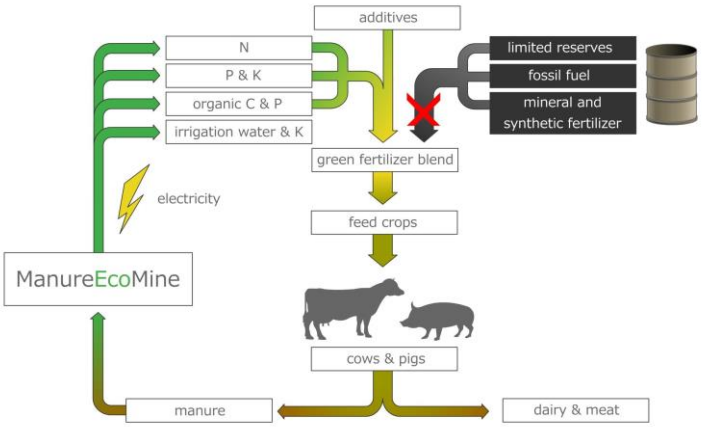


**Fig. 1 ManureEcoMine Project concept.**

Rather than only focus on the nutrient removal from manure, traditionally done before the direct application to the fields (Perez-Sangrador et al., 2012; Zeng and Li 2006; Parson and Smith 2008), the joint project also involved efforts for nutrient recovery and subsequent recycling of the recovered products. The recovery of nutrients in a form free of contaminants, with a stable composition, can promote its use as a fertilizer, contributing to being less dependent on rock reserves and fuel-derived fertilizers. The main objective of recovery is, therefore, to encourage recycling of recovered nutrients as fertilizers. The recovery of nitrate and phosphates and subsequent recycling will contribute to close the nutrient loop while reducing environmental impacts. Furthermore, this will still contribute to the traditional removal of nitrate and phosphates from waste, preventing the environmental risk of waste disposal without treatment.

Throughout this dissertation, different ways in which to increase fertilizer effectiveness of recovered nutrients delivered by the project partners were analyzed. Specific changes in nutrient management will be described for different plant species. The project partners recovered several nutrients at different steps of the manure treatment such as ammonium nitrate, ammonium sulphate, or phosphorus-ammonium-magnesium precipitates as struvite. The study case of this thesis will be focused on struvite and its use mainly as a phosphorus source. In the following

sections, it is explained why there is a focus on recycled phosphorus using struvite and which experiments were carried out to analyze the main factors that influenced fertilizer use efficiency. To conclude, some agronomical advice with specific suggestions to struvite management will be discussed, as well as how it is possible to translate this knowledge to other recovered products.

## 1.2 Why focus on recycling phosphorus?

Phosphorus (P) is an essential macro-element that plays a key role in many essential plant processes (Misson et al., 2005; Jouhet et al., 2007). After nitrogen (N), P is the most important nutrient for plant growth (Smil 2000; Filippelli 2008). Before the intensification of agriculture, farmers relied mostly on natural soil P reserves and on manure. Due to the growing demand for food and feed production, P is not available in the soil in sufficient concentrations for sustained plant production, resulting in the need to apply P in the form of fertilizers (Vance et al., 2003). In contrast to N, which can be assimilated by biological N-fixation or industrially made, the main source of P comes from rock phosphate mines - a non-renewable resource predicted to be depleted within a few hundred years (Fixen and Johnston 2012). Those mines are located only in a few places on Earth, with 75% of known reserves located in Morocco, with no reserves available in Europe, which thus relies heavily on imports (Schoumans et al., 2015). The demand for rock phosphate will increase not only due to increasing demand for food production, but also feedstocks, and bioenergy crops (Erb et al., 2009). On the other hand, the application of inappropriately high amounts of organic fertilizers as an alternative to rock phosphate, such as untreated manure, can cause P eutrophication of ground waters (Sharpley et al., 2015).

Even once it is applied in the soil, the P that is directly available for plants it is usually low. The plant available P is usually in the orthophosphate form, a small percentage of the inorganic P pool. The inorganic P is normally associated to other minerals (P absorption) like iron, calcium or aluminum depending on the soil pH (Gahoonia and Nielsen 1992). Plants cannot directly assimilate the organic P that constitutes between 30-80% of the total P in the soil (Dalai 1977). Organic P can be mineralized to orthophosphate by the microbes, however microbes are also responsible for the immobilization of P (Van Der Heijden et al., 2008). All this different reactions in the soil make that more than 80% of P becomes plant unavailable (Holford 1997)

The main phosphorus limitations that motivated this study are summarized:

i) Rock phosphate, as a non-renewable source, is predicted to be depleted in a few hundred years (Fixen and Johnson 2012).

ii) Moreover, the demand for phosphate rock will not only increase due to the higher demand for food-feed production but also due to the increasing demand for plants for energy production.

iii) The availability of P once it is applied to the soil is usually very low and despite the total amount of P in the soil often being high, due to years of over-fertilization, it is often present in unavailable forms for the plant as it is interacting with the cations present in the soil.

iv) Plants cannot directly take up organic P (that can constitute 20-80% of available P). The microbial conversion of organic P to orthophosphate is slow and does not usually provide enough P to support crop demands. Therefore recovery of applied P by plants is very low because of absorption, precipitation or conversion to the organic form.

v) Phosphate losses to waters derived from agriculture are a major source of contamination (Sharpley et al., 2015).

### 1.2.1 Struvite as an alternative P source

In the quest to find a more sustainable use of P, the European Union has recently proposed to implement a coherent package of strategies to address the broken P cycle. Some of the strategies include: recover P from wastes (Bonvin et al., 2015; Stutter, 2015), redefine the food systems, and reduce P losses (Schoumans et al., 2015; Withers et al., 2015). In this context, alternative sources such as manure, sewage, or wastewater for P recovery have been studied. Struvite, a crystal containing magnesium ammonium and phosphate ($MgNH_4PO_4$ $6H_2O$) is a promising example of P recovery product from these three sources.

Struvite crystals are created when magnesium, ammonia, and phosphate precipitate in a mole to mole ratio of 1:1:1. The crystallization process is affected by several factors: among others, pH, molar ratios of $Mg^{2+}$, $NH4^{+}$, and $PO4^{-}$ (Huchzermeier and Tao 2012), or the temperature of the solution (Bouropoulos and Koutsoukos 2000). Of interest for this thesis is that high pH will favor the precipitation of struvite, and therefore low pH will favor its solubilization.

Several studies on struvite have focused on improving the recovery from wastewater treatment plants (Zarebska et al., 2015), exanimating which physicochemical conditions and technologies are the most efficient for the struvite recovery regarding the source of input. The new technology developed within the ManureEcoMine project allowed implementing and improving those processes specifically for manure. The goal was to increase struvite purity in the most economically and sustainable approach. Moreover, the studies on struvite precipitation were

carried out with a focus to support the expected use of struvite as a fertilizer. As an example, Tarrago et al. (2016), ManureEcoMine project partner, described how to control the particle size of struvite to adjust it according to the requirements of fertilizer blending. This technology meant a distinct improvement on the current scenario of nutrient recovery from manure.

In recent decades, struvite has been tested as a fertilizer on different soils with different species: Lolium perenne (Johnston and Richards 2003), Zea mays (Antonini et al., 2012) or Triticum aestivum (Massey et al., 2009). In most of these studies, when using struvite as a fertilizer, similar crop yields and P uptake were found compared to the use of commercial phosphate fertilizer (as reviewed by Kataki et al., 2016). However, there are still some limitations. Besides the positive agronomic results in P uptake and yields, lower yields in struvite-treated plants have also been reported because of lower availability of nutrients compared to chemical fertilizer, as stated by Ackerman et al. (2013), who also found no significant effect of struvite on biomass yield of canola. It is important that we resolve the conflict in these reports by understanding to what extent species and growth conditions influence P recovery from struvite or similar recycled fertilizer. Once resolved, proper management advice may be given to farmers.

### 1.3 Adaptation of nutrient management to the application of novel recovered product

The importance of changing the management of P has been extensively discussed (Cordell and White 2011; Richardson et al., 2011). The main actions addressed in this thesis to move towards a more sustainable and efficient agriculture are:

i) Analyze the potential fertilizer effect of a recovered P source as struvite, free of contaminants, under different soil conditions.
ii) Adapt the nutrient management expressly to have a more tuned and efficient use of recycled P fertilizers.

The improvement in the nutrient management of recycled products is based on understand the crop needs and afterwards apply fertilizers with more efficient practices. The method used to have a more precise fertilization is an update of “4R” nutrient management stewardship (right form, right time, right place, and right amount) (International Plant nutrition institute, 2014); in this case with a special focus on closing the broken P cycle. For that, it is essential to explore the specific conditions of how and where the recycled fertilizer will be implemented. Understanding the plant strategies to mobilize nutrients, as well as analyzing possible long-term effects of soil microbiota on nutrient turnover, can be highly beneficial for increasing the use efficiency of the recycled

products, reducing at the same time the amount of fertilizer needed for optimum plant growth, and increasing plant yield with less inputs.

Updating the management practices of recovered nutrients by adapting them to the most suitable plant species, soil pH, and method and duration of implementation will increase the value of the recovered products and make them competitive with mineral fertilizers. Without recognizing the significant effects of those factors, struvite use efficiency cannot be improved and lower yields can be expected because of lower availability of nutrients from struvite compared to chemical fertilizers. Therefore, in order to identify plant traits that can enhance the mobilization of recovered P, plant-rhizosphere-soil processes were studied after the application of struvitre. A particular focus was put on rhizosphere traits that would have an effect on phosphorus bioavailability (root exudates of organic acids) and spatial availability (root architecture).

The different experiments throughout this dissertation were designed following the scheme in Fig. 2, based on the study of Shen et al., (2011) who stated that "effective strategies for P management involve multiple levels approaches." Therefore several analyses of plant, rhizosphere, and soil process after the application of the recovered products were carried out. This allows formulating the hypothesis of how each factor might influence P availability, always referring to struvite as an example of recovered P source. Each section stated in Fig. 2 will be expounded in depth.

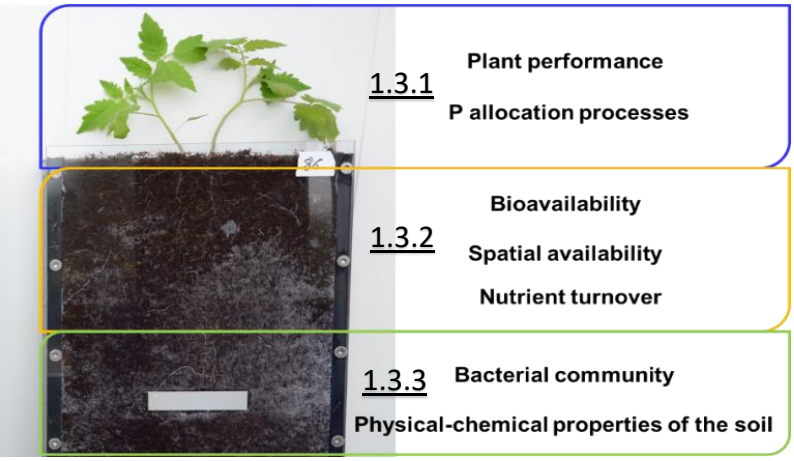


**Fig. 2 Conceptualization of the three main factors analyzed to study struvite availability**. 1.3.1 Crop productivity and nutrient uptake, 1.3.2: Rhizosphere traits such as organic acid exudation (bioavailability) or changes in root morphology (spatial availability) and 1.3.3: Soil processes such as changes in microbial community.

### 1.3.1 Plant performance and P recovery

The most direct way of determining nutrient availability from struvite and how it varies among plant species and environmental conditions is to measure plant biomass production. The comparison of this value with that of readily available fertilizers allows determination of the effectiveness of struvite in terms of plant yield. By realizing biomass allocation studies in species with different growth rates, the effect of struvite on final plant size will be easier to compare (Imo and Timmer 1992). Besides that, chemical analyses of selected mineral nutrients in leaves and other parts of the plant can be related to fertilizer efficiency (Marschner 2011). Plant nutrient analyses allow the calculation of P recovery (percentage of the nutrient that was applied that is taken up by the plant) (White and Hammond 2008). which is useful for comparing struvite with other P fertilizers. However, differences in P recovery are not always correlated with an increase in biomass. Growth responses are strongly modulated by interactions between mineral nutrients and other growth factors (soil pH, atmospheric $CO_2$, or phenological stage of the plant) (Jakobsen et al., 2016). Therefore, biomass response for a particular nutrient cannot be described as constant, and it is not easy to identify by analyzing only biomass and nutrient content in the plant. Deficiency or toxicity of some nutrients can also inhibit plant growth. Nutrient deficiency or toxic values are typically described in % ranges (Marschner 2011). Analyzing nutrient concentration rather than content or recovery might allow in some cases a more precise diagnosis of plant nutritional state.

Struvite has been previously proposed as a slow-release fertilizer (Rahman et al., 2014). The slow release can ensure steady nutrient supply for plants that might improve fertilizer efficiency and plant growth, promote root development in the early stages and avoid nutrient leaching. On the other hand, slow release of P have been reported to cause a delay in nutrient availability for plants after the application, with a consequent initial delay in the growth response. To analyze the effect of slow release fertilizers in plant growth, measurements of biomass at the final harvest would not be enough as those fertilizers can have different P availability levels along the growth period. Therefore, new approaches to dynamically observe plant biomass accumulation of the slow vs. quick P release fertilizers at different plant life stages are necessaries.

If struvite is used to fertilize horticultural plants, where the rooting media has a poor buffering capacity, a slow fertilizer release can be beneficial. Similarly, struvite fertilization is potentially a nutrient management option in sandy soils like those in Western Australia, where the water bodies of the Coastal Plain became eutrophic partly because of the low P retention of the sandy soils (Summers et al., 1993).

I hypothesize that P recovery (P content in plants related to the amount of P added) is comparable to the recovery of conventional P fertilizers derived from rock phosphate producing similar biomass yields. This P recovery might not differ between fertilizers; however, it would be specific for each plant species, due to differences in root morphological and physiological characteristics. I also hypothesize that slow-release properties of struvite will be reflected in the biomass accumulation differently at distinctive plant growth stages.

### 1.3.2 Root and rhizosphere processes and its effects on P availability

Plants are able to directly uptake P in the form of orthophosphate ($P_2O_4$) (Schachtman et al., 1998). As detailed before, it precipitates very quickly with other ions present in the soil and consequently the mobility of plant available P in the soil is low. That is the reason why, as a result of continued fertilizer use, many fertilized soils have accumulated significant amounts of P (Simpson et al., 2015).

P availability is influenced by the dynamic changes in the rhizosphere environment. Those changes can be caused by the plants or by the nutrients applied in the soil that can modify the chemical properties of the soil, such as pH (Marschner and Romheld 1983).

Plant strategies for efficient mobilization and acquisition of phosphorus involve physiological and morphological changes in different root traits. Studying possible effects of the applied fertilizers on the root morphology should help to identify those traits that are related to an increase in the P uptake. Phosphorus concentration in the rhizosphere and nitrogen source applied can act as growth regulators, which can significantly alter root system architecture.

The most significant morphological traits that affect P and N mobilization and uptake are:

i) Reduction of length and thickening of the primary root, and proliferation of lateral roots (Hammond et al., 2004).
ii) Plants growing with N applied as $NH_4^+$ might require a larger root surface area for N acquisition compared to when N is applied as $NO_3^-$ , as the ammonium is less mobile (Cambui et al., 2011).
iii) Placement of roots in regions with higher phosphorus availability (Postma et al., 2014a).
iv) Increased root surface area through denser and longer root hairs that enable foraging for plant-available P and its uptake from the soil solution. (Hammond et al., 2004).

One noteworthy plant physiological adaptation to increase the P recovery is the exudation of carboxylates (low molecular weight organic anions) into the rhizosphere when plant experience P

defficiency conditions (Richardson et al., 2011, Veneklaas et al., 2003). Because of this reason, it was expected that lupine, due to its high capability to accumulate carboxylates, will have generally a higher P uptake efficiency from struvite, even though struvite solubility is lower than other commercial P sources at certain pHs. This process can be beneficial not only in soils which accumulate high amounts of unavailable P but also in poor alkaline soils fertilized with struvite, where those plant mobilization strategies will likely improve P uptake.

Combining the analyses of morphological and physiological root traits, the phosphorus use efficiency of a specific fertilizer can be examined. Phosphorus use efficiency is defined as P recovery per unit of root length or unit of root surface area. Taking to account which root traits can enhance struvite-P use efficiency when apply it as fertilizer would increase struvite's value as a P fertilizer and might enable it to more easily compete with mineral fertilizers (Manske et al., 2001; Shenoy and Kalagudi 2005).

I hypothesize that the different mechanisms of modification in root morphology and physiology including root diameter, total root length and the capacity to exudate carboxylates are different between lupine and maize, but also that they will vary as a response to the P source applied.

### 1.3.3 Investigating plant-microbe interactions and physical-chemical soil properties

Besides the root-triggered processes mentioned above, plant-microbe interactions also affect nutrient availability and uptake. Many rhizosphere bacteria can enhance P solubilization and release P from rocks (as reviewed in Hunter et al., 2014) that might be relevant when related to struvite P availability analyses. Struvite, besides being a P source, is also an ammonium source for plants. As for other ammonium sources such as organic fertilizers, its availability will depend in a direct and indirect way on biological activity for mineralization into forms that can be easily absorbed by the plant. Bacteria will transform ammonium ($NH_4^+$) to nitrite ($NO_2^-$), which will be further converted into nitrate ($NO_3^-$) (Thion et al., 2016). For this reason, studying N-mobilizing bacteria is necessarily related to analyses of the nutrient availability from struvite.

The study of root-associated microbes has received significant attention since high-throughput sequencing has allowed measurement of relative abundance, potential activity, and function of the microbial community (Grunert et al., 2017). These analyses of microbial populations might be applied to manipulate the microbial community in order to increase N mobilization or P mineralization and solubility (Ulen et al., 2010),

Most of previous studies focused on interactions between different types of soils and soil textures (Girvan et al., 2003; Sessitsch et al., 2001). Plant species effect on soil microbial community has

taken some attention before (Garbeva et al., 2006). Nevertheless, the reports in the literature explaining the mechanisms of adaptation of microbial community to the fertilizers applied are few and have not yet fully elucidated the mechanisms behind. As an example, Lazcano et al., (2013), compares the effect or organic and inorganic fertilizers on soil microbial community structure, or Stark et al., (2007) that analyze the influence of organic and mineral amendments. Therefore, the study of the microbial community structure in the rhizosphere affected by specific fertilizers applications is required.

I hypothesize that different nutrients sources such as organic fertilizer and struvite would influence differently the microbial community composition in the rhizosphere associated with the substrate and plant species analyzed.

In addition to acting as growth regulators, the nutrient source applied can change the rhizosphere pH (Gahoonia et al., 1992). Changes in physical-chemical soil properties like soil pH, not only inherently modifies plant growth but also alter P availability. As an example, the effect of the nitrogen source applied together with the struvite will be studied. The uptake mechanisms for the main two ions, ammonium and nitrate, will have clear consequences for soil pH that will indirectly affect struvite availability. Alkalinization by $NO_3^-$ nutrition and acidification by $NH_4^+$ nutrition (Britto and Kronzucker 2002) can affect other processes such as i) inhibition of nitrification rates (Falkengren-Grerup 1995) and as mentioned before ii) changes in the availability of nutrients, as the increase of P availability (Ruan et al., 2000).

The solubility of many phosphorus compounds is highly dependent on the soil pH. Under acid conditions (pH < 5), phosphorus is precipitated as Fe or Al phosphates. With increasing soil pH, the solubility of Fe and Al phosphates increases but the solubility of Ca phosphate decreases, except for pH values above 8 (Hinsinger 2001). Chemical properties of struvite will make it more available at acidic conditions compared to alkaline or slightly neutral pH.

Highly soluble P sources such as triple superphosphate (TSP) normally add P to the soil in the form of the $H_2PO_4$ ion, which can acidify soil with a pH greater than 7.2 but has no effect on soil pH in acidic soils (Fertilizer technoclogy research center). Struvite, however, adds P to the soil in the form of $PO_4$, as Mg when in solution substitutes the $H^+$. Therefore struvite fertilization would increases pH.

I hypothesize that changes in soil pH induced by the addition of other nutrients (such as ammonium versus nitrate) would affect struvite P availability differently compared to TSP.

## 1.4 Possibility of the new technology to analyze biomass yield and rhizosphere processes

Plant phenotyping means the quantitative analysis of plant structures and functions. So far, most of the studies that analyze the slow release properties of struvite are based on analyses of the chemical and physical properties of the product itself, not the efficiency of plant uptake over time (Rahman et al. 2011; Yetilmezsoy et al., 2013). An alternative approach to only chemical analyses is now necessary to confirm the suggested positive effects of the slow P and N release from struvite on plant performance. Due to major progress in non-invasive shoot phenotyping achieved with imaging sensors (Fiorani and Schurr 2013), struvite phosphorus availability during the growth period and consequent dry matter accumulation can be investigated. Using a shoot-imaging platform (Screen-House) that captures shoot traits of plants via image analysis, it is possible to make precise observations of plant performance at different developmental stages (Fiorani et al., 2012; Hillnhütter and Mahlein 2008; Nakhforoosh et al., 2016).

In addition to allow dynamically measurements of shoot development, recently developed phenotyping technics allow to improve the analyses of the rhizosphere processes, also difficult to study since they vary in space and time. Rhizosphere, defined as the narrow region of soil in the vicinity of plant roots that is directly influenced by the roots processes, is consequently not easily accessible for measurements. Despite tremendous progress in method development in recent decades (Oburger and Schmidt 2016), finding a suitable method to monitor rhizosphere processes still represents a challenge. Growing plants in rhizotrons is a well-known technique for non-destructive root morphology measurements. Recently, it has been shown that it also allows the combined analyses of root architecture with the visualization of the rhizosphere processes via chemical imaging using optical pH sensors (planar optodes) (Blossfeld et al., 2013). Planar optodes offer a unique opportunity to monitor in situ the concentration changes of the analyte of interest (in this case the pH) in the vicinity of roots based on photoluminescence (Santner et al., 2015). The planar optode set-up consists of a foil containing the sensor, which is in contact with the rhizospheres as it is fixed to the inner side of the rhizotron's surface (Blossfeld et al., 2010). I therefore included in my studies noninvasive continuous analyses of pH changes in the rhizosphere produced by the plant-soil activities using optodes, of plants treated with two different fertilizers (organic N source and struvite).

When analyzing rhizosphere chemistry (like nutrient concentration, or plant exudates), if the sampling is done just one time at the end of the experiment in non-sterile conditions (such as an organic substrate), results can be influenced by the microbial activity and provide therefore wrong

conlusions (Kuijken et al., 2015). Using optodes, it will be possible to target specific changes in rhizosphere pH at different time points that might be afterward related to root activities. In this thesis, the optode pH measurements were used as a guide to sample the rhizosphere for later analyses of the microbial community.

## 1.5 Target plants

There is a large diversity of plant nutrient acquisition strategies that include modifications of different plant traits (Zemunik et al., 2015). In this thesis, to chose the target species I will focus first on two main trait adaptations:

i) Root morphology as an indicator of spatial nutrient availability adaptations (Richardson et al., 2011).
ii) Exudation of organic compounds such as organic acids as an indicator of modifications in the nutrient bioavailability (Pang et al., 2010).

Besides that, plant species are naturally adapted to grow better at specific pH conditions. Normally it is hard to separate the direct effect of pH on plant growth with the indirect effect associated with the solubility and subsequent availability of nutrients. Therefore, a second selection criterion to select the species will be plant species that are naturally adapted to grow under different pH conditions.

Four different species were chosen based on the above-mentioned root traits, and pH tolerances. For each species, there was a specific research goal: Lupine (*Lupinus angustifolius*) was used for the study of plant traits that would influence the availability of P uptake from struvite. Lupine has high physiological root plasticity, related to exudation of large amounts of organic acids, and is able to engage in symbiosis with N-fixing bacteria (Tang and Robson 1993). Lupine is adapted to grow under acidic pH conditions (Tang et al., 1998). Tomato (*Solanum lycopersicum*), a major plant for fruit development, was chosen to study the horticultural application of struvite. Tomatoes also have high root exudation capacity (Kuijken, 2015) and an optimal growth for high fruit yield between pH 5.5 and 6.5; however, it was shown that growth was not depressed by increasing the pH until 8.5 (Islam et al., 1980). Maize (*Zea mays*) has a large agronomic importance that will allow translating the results to the agricultural practices. It is known to exhibit extensive root morphological alterations to modify P acquisition (Postma et al., 2014b; Li et al., 2014), rather than increasing root exudation (Wen et al., 2017). Viola (*Viola cornuta*) was used due to its economic relevance as an ornamental plant. It was previously shown that viola grow and develop well in organic substrates consisting of a mixture of peat and recovered nutrients (Janicka and

Dobrowolska, 2013). Fig. 3 summarizes the influence of plant traits, soil chemical properties and fertilizer addition on nutrient turnover and the potential interactions

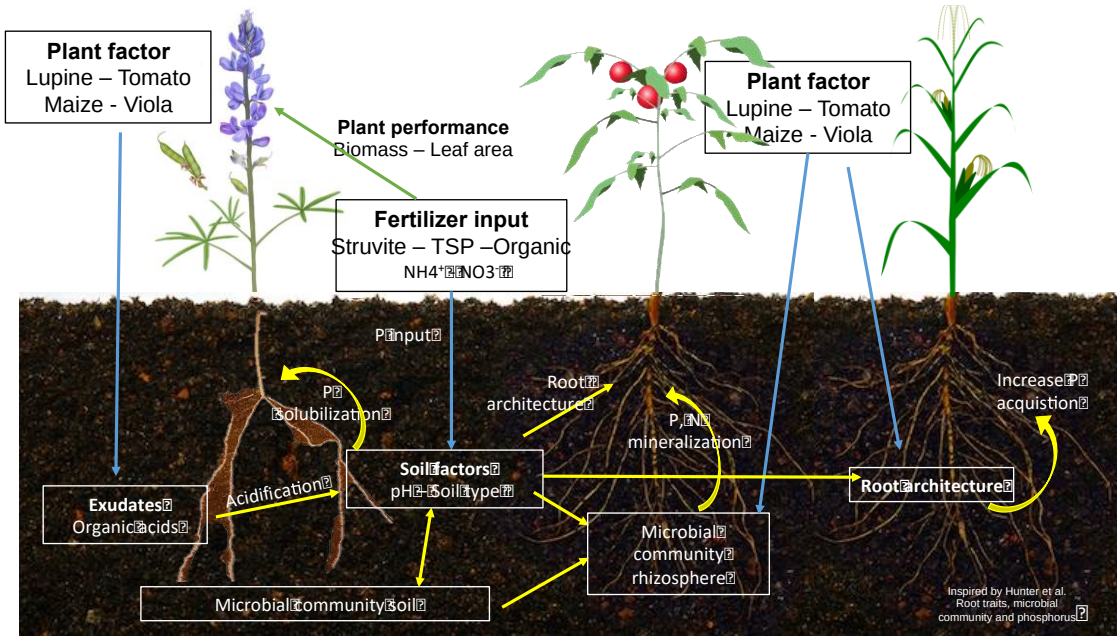


**Fig. 3 Influence of plant species, with specific root systems and exudation on nutrient turnover and microbial community, and their potential interactions**.

## 1.6 Objectives and hypothesis

The recycling of recovered products using them as plant fertilizers needs to be accompanied by individual case studies to improve the utilization efficiency in every case.

The objectives of this thesis are:

i) Study which plant-rhizosphere-soil factors have a major effect on struvite availability.

ii) Analyze which of those factors and plant traits increase the use efficiency of struvite.

iii) Translate this knowledge not only for struvite but also for other sources of recycled P.

The hypotheses, as described in each section are:

i) P uptake efficiency from struvite (P concentration in plants related to the amount of P added) would be comparable to the recovery of conventional P fertilizers derived from rock phosphate producing similar biomass yields. This P acquisition efficiency will not differ between fertilizers; however, it would be specific for each plant species, due to differences in root morphological and physiological characteristics.

ii) The different mechanisms of variation in root morphology and physiology including root diameter, total root length and the capacity to exudate carboxylates are different between lupine and maize, but also different between struvite commercial P fertilizers.

iii) Different nutrients sources such as organic fertilizer and struvite would influence differently the microbial community composition in the rhizosphere associated with the substrate and plant species analyzed.

iv) Changes in soil pH induced by the addition of other nutrients (such as ammonium versus nitrate) would affect struvite P availability differently compared to TSP.

## 2. Material and Methods

Throughout this dissertation a variety of experiments were done to study i) which factors have an effect on struvite availability, ii) which of those factors and traits increase the use efficiency of struvite.

### 2.1 Model Plants

Four plant species with different strategies to mobilize nutrients, as well as various market applications, were selected for the experiments performed in the course of this dissertation (Table 1).

**Table 1 List of plant species, indicating the interest and the identification name along the dissertation**

| Specie | Variety | Interest | Identification name | Producer company |
|---|---|---|---|---|
| *Lupinus angustifolius* | Subs. angustifolius | High exudation of organic acids, C3, N fixing | Lupine | Kiepenkerl |
| *Solanum lycopersicum* | Variety Maxifort | Major crop plant for fruit development, C3 | Tomato | Monsanto |
| *Zea mays* | Badischer Gelber | High agronomic importance, C4, monocotyledon from Poaceae family | Maize | Kiepenkerl |
| *Viola cornuta* | Hornveilchen Gelb | Economic relevance as an ornamental plant, C3 | Viola | Kiepenkerl |

### 2.2 Fertilizers

The fertilizers studied in this dissertation provided phosphorus (P) and nitrogen (N) (Table 2). The specific concentrations were adapted to each setup and are detailed for each experiment in section 2.5.

The struvite ($NH_4MgPO_4$) was mainly investigated as a P source. The Laboratory of Chemical and Environmental Engineering (LEQUIA Girona, Spain) supplied the different types of struvite used in this

thesis. Struvite was recovered from wastewater or manure after anaerobic digestion and solid-liquid separation before the biological nitrogen was removed. The composition of struvite samples were: ammonium-N ($NH_4$-N), 6.64 ± 0.17 %; phosphate ($P_2O_5$), 30.2 ± 0.8 %; magnesium (MgO), 17.5 ± 0.4 %; total organic carbon (TOC) of 0.03% of dry weight. The contents of nutrients in the different sets of struvite remained the same, but the purity and color were subject to changes. Struvite as P source was used in experiments 2-3-4-5-6-8-9. Additionally, one sample of potassium struvite ($KMgPO_4$) provided by a Water Treatment Plant from Netherland (www.smg.nl) was also tested as part of a fertilizer blend in experiment 10.

The highly soluble mineral P fertilizers used as positive controls were i) triple superphosphate (TSP) and ii) potassium phosphate ($KH_2PO_4$, KP) (Table 2). The N fertilizer used as a control when struvite was analyzed as an N source in experiment 7 was a commercially available organic fertilizer (8 % organic-N, 2.18 % P and 4.97% K), (Frayssinet, France) (Table 2).

Besides using struvite as P or N source, struvite was also used as part of a fertilizer blend at experiment 10. BOKU-IFA (University of Natural Resources and Life Sciences, Vienna) provided samples of ammonium sulphate and ammonium nitrate recovered from the wastewater and manure batches during lab-scale experiments.

**Table 2 List of fertilizers used and provider**

| Recovered products and fertilizers | Provide |
|---|---|
| Struvite (recovered at lab scale from digested manure) | Lequia - ES |
| Struvite WWTP (influent from a Wastewater treatment plant) | Lequia - ES |
| Ammonium Nitrate | BOKU - AT |
| Ammonium Sulphate | BOKU -AT |
| Potassium Struvite (recovered from a water treatment plant) | (www.smg.nl) - NL |
| Potassium Phosphate | UWA - AU |
| Triplesuperphosphate | (www.vanloonhoeven.nl) - NL |
| Organic fertilizer | Frayssinet - FR |

When struvite was used as P source, and other essential nutrients were necessaries for plant growth (Exp. 2,4,5,6,8 and 9), they were provided as nutrient solutions. The final nutrient concentration depended on the duration and goal of the experiment:

i) Experiments longer than ten weeks: nutrient solution concentration (g $L^{-1}$) 0.1 N, 1.2 K, 0.65 Ca, 0.22 Mg and 1.1 Cl (corresponding to 1/3 modified Hoagland solution) (Hoagland, 1920). Three increasing concentrations of those nutrients (15, 30 and 55% of total concentration) were applied via fertigation to the soil in the 1st, 3rd and 5th week of the experiments 4 (2.5.4) and 5 (2.5.5) and 6 (2.5.4). N was applied either as ammonium or as nitrate.

ii) Experiment shorter than ten weeks: nutrient solution concentration (µg $g^{-1}$ dry soil) 30 N, 40 S, 24 Ca, 11.5 Mg, 0.5 Cu, 5 Fe, 55 Cl and 80 K. The supply of appropriate concentrations of $NH_4NO_3$ and $K_2SO_4$ to the P treatments balanced the potassium and nitrogen provided by the struvite. For experiment 9, the pH of the nutrient solution was adapted two times from the original pH 4.5 to a neutral solution (pH 6.5) by the addition 10 ml KOH $L^{-1}$ solution (200mM), and alkaline solution (pH 8), by adding 25 ml KOH $L^{-1}$ solution (200mM). The pH of the nutrient solution was adapted to keep the desired pH conditions in the sand (2.5.8).

When struvite was used as an N source (Exp. 7) nutrient solution iii) was provided, which is identical to the nutrient solution i) but without N.

## 2.3 Rooting media

Plants grew in different rooting media (defined as any sand, substrate or soil where plants were growing) with specific physical and chemical properties (Table 3 and 4).

### 2.3.1 Sand

Sand is as a rooting media with very low organic matter content (<0.5%), which reduces the biological process and therefore might reduce nutrient availability. On the other hand, the air space generated by the sand structure should retain water as well as nutrients in the solution, avoiding dehydration and supporting plant growth. The P concentrations used in all sands were indicated as suboptimal P supply for plants (<1mg 100g $^{-1}$ soil, analyzed by LUFA) according to the German soil P classifications (Düv.,2009). The sands used were: i) Acidic sand (pH 4.8) imported from the substrates company Peltracom, Belgium, ii) Alkaline sand (pH 8.1) imported from the company Natursteinbrüche Bergisch Land GmbH, Wuppertal and iii) a neutral course river sand from Western Australia (pH of 6.5) provided by the University of Western Australia (UWA) (Table 3).

**Table 3 Chemical composition of different rooting media used**

| | Acidic sand | Alkaline sand | River sand | Organic substrate | Null Erde | Spanish acidic | Spanish alkaline |
|---|---|---|---|---|---|---|---|
| Nt (mg/l) | <0.01 | <0.01 | <0.01 | 1.7 | <2 | <2 | 43 |
| P (mg/l) | <0.01 | <0.01 | <0.01 | 18.7 | 6 | 83 | 142 |
| K (mg/l) | <0.01 | <0.01 | <0.01 | 115 | 13 | 59 | 426 |
| O.M (%) | <0.01 | <0.01 | <0.01 | 96.5 | 35.4 | 2 | 5.3 |
| pH | 4.8 | 8.5 | 6.5 | 5.5 | 6.1 | 5 | 7.2 |

### 2.3.2 Substrate

A substrate, often also referred as “potting soil,” is a material different to soil used to supply nutrients, air, and water to the root (Mofidpoor, 2007). Substrates are formulated as a blend of different elements like peat, coconut, wood fiber, bark, and composted materials; and are usually enriched with lime to get the desired pH. In this dissertation, the objective of using substrates was to control the initial chemical condition (Table 3) as well as including a more realistic physical structure for the plants to grow (Table 4). The substrates used were: i) an organic substrate (GB, Grow Bag, Peltracom, Belgium), consisting of a mixture of white peat [80% v/v], and coconut fiber [20% v/v] ii) Null-Erde (Einheits Erde- Classic, Substrat, Germany).

### 2.3.3 Soil

In order to test the effects of struvite in natural agricultural soils, the ManureEcoMine project partners provided agricultural soil samples with contrasting pH from Galicia, Spain (acidic) and Catalunya, Spain (alkaline). Both soils were sieved and dried before the fertilizers were added. Soil samples were analyzed to confirm the differences in the pH between both soils (Table 3), (pH analyses by $CaCl_2$ extraction, see section 2.8.3). Both, the acidic and the alkaline soils were treated with a chemical P buffer (Compalox®, Martinswerk GmbH) before adding the fertilizers to avoid clouding effects of the initial high P content on the investigated fertilizer. The amount used was 5% buffer (weight) for the acidic and 10% (weight) for alkaline soil. Nevertheless, the P content in the soils could only be reduced by around 50% in both cases. The final amount for each soil is described in Table 3.

**Table 4 Physical characteristics of the rooting media.** Values are mean (n=4) ± SD. Na= not analyzed.

| Characteristics | Organic substrate | Spanish Acidic | Spanish Alkaline |
|---|---|---|---|
| Ash (% Dry matter) | 3.5±0.6 | na | na |
| Available water (%) | 18.75±3.9 | 32 | 25 |
| Dry matter (%) | 41.5±0.6 | 80±0.4 | 88.2±0.1 |
| Air content (%) | 33±4.4 | na | na |
| Density (kg/ $m^3$) | 225.04 | 1094 | 991 |
| Total pore space (%) | 92±0.8 | na | na |
| Humidity (%) | 58.5±0.6 | 28±3 | 32±1 |

2.4 Cultivation condition and location

Plants were grown in three different cultivation conditions depending on the site of the experiment.

i) Experiments conducted in a temperature-controlled greenhouse in Forschungszentrum Jülich (Germany, 50.89942°N 6.39211°E). Greenhouse was covered with low iron float glass with a predominant diffuse light transmission that allows plants to grow under natural light during the day, with additional assimilation lighting supplied by mercury lamps (SON–T AGRO 400, Phillips) whenever natural light intensity was below 400 µmol $s^{-1}$ $m^{-2}$, providing a total daily light period of 16 hours. Average temperature during the experiment was 25°C during the day and 17°C at night, with a relative humidity of 60% during the day and 50% at night.

ii) Experiments conducted in a climate chamber in Forschungszentrum Jülich. The conditions were: day length of 16 h, day/night temperatures of ~24/18°C and illumination was <400 µmol m–2 s–1 between 0600 and 2200 hours local time.

iii) Experiments conducted in a temperature-controlled glasshouse at the University of Western Australia, Perth, Australia with an average daytime temperature of 24°C and average night temperature of 21°C.

## 2.5 Description of experiments

The experiments of this dissertation are listed below with identification number. Table 5 summarizes these experiments indicating the species, fertilizer treatments, measurements and main goal, showing the reference to the section where each factor will be further explained.

1. Dose response
2. Germination test
3. Nodulation test
4. Screening of factors driving higher biomass variation in sand
5. Phosphorus availability from struvite modified by N source applied and consequent effects on plant performance (A&B)
6. Phosphorus availability from struvite in different agricultural soils with different pH values. Comparison between lupine and maize
7. Rhizotron experiment to analyze root architecture and rhizosphere dynamics
8. Testing citrate flushing on struvite P release
9. Effects of pH and P fertilizer (struvite vs. potassium phosphate) on lupine morphological and physiological traits
10. Growth of *Viola cornuta* in response to fertilization with a novel recovered nutrient blend containing struvite

**Table 5 Summary of conducted experiments along the thesis**. The treatments, measurements, experimental goal and growth conditions are indicated. In brackets is specified the sections where the species used, the measured traits and the growth conditions are explained more in details.

| Experiment | | Treatment | | Measurements | | | Experiment goal | Growth conditions | |
|---|---|---|---|---|---|---|---|---|---|
| n° | Tittle | Species (2.1) | Fertilization (2.2) | Biomass (2.6) | Root morphology (2.6 & 2.7) | Other main specific traits (2.6 to 2.9) | 2.5 | Soil (2.3) | Location (2.4) |
| 1 | Dose response | Lupine, Maize | 1/3 Hoagland solution | x | | Visual assessment of nutrient deficiency symptoms in leaves | Optimum dose of nutrient application | Organic substrate | i |
| 2 | Seeds Germination | Lupine, Maize | Struvite-water | | | Count the germination rate | Struvite effect on seed germination | Acidic, Alkaline sand | i |
| 3 | Nodulation of Lupine | Lupine | Struvite (with N/without N)-no nutrient | | x | n° of nodules and nodulation effectiveness | Struvite, N and pH effect on nodulation | Acid/alkaline sand, Null Erde substrate | i |
| 4 | Screening pH, P and N effect on biomass | Lupine, Maize | Struvite-TSP-NoP | x | | Nutrient content plant | % biomass variability driven by each factor | Acid/alkaline sand | i |
| 5 | Struvite-P Availability | Lupine, Maize | Struvite-TSP-NoP | x | x | Nutrient content plant, P uptake, Physiological use efficiency | Struvite availability modified by N source and plant species | Acid sand | i |
| 6 | Agricultural Spanish soils. | Lupine, Maize | Struvite-TSP-NoP | x | | | pH and soil physic effect on struvite availability | Spanish acid/alkaline | i |
| 7 | Rhizotron test for microbial analyses | Lupine, Tomato | Struvite-Organic fertilizer | x | x | Microbial Abundance, activity and diversity | Plant and fertilizer effect in microbial community | Organic substrate | ii |
| 8 | Flushing with citrate | - | Struvite-citrate | | | P content in sand and leachate | Increase struvite solubility by external citrate addition | Acid/alkaline sand | i |
| 9 | Organic acids extraction in Australia | Lupine | Struvite-Potassium phosphate | x | x | Root carboxylates exudation | Differences in the organic acids exudation between P sources | River sand | iii |
| 10 | Blend experiment | Viola | Blends of recovered products | x | | Chemical properties soil | Analyze struvite as part of a fertilizer blend | Organic substrate | i |

### 2.5.1 Dose response (Experiment 1)

The goal of experiment 1 was to identify the optimum P and N concentrations in the modified Hoagland solutions, suitable for tomato and maize growth. The dose-response curve characterized plant growth varying from deficient to optimal nutrient application. Plants were grown in 1.45L Pots (11cm x 11cm x 12cm), and each pot was filled with the corresponding amount of organic substrate (225g) regarding the density (Table 4). Germinated seeds of maize and tomato were individually transplanted into the pots one week after germination. Plants were grown under the conditions explained in 2.4 and with six different nutrient doses of N:P:K detailed in Table 6. The soil was supplemented with basal nutrients (mg plant$^{-1}$): 69.6 $Ca(NO_3)_2 \cdot 4H_2O$, 19.2 $MgSO_4 \cdot 7H_2O$, and 3.3 EDTA-FeNa to ensure that the supply of other nutrients was adequate for plant growth.

Fertilizer content for the 100% dose was calculated according to 1/3 Hoagland solution modified to reach a N addition comparable to what is added in the field (200kg Ha$^{-1}$). The pot volume was 1.45L, which entails an amount of 100mg N pot$^{-1}$ with a fixed 1:1 $NH_4$: $NO_3$-N ratio, 12mg P pot$^{-1}$, and 80mg K pot$^{-1}$. The other doses (200, 50, 25, 10 and 0%) were prepared from dilutions of the 100%, except the dose 200, which was developed from the formulations. The different fertilizer doses were applied with the irrigation water six days per week during four weeks in an amount of 50 ml day$^{-1}$ with a total of 1.2L of water and fertilizer per pot. The amount of water was calculated to avoid leaching of water and nutrients and to keep soil moisture at ~50% of water holding capacity. Five plant replicates were cultivated per each dose and pots were arranged in a randomized design. Finally, plants were harvested 28 days after planting (DAP), when visible growth differences among the P doses were observed.

**Table 6 Nutrient doses (mg N-P-K plant$^{-1}$) applied in experiment 1**

| Dose (%) | N (mg plant$^{-1}$) | P (mg plant$^{-1}$) | K (mg plant$^{-1}$) |
|---|---|---|---|
| 200 | 194.97 | 24.08 | 167.4 |
| 100 | 97.48 | 12.04 | 83.7 |
| 50 | 48.74 | 6.02 | 41.85 |
| 25 | 24.37 | 3.01 | 20.92 |
| 10 | 9.74 | 1.20 | 8.37 |
| 0 | 0 | 0 | 0 |

### 2.5.2 Germination test (Experiment 2)

The effect of soil pH and struvite concentration on lupine and maize seeds germination was evaluated in two different types of sand: alkaline (pH 8.5) and acidic (pH 4.8). Each type of sand was treated with i) nutrient solution with no extra P, ii) nutrient solution plus P as struvite (60 mg $kg^{-1}$ sand), and iii) no fertilizer addition (control). Each treatment was prepared in three kg of sand and was distributed in individual trays (Fig. 4). Once in the tray, half of the sand was sown with lupine seeds and the second half with maize seeds (70 seeds per treatment). Germination rate was determined visually at 2, 3, 6, 7 and 8 days after sowing.

**Fig. 4 Experimental design of the germination test.** Representative tray with alkaline sand under control condition (without fertilizer). Lupine seeds on the left, maize seeds on the right.

### 2.5.3 Nodulation test (Experiment 3)

The effect of different soils, pH values and the presence or absence of N on nodulation was evaluated for lupine in experiment 3. Four different soils were used: acidic and alkaline sand, Null Erde, and a mixture of sand and substrate (1:1, v/v). Plants were grown in pots (9x9x9.5cm). The N applied was equivalent to what is implemented in field practices: 200kg $Ha^{-1}$ (2m depth). The resulting amount was 0.076g N in each pot that was added via fertigation within 50 ml water, and the amount per liter of solution was 1.52g (0.76 ammonium-0.76 nitrate).

Ten days after germination the nodulation was visually assessed every other day over the course of 2 weeks, with a total of 6-time points. At each time point, three randomly selected plants were removed from each rooting media with and without N addition, making a total of 24 plants per time point, and a total of 144 plants at the end of the experiment. To identify the nodules, the sand or substrate was carefully washed off the roots. For the assessment of nodulation, five parameters were studied following a nodulation guide (Field guide for nodulation and nitrogen fixation, 1991). The parameters were i) the plant vigor, ii) nodule number, iii) position, iv) color, and v) appearance. Each parameter was evaluated and graded with a score of 1 to 5. The sum of scores for all parameters indicates the effectiveness of nodulation: effective nodulation (20- 25), low nodulation (15-20), and unsatisfactory nodulation (0-14).

### 2.5.4 Screening the main factors driving biomass variation (Experiments 4 & 6)

To study struvite effect, it is necessary to know beforehand how other factors that define the current setup will influence the plant growth. Knowing this, there will be less risk attributing a right or harmful effect on the plant to the struvite when it was caused by another factor. Therefore the effect of soil pH and soil chemical composition on lupine and maize growth were analyzed in Experiment 4 and Experiment 6 under the conditions i) described in section (2.4). At the same time, it was analyzed for each condition the effect of struvite, TSP and no P application in the plant performance. Each factor (pH and P source) was tested with ten different replicates and a total of 60 pots per each species resulting in a total of 120 pots.

The different P sources (struvite or TSP) were thoroughly mixed with the rooting media in an end-over-end mixer (Table 9) for 10 minutes to produce a homogeneous fertilizer distribution. The fertilized mixtures were left undisturbed for a period of three days prior to the start of the experiment. A corresponding amount of each rooting media was left unamended and used for the unfertilized control treatment (NoP). Lupine and maize seeds were pre-germinated on filter paper at different times according to the pre-calculated germination time for each species determined as described in 2.5.2.

In experiment 4 the rooting media used were acidic sand and alkaline sand (Table 3). Pots, with a volume of one liter were filled with the sand-fertilizer mixture. The acidic and alkaline sands were amended with the recycled P as struvite or highly soluble mineral P in the form of TSP, both as solid powder, at a rate equivalent to 0.010g P $plant^{-1}$ and L $soil^{-1}$. Plants were harvested 4 weeks after transplanting. In experiment 6 two Spanish agricultural soils with different pH and chemical composition (Table 3) were used as rooting media. Both soils were amended with the recycled P as struvite, or highly soluble mineral P in the form of TSP, both as solid powder at a rate equivalent to 0.036g P per plant (0.010g $Kg^{-1}$ soil). The P amount applied was higher than in experiment 4 as plants were growing for a longer period (6 weeks instead of 4 weeks), as it was the volume of the pots used. Subsequently, each 3L pot received the corresponding amount of the soil-fertilizer mixture (3.1kg for the acidic and 2.9 for the alkaline) regarding the soil density (Table 4). Plants were harvested after 40 days of growth.

In both experiments plants were fertigated manually 3 times per week to supply the necessary amount of nutrients described in section 2.2 to fulfill plant demands and retain 50% water holding capacity. Pots were placed in a complete randomized design and randomly rearranged 3 times per week.

### 2.5.5 Struvite P availability modified by the nitrogen source applied and its effect on lupine and maize growth (Experiments 5A and 5B)

Experiment 5A followed a three-factorial (P-fertilizer, N-fertilizer and plant species) completely randomized design with 10 repetitions. Fertilizer treatments comprised of struvite and an unfertilized control (no P) and each of them was combined with either ammonium or nitrate as the N form applied. The two different plant species in this study were lupine and maize. Plants were grown in 3.5L pots filled with the corresponding amount of sand-fertilizer mixture and were continuously monitored in the automatic shoot phenotyping platform ScreenHouse (Nakhforoosh et al. 2016) under condition i) described in 2.4

Seeds were pre-germinated on filter paper at staggered times according to their pre-determined germination time. Two seedlings were transplanted into each pot at a depth of 2cm to ensure uniform seedling growth in the experimental pots. After one week, one seedling was removed. The automated watering system in both experiments (costume-made, Hellmuth Bahrs GmbH & Co. KG, Brüggen-Bracht, Germany) was adjusted to remain at 50% of the water holding capacity calculated by pot weight.

A follow-up experiment (experiment 5B) was conducted following the set up from experiment 5A with minor modifications. In this case the no P treatment was replaced with a positive control: fertilization with highly soluble triple superphosphate (TSP). In experiment 5B, the number of replicates was reduced to five based on the level of variability we found in experiment A. The recycled P as solid struvite powder or highly soluble mineral P in the form of solid powder TSP were applied at a rate equivalent to 0.036 g P per plant (0.010g $Kg^{-1}$ soil). Plants were harvested after 40 days of growth in experiment 5A and 48 days of growth in experiment 5B Automated randomization was done three times per week.

### 2.5.6 Effect of struvite applied as a nitrogen source on lupine and tomato root architecture and microbial community in the rhizosphere (Experiment 7)

In experiment 7, lupine and tomato plants were grown in rhizotrons filled with organic substrate (Table 3 and 4) blended with organic fertilizer, and struvite (Table 2). The aim of the study was to determine the effect of recovered nutrients (struvite and the control organic fertilizer) as a nitrogen source blended with an organic substrate on i) the plant performance (section 3.3.3) ii) nitrogen dynamics (3.4.3, 3.5.2) and iii) microbial community (3.8). Plants were grown under the conditions ii) explained in section (2.4). Over the course of the experiment, plant growth, rhizosphere pH and bacterial community associated with plant roots were monitored.

The fertilizers (struvite and organic fertilizer) were mixed with the organic substrate at a dose of 100mg N $L^{-1}$ substrate and left undisturbed for three days. Each rhizotron (5L volume) was filled with 1.1 kg of the substrate-fertilizer mixture. The rhizotrons, with dimensions of 60cm x 30cm x 2cm, consist of a black polyethylene with one removable side of transparent polycarbonate plate (Plexiglas) (Fig. 5). The substrate of the control treatment was sterilized using gamma–irradiation (BGS, Wiehl, Germany) at minimal doses of 50kGy. The use of gamma-irradiation as a method for soil or substrate sterilization for laboratory experiments has been recommended over other sterilization techniques (McNamara et al., 2003).

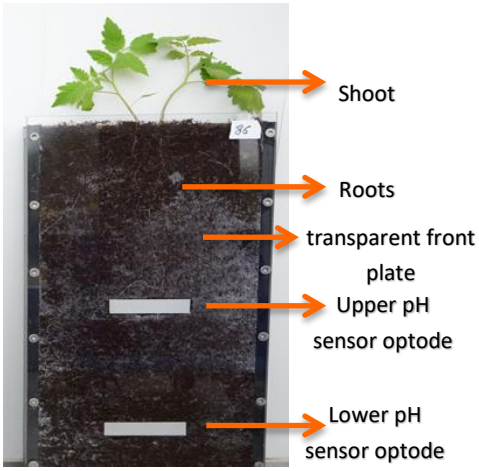


**Fig. 5 Rhizotron of 60cm x 30cm x 2cm planted with tomato, filled with organic substrate.** The position of the planar pH optode fixed in the inner side of the transparent plate is also indicated**.**

In order to monitor the pH in the rhizosphere, planar optodes (pH sensors, Presense GmbH, Regensburg, Germany) were placed on the Plexiglas with special glue (GE Bayer Silicone, Leverkusen, Germany) at 27cm from the top and a second optode was placed at a depth of 16.5 cm measured from the first optode or 43 cm from the top (Fig. 5). The optodes had a sensitive side directed to the substrate, whereas the glue side was headed to the glass. The protocol of how the measurements were done is explained in non-invasive measurements section 2.6.3 (Fig. 8). To place the optodes, the Plexiglas was removed carefully from the rhizotron (in horizontal position) after filling the rhizotrons and screwed back after placing the optodes. Afterward, the seedlings were transplanted.

Tomato and lupine seeds were germinated on filter paper and transplanted two days later (two seedlings of tomato and one seedling of lupine, per rhizotron). The seedlings were planted at a depth of 2cm and in contact with the Plexiglas. After the introduction of the seedlings, the rhizotrons were kept at an angle of 45°C during the whole growing period to allow root visualization. Each rhizotron received at the beginning of the experiment 100mL of nutrient solution as described in section 2.2. All plants were supplied with 60mL deionized water three times per week to maintain the moisture of the substrate at 30% of water holding capacity.

Two harvests were performed at two different time points. Time point 0 was defined as the time when the rhizotrons were filled, and seeds were sown on the top. When the roots reached the level of the upper optode (20DAS, Fig. 5) 50% of the rhizotrons were opened, and samples were collected (time point 1). Samples from bulk zone (the substrate not influenced by the root) (± 0.5g) and rhizosphere (zone near the root) (± 0.2g) were collected within the area of the upper pH sensitive optode for microbial community analyses (Fig. 5). Optodes were used for guided sampling to determine optimal sampling times and locations with pH changes in the bulk zone and the rhizosphere. Two weeks after the first harvest (34DAS), the remaining 50% of the rhizotrons were opened, and plants were harvested as most of the roots had reached the bottom of the rhizotrons (time point 2).

### 2.5.7 Percolation experiment to determine struvite solubility as affected by the external addition of citrate (Experiment 8)

To determine P mobilization form struvite by organic acids, acidic or alkaline sands mixed with struvite were flushed with citrate. The experiment was conducted in Falcon tubes which were filled up with 10 g of alkaline sand or acidic sand (Table 3) occupying a volume of 20ml. The sand was previously mixed with either high (0.5g of struvite $kg^{-1}$ soil) or low (0.05g struvite $kg^{-1}$ soil) dose of struvite. The amount of P added to the sand with the high dose was 60mg $soil^{-1}$, i.e. 600µg P in 10g and 6mg$soil^{-1}$, i.e. 60µg P in 10g with the low dose. After the tubes had been filled with the fertilized sand, they were flushed once per week over a three-week course with 50 mL of water or citrate solution with either 10mM, 1 mM or 100µM concentrations. The leachate was collected after the water or citrate solution passed through the sand, using medical infusion syringes (Henry Schein®) (Fig. 6).

The collected liquid samples were analyzed by ICP-OES (section 2.8.1). The infusion syringes allowed us to regulate the collection of the leachate by closing the system. Therefore the collection was conducted once per week at a specified time point. Further, the P content of the sand was analyzed at the end of the experiment

**Fig. 6 Percolation tubes to analyze P mobilization from struvite after flushing the soil with citrate.** The orange syringes regulate the leachate that is collected in the tubes shown in the right picture**.**

### 2.5.8 Phosphorus mobilization by root-exuded organic acids (Experiment 9)

In experiment 9 it was determine the morphological and physiological changes in the root of lupine grown at three different pHs and fertilized with two P sources (struvite and potassium phosphate). The experiment was carried out during six weeks under the conditions (iii) explained in section 2.4. Pots (8.5cm x 8.5cm x 18cm) were filled with 1.3kg coarse river sand adapted to the correspondent pH (acidic-neutral-alkaline) (Table 3). Two pots without plants were used as controls for each treatment to monitor the sand pH development during the experiment. Three germinated seedlings of lupine were planted in each pot and thinned to one plant after one week. All seedlings were inoculated with appropriate rhizobia at planting. The inoculation was done by applying a few drops to the seedlings using a plastic syringe of a mixture of rhizobia in peat and water. Rhizobia was provided by Rutherglen Centre (Department of Primary Industries, Victoria, Australia).

The initial pH of the sand was 6.5; it was modified to an acidic pH of 4.5 and an alkaline pH of 7.8 by the addition of 3g $Kg^{-1}$ sand of iron sulphate ($FeSO_4$) or 50g $Kg^{-1}$ sand of calcium carbonate ($CaCO_3$), respectively. The sand was mixed in an end-over machine (Table 9) during 15 minutes at 99 rpm. Three P treatments were applied: i) $KH_2PO_4$ added to the original nutrient solution (2.2); ii) Struvite mixed with sand, both to a final concentration of 15µg P $g^{-1}$ of dry sand; and iii) no P addition, as a control. All essential nutrients, other than P, were provided at the final concentrations of nutrient solution ii) described in section 2.2. After filling the pots, the sand was moistened to 50% water-holding capacity by adding 130ml of the corresponding nutrient solution previously adapted to the respective pHs (acidic, neutral and alkaline). Sand water content was maintained during the rest of the experimental time by watering pots to weight with deionized water. Plants were harvested after six weeks (being this the reason to reduce the amount of P from 36 mg $plant^1$ in experiment 5 to 20mg $plant^{-1}$ in this experiment).

### 2.5.9 Assessment of struvite as part of various fertilizer blends (Experiment 10)

Using the different recovered nutrients from the ManureEcoMine processing activities (Table 7), 15 fertilizing blends were prepared (Table 8) and tested in experiment 10. Two controls were used: a blend without fertilizer and a blend with a slow release commercial fertilizer (Osmocote, Table 2).

TSP, a commertial highly soluble phosphorus source (applied in blends 1 and 2) and potassium sulphate ($K_2SO_4$) (applied in all the blends except for the no fertilizer and the osmocote) were used together with the recycled nutrients in order to fulfill the nutrient requirements of viola and test the effect of the recovered nutrients in the plant performance without having other nutrients deficiency.

The blends had an N:P:K ratio of 1:0.26:0.61 and the concentrations were 900mg N $L^{-1}$ 235mg P $L^{-1}$ and 548mg K $L^{-1}$. Plants were harvested at the onset of flowering (five weeks after planting).

Five replicates of a total of 15 treatments were tested. The volume of the pots was 1 L with a final weight of 250g. For the preparation of the substrate, 33.75 Kg of organic substrate was mixed with 0.375 Kg of lime to raise the pH to the desired value of pH 6. Viola seeds were placed at 3.5 cm depth in the middle of the pot. The dry weight of the seeds was measured before sowing.

**Table 7 Overview and coding of the different recovered nutrients and fertilizers used in experiment 10**

| Code | Description |
|---|---|
| A | Potassium Struvite |
| B | Potassium Struvite |
| C | Struvite (Product recovered from digested manure) |
| D | Struvite WWTP (Product recovered from Waste water treatment effluent) |
| E | Struvite (Struvite recovered from digested manure) |
| F | Ammonium Nitrate |
| G | Ammonium Sulphate |
| H | Triplelsuperphosphate |
| I | Potassium sulphate |

The fertilizer blending process in the organic substrate was as follows: i) Preparation of the different recovered nutrients, ii) blend preparation by weighting the correspondent amount of the recovered products, iii) blend grinding, iv) labeling of the combinations, v) mixing the blends on the organic substrate and vi) liquid ammonium sulphate addition in the necessary amounts (Fig. 7).


**Fig. 7 Overview of the fertilizer blending process in the organic substrate**: a) Recovered nutrients as delivered; b); blend preparation before grinding; c) overview of the different blends before mixing with the substrate

The physical characteristics of the organic substrate (Table 4) were analyzed before the experiment setup, and the chemical analyses of the different blends were done after the mixing (section 3.5.3).

**Table 8 Overview of the different recovered nutrients and fertilizers that form the blends for the growth of viola in experiment 10.** Values are mean (n=3) g $L^{-1}$ substrate

| | Code for the recovered nutrient | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Blend | A | B | C | D | E | F | G | H | I |
| 1 | - | - | - | - | - | 5.5 | - | 1.2 | 1.3 |
| 2 | - | - | - | - | - | - | 4.6 | 1.2 | 1.3 |
| 3 | - | 1.7 | - | - | - | 5.5 | - | - | 1.3 |
| 4 | - | 1.7 | - | - | - | - | 4.6 | - | 1.3 |
| 5 | 4.1 | - | - | - | - | 5.5 | - | - | 1 |
| 6 | 4.1 | - | - | - | - | - | 4.6 | - | 0.6 |
| 7 | - | - | - | 1.8 | - | 4.7 | - | - | 1.3 |
| 8 | - | - | - | | | | | | |
| 9 | - | - | 4.2 | - | - | 4.2 | - | - | 1.3 |
| 10 | - | - | 4.2 | - | - | - | 3.6 | - | 1.3 |
| 11 | - | - | - | - | 2.6 | 4.1 | - | - | 1.3 |
| 12 | - | - | - | - | 2.6 | - | 3.6 | - | 1.3 |
| 13 | 0.7 | - | - | 1.5 | - | - | 4.1 | - | 0.08 |
| 14 | Osmocote (6kg / $m^3$) | | | | | | | | |
| 15 | without fertilizer | | | | | | | | |

## 2.6 Non-invasive, repeated measurements

### 2.6.1 Shoot measurements

For each individual plant, leaf area and plant height were recorded automatically for those experiments carried out in the automatic phenotyping platform SCREEN-House (Nakhforoosh et al., 2016) (Experiment 5 and 6). In the experiment 10 (2.5.9) the phenological stage of the viola plants, as well as nutrient deficiency symptoms (color and appearance of leaves), and time of flowering, were recorded manually on a weekly basis.

### 2.6.2 Root measurements

In experiment 7 (2.5.6), the measurement of the roots started six days after sowing, when the first roots were visible at the plexiglas of the rhizotrons. In total, the roots were measured at six-time points using the root phenotyping pipeline (GROWSCREEN-Rhizo) described by Nagel et al. (2012). Roots were measured three times per week.

The total root length (summary of main root and lateral roots length) was measured non-invasively by tracing the root pictures taken at the transparent surface of the plexiglas. The GROWSCREEN-Root software previously described by Nagel et al. (2012) was used for data analysis. Traits resulting from performance of individual roots comprise lengths of different root orders, such as main roots and lateral roots. The root measurements were done at the point times described at 2.5.6. The visible root length at the surface of the rhizotron should represent approximately 30% of the total root system length, as published by Nagel et al. (2012). The experiment was finished at this point because the roots of plants had reached the bottom of the rhizotrons.

### 2.6.3 Soil and rhizosphere measurements

In plants growing in pots, water content, temperature, and electrical conductivity (EC) was measured twice per week after watering using a portable system (Table 9), to ensure that the pots were kept at approximately 50% water holding capacity.

At the rhizotron experiment, the pH was analyzed with optodes three times per week, non-invasively. Planar optodes, (Gansert and Blossfeld 2008), (Blossfeld et al., 2013) were used for pH measurements and guided sampling for later microbial community analyses. A camera that is sensitive to the emission range of the optode detects the emission signal, which serves as information carrier. Furthermore, using light as an information carrier allows for separation of the sensor (the optode) and detector (the camera) (Fig. 8).

In this experiment, the planar optodes were used as non-invasive *in situ* measurement of pH dynamics in the rhizosphere and the bulk zone. The used planar optodes had sensitivity in the range of pH 5.5 to 8.30.

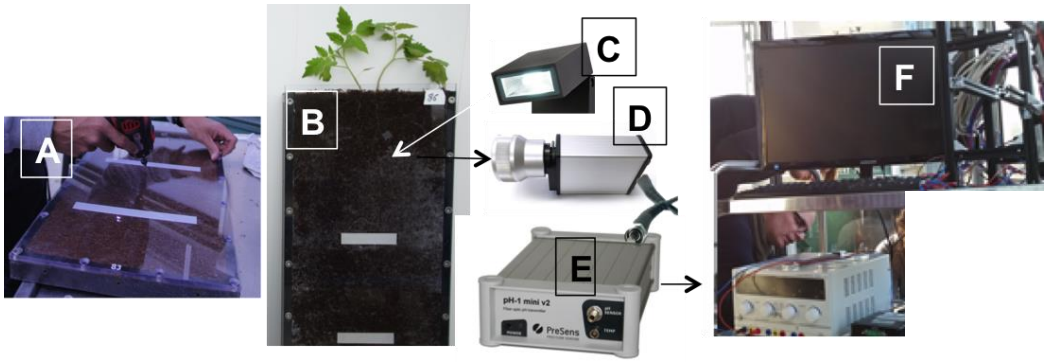


**Fig. 8 Scheme of the experimental design for the optodes pH measurements** A) Screw the acrylic front plate of the rhizotron after the optodes (white stripes) were fixed at the inner side B) Rhizotron with tomato plants illustrating the positions of the planar pH optodes. C) Excitation light that will excite the optodes D) Special camera that will record the emission light and transported through a special wire to E) Optical sensing device. F) Computer where the software is installed.

Calibration of the planar optode was done by the use of six different conventional pH buffer solutions, ranging from pH 4 to 9 (Riedel-deHaën; Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) which make possible to convert the value of the optode measurement into real pH values (Fig. 9).

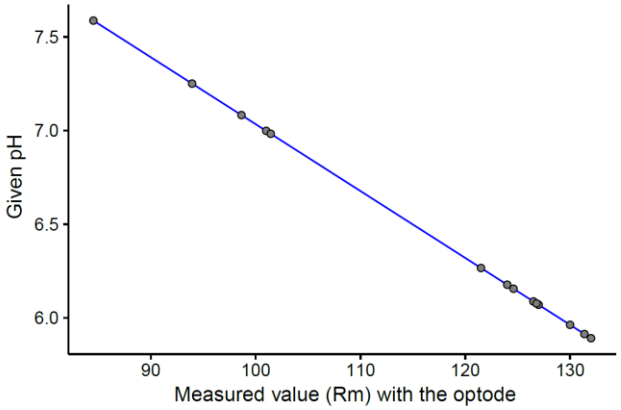


**Fig. 9 Calibration curve to extrapolate measured values from the optodes to pH values.** Rm is the measured R-value,i.e.,the ratio of red to green in the emitted fluorescence response (Gansert and Blossfeld, 2008).

Two 4 x 4 mm sections were cut from the original planar pH optode and were used as calibration replicates. Each part was fixed with silicone grease to a Petri dish. For each pH buffer solution, the Fm (imaged pH value) of 10 measurements per replicate was recorded. The mean of all 60 measurements of each buffer solution was used as input data for a calibration curve, and the derived calibration values of the coefficients were used for the calculation of the pH values. Recalibration at the end of the experiment was conducted in the same way. The Formula 1: $pH= 0.0357\ Fm + 10.604$, $R^2=0.9425$, was obtained from the calibration curve (Fig. 9).

## 2.7 Destructive measurements

### 2.7.1 Assessment of height, fresh and dry weight

The height of the plants, the number of leaves, and plant developmental stage were determined visually before harvest. Harvesting was done using secateurs. Plants were cut below the soil surface such that the basal internode remained attached to the shoot. Shoots were separated into leaves and stems, and fresh weight was measured by balance (Melter Toledo XS205) directly after harvesting. Subsequently, leaf area was measured using a leaf area meter (Li- 3100, Li-cor, Nebraska, USA). Maize blades were unwound, and all the leaves were cut at the ligule position before scanning. Plant samples were dried at 65 °C in a forced-draft oven until dry weights were stable.

### 2.7.2 Assessment of root traits

The root samples were carefully washed under running water with the help of sieves, forceps, and tweezers. All rooting media attached to the roots as well as dead roots (identifiable by the darker color and lack of elasticity and flexibility) were removed manually. The root samples were stored in 50% ethanol in water for a maximum of three weeks until root scanning.

Cleaned roots samples were scanned with a transparency adapter (Epson Expression Scan 1680, WinRHIZO STD 1680, Long Beach, Canada). Roots were spread in an acrylic box (size A4) with tap water to minimize the number of overlaps. Roots were scanned at 400 dpi resolution and 256 grays contrast. When the root samples were too large to complete in one scan, two or more scans were performed. On the scanned images of the root systems the total length, mean diameter, total area, and volume were measured, using the WinRHIZO regular V.2009 software (Regent Instruments Inc., Quebec, Canada). The debris removal filter was set to discount objects less than 1 $cm^2$ with a length/width ratio less than 10. The root length measurements were partitioned into 11 diameter classes: <0.25, 0.25–0.5, 0.5–0.75, 0.75–1.0, 1.0–1.25, 1.25–1.5, 1.5–2.0, 2.0– 2.5, 2.5–3.0, 3.0–3.5 and >3.5mm.

Data for various root traits, such as total root length, root surface area, root volume, average root diameter, and Diameter Class Length (DCL, root length within a diameter class) were generated in WinRHIZO from root images for each root section. Subsequently, the root dry weight was recorded after drying for 48 h at 65 ºC. The following parameters were based on observed and/or computed data: Root-to-shoot mass ratio (root dry mass/shoot dry mass), specific root length (SRL) = root length/root dry mass (m $g^{-1}$) and relative diameter Class Length (rDCL) = DCL/root length (yielding a proportion of root length to normalize disparity between plants sizes). In lupine roots, visual scoring of nodulation was done at harvest as described in section 2.5.3.

### 2.7.3 Soil measurements

After the shoots and roots were harvested, soil samples from individual pots were collected. Samples were dried at 40 °C in a forced-draft oven and ground (Table 9). Plant available P, K, Ca, and Mg were analyzed in the milled samples by inductively coupled plasma atomic emission spectroscopy (ICPAES). Soil pH was also measured (CaCl2 extraction). In experiment 7, soil samples were collected at the beginning of the experiment as well as at each harvest point (time point 0, 1 and 2).

### 2.7.4 Soil microbial community sampling

In experiment 7 soil samples from the bulk zone (± 0.5 g) and rhizosphere (± 0.2 g) were collected at the time points indicated in (2.5.6).

To collect the soil samples, the plexiglas with the attached optodes was carefully removed from the rhizotron ensuring minimal disturbances to the root system. A tissue with the dimensions of the plexiglass was placed on the now exposed soil in the rhizotron leaving sampling spaces open where the optodes were initially located (Fig. 10).

The samples were taken with surface sterilized material (70% (v/v) ethanol in water). Two sampling areas were considered, i) the rhizosphere, which is defined as the substrate attached to the roots - with an approximate distance < 2 mm from the roots and ii) the bulk zone, it is the substrate not attached the roots and with a distance of more than > 10 mm from the roots was sampled. The fresh weight of the substrate sampled was every time determined, and these samples were immediately stored at -80°C for microbial community analysis

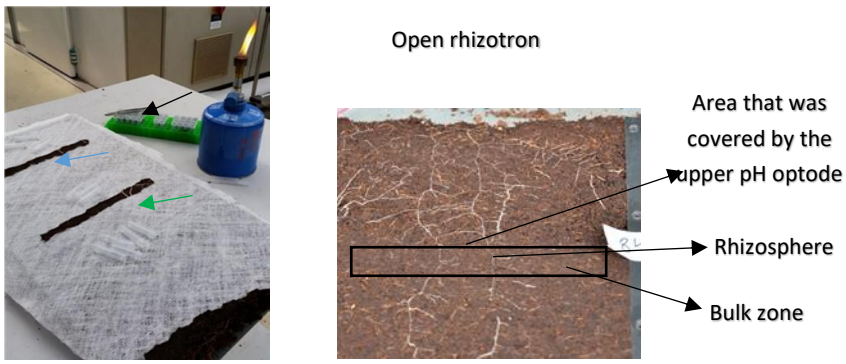


**Fig. 10 Sampling methodology for microbial community in the rhizosphere**: white tissue is placed over the substrate leaving the space open where the upper (green arrow) and lower optodes (blue arrow) were located, indicating the sampling area. Black arrow shows the vials in which samples were collected.

In total 203 samples were collected for determination of the microbial community composition. Samples were assessed by running them on an agarose gel and subsequently staining the gel with a fluorescent dye. Visualization was performed with a GelDoc containing a UV trans illuminator enabling excitation of the dye.

### 2.7.5 Sampling rhizosphere exudates and measurements of rhizosphere pH

In experiment 9, rhizosphere extracts for the analyses of organic acids were collected from lupine plants treated with two P sources at three different pH levels as described in section 2.5.8. The sand was carefully tipped out of the pots and the root systems gently shaken to remove excess sand. What still keep attached to the roots was defined as rhizosphere (Veneklaas et al., 2003) and together with the roots were transferred to a beaker containing a known volume of 0.2mM $CaCl_2$. A subsample of the rhizosphere extract was taken and filtered through a 0.22µm syringe filter into a 1 mL HPLC vial. HPLC samples were acidified with orthophosphoric acid and frozen at −20°C, until analysis.

## 2.8 Chemical and microbial analyses of plant, rhizosphere and soil.

### 2.8.1 Nutrient content in the plant and soil

Soil nutrient content in all experiment except 7 and 9 was analyzed by LUFA (Germany) by the method (VDLUFA Method Band I, A 13.1.1 bzw. A 6.4.1 (Akkr). pH-value was analyzed in CaCl2 (VDLUFA Method Band I, 1991, A 5.1.1).

In experiment 7, soil chemical analysis (pH, electrical conductivity, and total nutrient content) were performed. Nutrients were extracted (1:5 vol/vol) in ammonium acetate and measured with ICPAES. The electrical conductivity (EC) and pH, ammonium ($NH_4^+$), nitrate ($NO_3^-$), sulphates ($SO_4^{2-}$), and sodium (Na) were measured in a 1:5 v/v water extract according to EN 13038, EN 13037 and EN 13652, respectively (Soil improvers and growing media, ISO). Nitrate was measured with an (IC) ion chromatograph. Ammonium was measured by steam distillation

Nutrient contents of plant samples in all experiments except experiment 9 were determined by element analysis via inductively coupled plasma optical emission spectrometry (ICP-OES) (VarioELcube, Elementar). Soil pH was determined using standard electrodes (Hanna Instruments pH 209 pH meter), using 1:5 distilled water extract at 20°C.

In experiment 9 (2.5.8) samples were dried and ground to a fine powder using stainless steel ball mill to determine shoot and root P concentrations. Weighed subsamples of approximately 200 mg were digested using a hot concentrated nitric-perchloric (3:1) acid mixture. Total P concentrations

in root samples were determined using a UV-VIS spectrophotometer (Shimadzu Corporation, Japan) by the malachite green method (Motomizu et al., 1983).

### 2.8.2 Carboxylate exudation

At experiment 9 (2.5.8), HPLC analysis of the elution liquid were performed using a 600 E pump and 717 autosamplers (Waters, Milford MA, USA). Working standards of malic, malonic, lactic, acetic, maleic, citric, succinic, cis-aconitic, and trans-aconitic acid (ICN Biomedicals Inc, Aurora OH, USA) were used to identify carboxylates on an Alltima C-18 reverse phase column (250× 4.6 mm, Alltech, Deerfield IL, USA) (Cawthray, 2003).

### 2.8.3 pH analyses

Rooting media pH was analyzed by LUFA in CaCl2 (VDLUFA Methodenbuch Band I, 1991, A 5.1.1). For experiment 9, carried out in Australia, pH was analyzed in a solution of 0.01M $CaCl_2$ (Ahern et al., 1995).

### 2.8.4 Microbial community analyses

Total DNA was extracted from the substrate samples using the Power Soil® DNA Isolation Kit (MoBio Laboratories Inc.). Five hundred milligrams were used from the bulk and 100 mg from the rhizosphere sample. The abundance of total bacteria, NOB (Nitrobacter and Nitrospira), AOB, Archaea, and AOA of the bulk zone and rhizosphere were also analyzed. Quantification was performed using a standard curve based on known concentrations of DNA standard dilutions.

Richness, Fisher's diversity, Shannon, Simpson, and inverse Simpson indices were calculated to assess alpha diversity within each sample. Pielou's index was used as an indicator of evenness in the community (Pielou, 1966). Differences in alpha diversity and evenness measures among treatments were compared using a repeated measures mixed model in SAS (version 9.4, SAS Institute, Cary, USA), with fertilizer (no fertilizer, organic fertilizer and struvite), plant (no plant, lupine, and tomato), location (bulk versus rhizosphere) as a fixed effect for the third time point. Hence, the differences in the diversity measures could be attributed to plant, fertilizer, and location or to the interaction of the three factors.

## 2.9 Calculations

### 2.9.1 Struvite solubility using the modeling program MinTeQ

The calculation of P speciation from struvite in solution was modeled using the chemical equilibrium-modeling program Visual Minteq 3.1. (Version 3.0, Environmental Protection Agency,

Washington, D.C., EPA/600/3-84/032). Different pHs of the solution were simulated to make a solubility curve (pH from 2 to 10).

### 2.9.2 Allometric analyses of shoot root biomass allocation

During whole-plant growth in a stable environment, roots and shoots maintain a dynamic balance such that $y=bx^k$, where y is root biomass and x is shoot biomass. This allometric equation was formalized by Huxley (1924) and can be ln-transformed to become $\ln y=\ln b+k\ln x$. This formulation enables to plot ln *y* as a function of ln *x* with slope *k* (i.e. the allometric coefficient). This model allows to visualize differences from the common root: shoot relationship and is not confounded by plant size.

### 2.9.3 Analyses for nutrient use efficiency and recovery

P-recovery and P-use efficiency of plants growing under the different P sources was calculated as described by Hammond et al. (2009) with modifications using the formula F2: P-recovery = ((plant P concentration × DW)/ P applied) and F3: P use efficiency = (P-uptake efficiency/unit of root).

## 2.10 Statistical Analyses

Statistical analyses were performed using the statistical program R.2.16.3 (R: A Language and Environment for Statistical Computing (2012) http://www.R-project.org/). Measurements were compared with two-way or three-way analysis of variance (ANOVA). Tukey´s HSD posthoc test after ANOVAs at a significance level of $p < 0.05$ was used to see which level of a factor differs from one another. Data were calculated as arithmetic means ± standard error of the mean of the indicated replicates

In experiment 7, differences in physicochemical characteristics among substrates supplemented with different fertilizers were compared using a mixed model in SAS. Pearson correlations were used to determine the interactions between the physicochemical characteristics; significance was assumed at $P < 0.05$. Multiple Factor Analysis (MFA) was used to detect how the relative abundances of bacterial genera differed in substrate harboring either of the two plants. Statistical differences in ammonia oxidation rate were analyzed using a longitudinal mixed model in SAS. A random slope model was used with time point, fertilizer and location (bulk or rhizosphere) as fixed factors and all interactions were considered. Technical replicates (n=6) were nested within each biological replicate (n=4). Unstructured covariance structure was used, assuming that the variance differed between the rhizosphere and the bulk substrate

## 2.11 Summary of technical equipment and software programs

**Table 9 List of technical equipment**

| Equipment | Type |
|---|---|
| Milling machine | Retsch MM 400 |
| Balance | Melter Toledo XS205 |
| Leaf area scanner | Li- 3100, Li-cor |
| PH meter | Decagon's 5ST |
| Optodes | pH sensors range 3-8 Presense GmbH, Regensburg, Germany |
| Camera | Nikon D5300 |
| Root scanner | Epson Expression Scan 1680, WinRHIZO STD 1680, Long Beach, Canada |

**Table 10 List of software programs**

| Program | Developer |
|---|---|
| Image J | NIH |
| WhinRhizo | Regent Instruments Inc. |
| MinTeQ | Environmental Protection Agency, Washington D.C |
| Rstudio | www.R-Project.org |

# 3. Results

## 3.1 Diagnosis of visual deficiency or toxicity symptoms after different fertilization regimes

To get insight into the first visual symptoms of applying different fertilization regimes, a germination test, a nodulation count, and a dose response experiment were performed.

### 3.1.1 Dose response

Results from the dose response experiment (experiment 1, see 2.5.1) showed an increase in maize shoot biomass from 0.59g to 4.15g and tomato from 0.01g to 3.88g (Fig. 11 for tomato) along the dose response. There was a significant relationship between shoot biomass and nutrients applied, with shoot biomass increasing linearly with the dose applied until nutrients applied surpassed 100% dose ($P<0.05$).

**Fig. 11 Tomato plants growing under increasing nutrient doses.** From left to right 0-25-50-75-100-200% modified Hoagland solution.

Plants exposed to 0-10% fertilization dose (10mg N and 1.2mg P plant$^{-1}$) presented growth disorders such as chlorosis and necrosis (Fig. 11). On the other hand, plants exposes to 25 to 200% fertilization dose (195mg N and 24mg P plant$^{-1}$) (Table 6) grew without deficiency symptoms.

For both species the appropriate rate of nutrients correspond to doses higher than 100% (N 100 and P 12mg plant$^{-1}$). Plant height did not increase from 100 to 200 % dose. Leaf area and biomass increased when the percentage of fertilization dose reached 200 % (Fig. 12 for biomass).

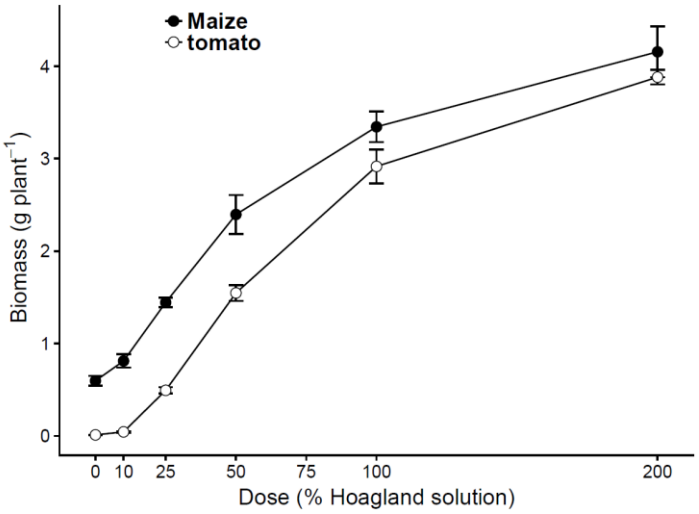


**Fig. 12 Dose response curve of tomato and maize plants.** Biomass (g dry weight) measured 28DAS for the different nutrient dose applications (% of modified Hoagland solution). Values represent the mean of five biological replicates and error bars represent the standard error of the mean.

### 3.1.2 Struvite effect on germination

The effect of struvite-P application on seeds germination was analyzed for lupine and maize in experiment 2 (2.5.2). Seeds were sown into two types of sand with different pH values (acidic and alkaline sand, Table 3) previously mixed with the correspondent P amount (60mg P kg$^{-1}$ soil, calculated for the 100% dose at experiment 1) applied as struvite.

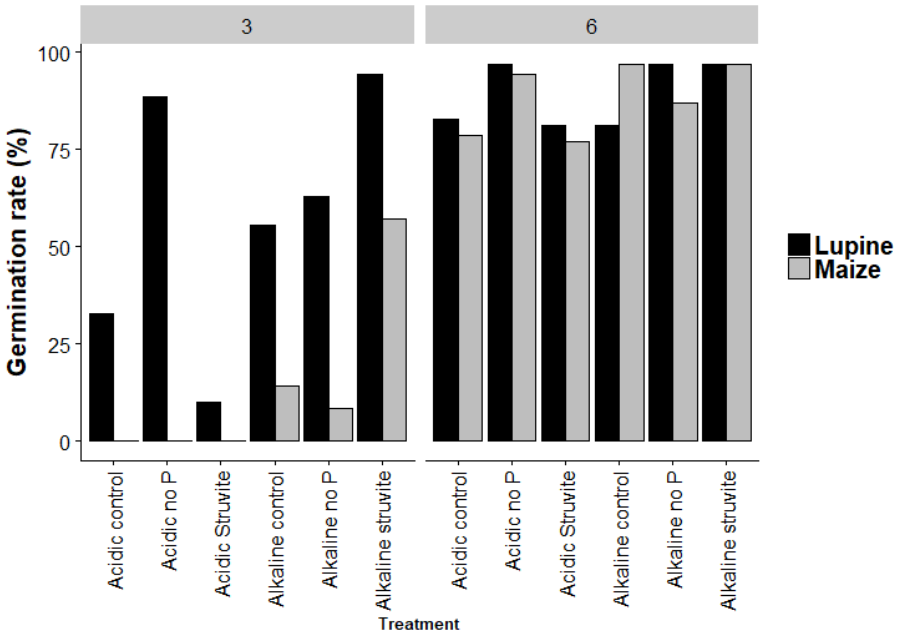


**Fig. 13 Germination rate of lupine and maize seeds (n=70 seeds/ species).** Germination rate (%) counted 3 days after sowing (3) in all the treatments, showing a delay in the germination when struvite is applied and 6 days after sowing (6) with no significant differences in the final germination rate.

A total number of 140 seeds were sown in 3kg of the sand-fertilizer mixture, with a correspondent amount per seed of 1.2mg P (calculated to have 10 fold less than for the whole growing period). The incentive to use the 100% dose and not the 200% (with a higher biomass production in the dose response experiment) was to analyze if at lower dose applications negative effects were already observable.

Fig. 13 shows germination rate at three and six days after sowing (DAS). In acidic conditions, struvite delayed the germination of lupine and maize, and the no phosphorus treatment had a positive effect in comparison with total absence of nutrients (control). In alkaline condition struvite accelerated the germination in comparison with the other treatments. 6 DAS the differences in the germination between the two soils were smaller.

Struvite in alkaline sand is the only treatment in which both species reached 100% germination. In acidic condition, germination was 10 to 15% lower. At the end of the experiment (8 DAS) 100% of germination was reached, indicating that the germination was not inhibited but only delayed by the application of struvite. To reach the 50% of the germination, lupine seeds needed approximately four days, and maize an average six days (in the favorable conditions). This information was taken into account for future experiments, where seeds from each species were pre-germinated at different time points and transplanted three to five days after mixing the struvite with the correspondent rooting media.

### 3.1.3 pH effect on the nodulation of lupine

The effects of the rooting media (sand and substrate), pH (acidic and alkaline), and presence or absence of nitrogen (N) on nodulation of lupine were evaluated in experiment 3 (2.5.3). At the end of the experiment, none of the plants growing in alkaline sand developed nodules, instead all plants presented reduced root growth (Fig. 14) as well as nutrient deficiency symptoms in the shoot. For lupine plants grown in acidic sand, first nodules were visible 12 DAS.


**Fig. 14 (A) Representative sample of roots of lupine plants 24 days after sowing under different treatments.** From left to right, Acidic pH with Struvite + N, Acidic pH Struvite with no N, Alkaline pH Struvite + N, Alkaline pH Struvite with no N. **(B) Score of crown nodule.**

Plants showed effective nodulation exclusively in the substrate Null Erde, or the substrate:sand (1:1, v/v) mix only 14 DAS. There were no differences with or without the application of N in the nodulation rate even though N treated plants had a higher root length.

To summarize the section 3.1, model plants showed the highest biomass production at 200% dose (900mg N and 100mg P $kg^{-1}$ soil) however application of lower amounts of P and N as struvite (60mg $kg^{-1}$ soil) already reduced germination compared with no P and no N applications in acidic conditions. This indicates that at acidic pH struvite was able to modify the sand chemical conditions affecting germination. Nodulation was highly inhibited by alkaline pH and was affected by the rooting media, with the most successful nodulation was measured in the substrate compared with the sand. However, N application did not affect the nodulation.

## 3.2 Struvite solubility analyses

The predominant parameters controlling solubility of struvite are temperature and pH (Doyle and Parsons, 2002). To analyze the solubility of a specific concentration of struvite with increasing pH, a model simulation and a flushing experiment were performed.

### 3.2.1 Modeling struvite P speciation

The effect of pH on struvite solubility was simulated using the program Visual MinTeQ (Environmental Protection Agency, Washington, D.C) (2.9.1). The concentrations of the different P species from 1.5mM struvite were analyzed at increasing pH values from 2 to 10. These analyses allowed observing in which form the P from the struvite might be release depending on the pH of the different experimental conditions (Fig. 15). At pH lower than 6 only the $H_2PO_4^-$ form would be present in the solution, and at pH higher than 7 $H_3PO_4$. Triple superphosphate (TSP) add P to soil in the form of the $H_2PO_4^-$ ion. Struvite however, add P in the soil in the form of $PO_4$, as the $H^+$ that is present for example in the Mono ammonium phosphate (MAP), is substituted in the struvite by Mg. Therefore, struvite fertilization will slightly increase pH.

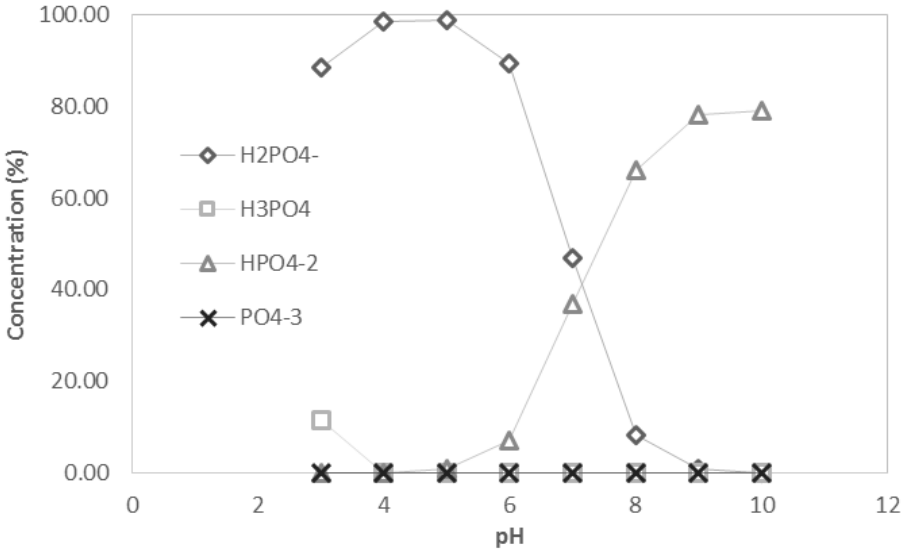


**Fig. 15 Concentration of struvite-P speciation forms at different fixed simulated pH values using MinTeQ.** Concentrations are obtained with a simulation of 1.5 mM struvite added to a pH buffered water solution. At pH> 7 not all struvite dissolved, this effect will be stronger at higher struvite doses.

### 3.2.2 Struvite solubility as affected by the external addition of citrate

Besides the modeling, the effect of citrate addition on struvite solubility was also analyzed in experiment 8 (2.5.7). Different citrate concentrations (0, 0.1, 1 and 10mM) were added to 10g of sand mixed with two doses of struvite (0.5 and 0.05g of struvite kg-1 soil that entails 60 and 6mg P kg-1 soil).

In the alkaline sand with high dose of struvite the first flush was the most effective (Fig. 16). The following flushes reduced the amount of soluble P found in the solution. The highest citrate concentration (10mM) led to the highest amount of P in leachate, however there were no differences between water (shown as 0.0mM), and the lower concentrations of citrate. There were significant differences between high and low struvite, leading to a highest soluble P when the initial concentration of struvite in the soil was higher.

Highest amount of P in solution measured when flushing with 10mM citrate was around 8mg $L^{-1}$, i.e. 400µg P in 50ml solution (the total flushing volume), which corresponds to 66% of total P added. The water was able to mobilize 200µg of P, only 30%. In acidic sand, there were no significant differences between the different doses of citrate flushing. The amount of water extractable phosphate that remained in the sand was also measured after all the flushings. When the highest concentration of citrate was used it was measured 22µg phosphate $ml^{-1}$ sand. The

sand samples were 20ml and therefore the amount of phosphate was 440µg of phosphate per sample (approximately 140µg of P in the sand).

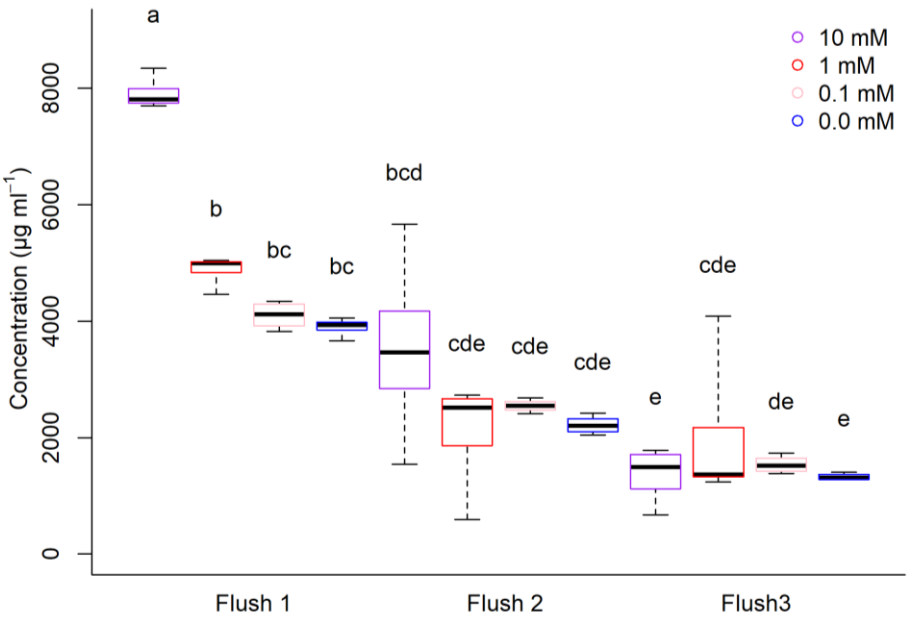


**Fig. 16 Concentration of phosphate (µg ml $^{-1}$) in the leachate after flushing 10g alkaline sand containing 60mg kg$^{-1}$ P in struvite form with 50ml of different citrate concentrations (10, 1, 0.1 mM and water (0mM)).** Higher amount of phosphate in the leachate was measured after flushing with 10mM citrate. Concentration is analyzed using a Two-Way ANOVA and Tukey, P<0.05. Mean ± SE, n=4. Different letters mean to significant differences (p<0.05).

## 3.3 Plant biomass development

### 3.3.1 Understanding the factors driving biomass variation: Influence of pH, fertilizer applied and rooting media condition depends on the plant species

Lupine and maize biomass in response to i) fertilizer applied, ii) pH, and iii) rooting media condition, were analyzed to verify differences between the two species in two different types of rooting media (sand and soil)

#### 3.3.1.1 Influence of each factor in sand as nutrient controlled rooting media

In experiment 4 (2.5.4), the effect of the P source on biomass and nutrient concentrations in shoot and roots of lupine and maize was analyzed, as well as how those differences are modulated by the initial pH of the rooting media. Table S1 (supplementary material) shows the biomass (g plant$^{-1}$) and p value of the effects of P source, soil pH and all interactions.

The goal of experiment 4 was to compare the fertilizer effect on the biomass of the two species. To analyze the percentage of influence on biomass variability that was induced by each factor (the pH and the P and N source applied), a coefficient of determination was calculated (Table 11). In the model two ANOVAS were run independently for each part of the plant (shoot and root).

Table 11 shows that lupine biomass was more influenced by the soil than by P treatment. For lupine, only 13% of the shoot biomass variation was explained by the P source applied; however, the sand pH explained the 69% of the variability. For maize plants, the sand pH also had higher influence in the biomass (42%) than the P source (37%), but significantly less than in lupine plants.

**Table 11 *=$R^2$: coefficient of determination (adjusted) based on mean squares of each factor and error according to ANOVA model (%) in experiment 4.** 36.97% of maize shoot biomass is explained by the P source applied.

| Plant part | Maize | | | Lupine | | |
|---|---|---|---|---|---|---|
| | P | Soil | PxSoil | P | Soil | PxSoil |
| Shoot | 36.97* | 42.3 | 16.68 | 13.15 | 68.8 | 8.04 |
| Root | 15.55 | 39.8 | 23.17 | 9.85 | 68.5 | 15.22 |

3.3.1.2 Influence of each factor in natural agricultural soils with contrasting pH

The objective of the experiment 6 (2.5.4) was to evaluate differences in plant performance of lupine and maize growing in two agricultural soils (Spanish alkaline and Spanish acidic, Table 3) with distinctive physical and chemical properties.

The initial phosphorus content in the Spanish alkaline soil (790mg $kg^{-1}$ soil) was higher than the acidic (110mg $kg^{-1}$ soil). Available P in both acidic and alkaline soils was reduced via the application of a P buffer to half of the original amount (Table 3). Biomass analyses showed no effect of the P treatments, as the initial P levels were sufficient for lupine and maize growth. Differences were only due to the effect of the soil properties (Table 12).

The chemical and physical characteristics of the soils explained more than 90% of the variation after calculations done with the R2: coefficient of determination (adjusted) based on mean squares of each factor and error according to ANOVA model. In maize plants, the Spanish alkaline (with a higher P content than the Spanish acidic) allowed a higher biomass production. For lupine plants, with a higher biomass production in the acidic soil, with low P content, the pH was the main growth-

limiting factor. Results showed that natural soils with high variety of physical and chemical properties are difficult to manage under control conditions. When the goal is to analyze the nutrient availability of certain specific compounds added to plants, the election of a sand with an initial low nutrient content can provide the necessary condition to control the turnover of the added nutrients.

**Table 12 Biomass of lupine and maize plants grown in agricultural soils at experiment 6.** Values are mean (n=10) ± SD Different letters means to significant differences in the biomass value.

| Sand pH | Biomass (g Plant $^{-1}$) | | |
|---|---|---|---|
| | No P | Struvite | TSP |
| **Maize** | | | |
| Acidic | 5.16c | 6.44bc | 6.38bc |
| Alkaline | 12.14a | 11.05a | 11.72a |
| **Lupine** | | | |
| Acidic | 1.25a | 1.26a | 0.98a |
| Alkaline | 0.32b | 0.47b | 0.49b |

Both experiments described in this section allowed to describe the differences between lupine and maize regarding pH and P as factors affecting plant performance. Lupine plants grew better on acidic soils, whereas maize grew better on alkaline soils indicating that both species may have different belowground adaptations.

### 3.3.2 Struvite fertilizer effect in plant biomass. Comparison with inorganic fertilizer

Struvite availability will be highly influenced by the soil conditions that can be modified by the nutrients added together with the struvite or by the initial soil pH. These are essential factors to take into account when studying the fertilizer use efficiency of struvite. Otherwise, lower yield in struvite treated plants can be expected (Ackerman et al., 2013) because of lower availability of nutrients compared to chemical fertilizers that are designed to be highly soluble in most conditions.

#### 3.3.2.1 P fertilizer effect modulated by the N source applied

In Experiment 5A (2.5.5), struvite was applied with either ammonium or nitrate. The combined fertilizer effect was analyzed in the shoot and root biomass of lupine and maize grown in acidic condition. The effect of struvite on plant growth was compared with growth of plants treated with no P or with a low dose of highly soluble P (TSP-low).

Struvite treated plants had significantly higher biomass than no P or TSP-low treatments (Table 13). Lupine plants had a higher biomass when struvite was applied with ammonium than with when it was applied with nitrate; however it was not possible to establish statistically significant differences. No differences in the biomass were observable in maize treated with struvite when applied with ammonium or nitrate (Table 13). Experiment 5A did not show any clear evidence of struvite effect modified by N source.

**Table 13 Effect of P and N sources and P dose applied on shoot and root biomass of lupine and maize grown in acidic sand in experiment 5A.** Values are mean (n=10). Different letters mean significant differences.

| Species | P source | N source | P applied (mg P $kg^{-1}$ soil) | Shoot biomass (g) | | Root biomass (g) | |
|---|---|---|---|---|---|---|---|
| Maize | struvite | $NH_4^+$ | 10 | 3.2 | a | 0.57 | ab |
| | struvite | $NO_3^-$ | 10 | 3.23 | a | 0.66 | a |
| | NoP | $NH_4^+$ | 0 | 0.48 | ef | 0.2 | de |
| | NoP | $NO_3^-$ | 0 | 0.51 | ef | 0.22 | de |
| Lupine | struvite | $NH_4^+$ | 10 | 1.87 | b | 0.47 | bc |
| | struvite | $NO_3^-$ | 10 | 1.73 | bc | 0.43 | bc |
| | NoP | $NH_4^+$ | 0 | 0.31 | f | 0.11 | e |
| | NoP | $NO_3^-$ | 0 | 0.54 | ef | 0.19 | de |

The effect of N source was again analyzed experiment 5B. In this setup, struvite effect was compared with a highly soluble P source (TSP) applied at the same dose as struvite (2.5.5). The ANOVA analyses showed no effect of nitrogen source ($p= 0.78$) on the biomass, however it shows a significant effect of P fertilizer ($p= 0.004$) and plant species ($p< 2.2e^{-16}$) (Fig. 17).

This data shows that in maize, struvite application can increase significantly the biomass compared with TSP application. The lupine plants showed no significant differences in the biomass between struvite and TSP or between the two N sources applied. Shoot dry weight of lupine increased from no P application to P treatments from 0.3 to 2.7g per plant in average, while maize showed a variation from 0.5 to 10g per plant. There are obviously differences in biomass between plant species. The root biomass of lupine plants showed no significant differences between struvite and TSP (average of 0.82 and 0.80g for nitrate, and 0.77 and 0.61 for ammonium). Lupine plants treated with nitrate had higher root biomass compared with those treated with ammonium, however not statistically significant effect of the N source was

established.

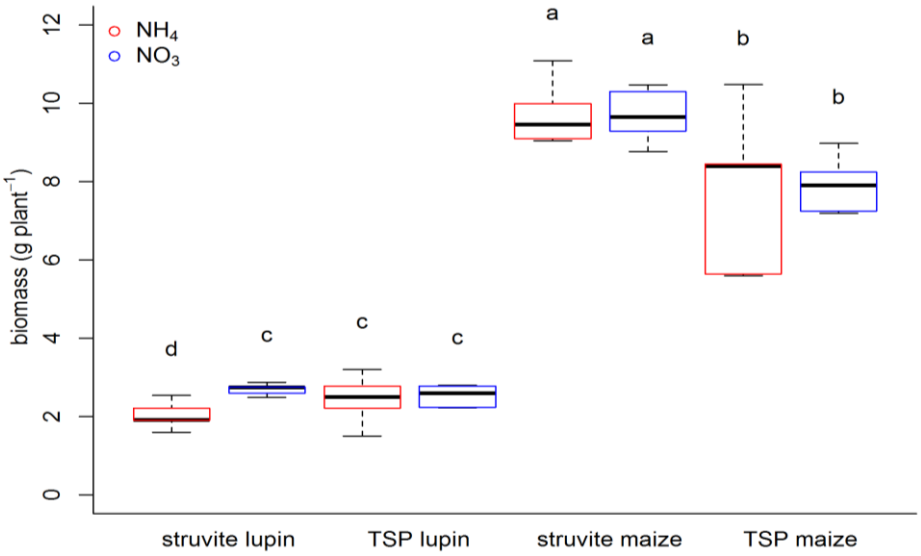


**Fig. 17 Plant biomass of lupine and maize (g plant $^{-1}$) under the different P (struvite and TSP) and N sources ($NH_4$ and $NO_3$) at experiment 5B.** Biomass is analyzed by a Three-Way ANOVA, P<0.05. Mean ± SE, n=5. Different letters mean significant differences.

To summarize, struvite fertilizer was as effective as TSP for lupine, and showed higher biomass production than TSP in maize. The N source had no significant effect on biomass, however a tendency of higher biomass production in response to ammonium application was noticeable.

3.3.2.2 Struvite-P fertilizer is modulated by the initial pH conditions

In experiment 9 (2.5.8), the effect of struvite P fertilization in comparison to a highly soluble P source was evaluated on biomass yield of lupine growing at different pHs. P treatments were struvite, potassium phosphate (labeled KP), both applied at a level of 15mg kg $^{-1}$ soil, and no addition of P (noP). When P was supplied, biomass varied significantly among pHs (Fig. 18 and 19a).

The ANOVA showed significant effects ($p<0.001$) of both factors (P treatment and pH), as well as the interaction between both ($p<0.001$). There were no significant differences at any pH between struvite and KP treatments (Fig. 18). The leaf area measurements (data not shown) are comparable to the biomass results. Soil pH had a big effect on plant growth, as the differences between P and no P addition were observable only at neutral pH with both P sources. The similar effectiveness of struvite compared to KP was confirmed at neutral and acidic pH. Here, both P

treatments showed higher total biomass than when no-P was supplied, with no significant differences between them (P<0.05) (Fig. 19b). 19b).

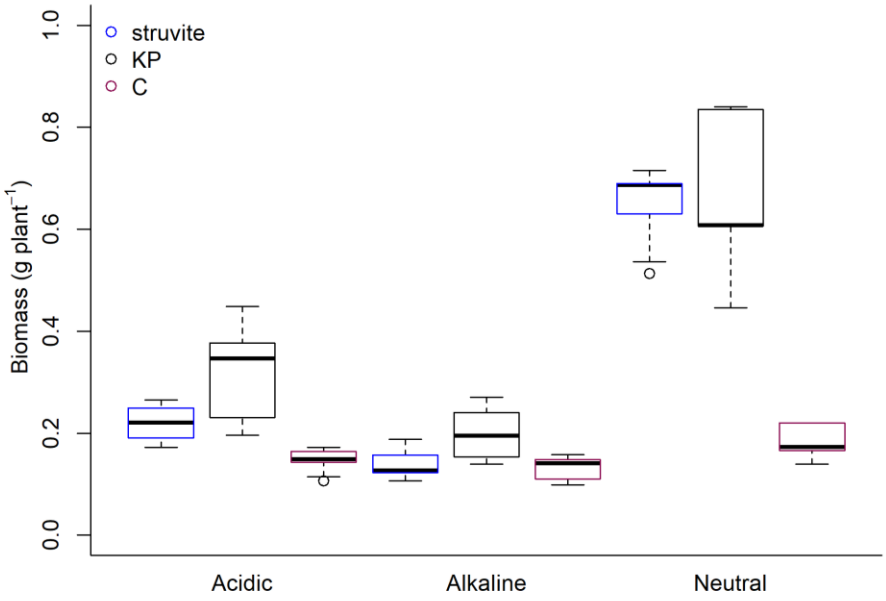


**Fig. 18 Plant biomass of lupine (g plant $^{-1}$) under the different P sources: struvite, potassium phosphate (KP) and Control with no P(C) applied at different adapted sand pH (acidic 4.5, neutral 6.5 and alkaline 7.8).** Biomass is analyzed by a Two-Way ANOVA. Mean ± SE, n=5.

The analyses showed again that the pH had a bigger effect on the plant performance than the P source for lupine. The biomass yield of lupine was decreased by 80% following the increase of the pH from 6.5 to 7.8, and 65% by decreasing the pH from 6.5 to 4.5 (Fig. 19a, for struvite treatment at different pHs). The optimum pH growth for lupine was slightly neutral (5.5 to 6.5).

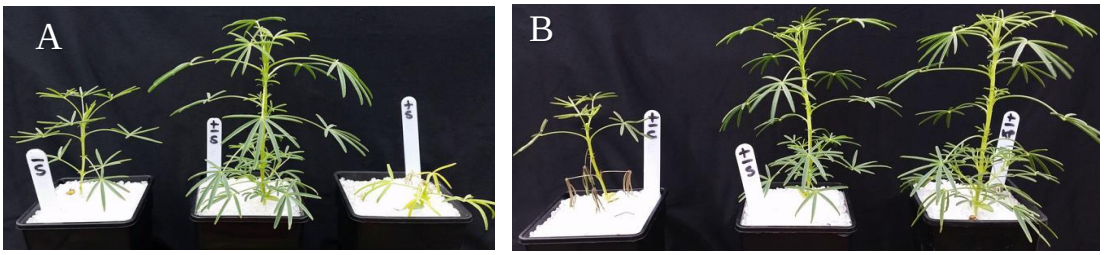


**Fig. 19 (A) Effect of pH on lupine growth under struvite (S) treatment in the acidic (-), neutral (±), and alkaline (+) sand. (B) Effect of P source on lupine growth in neutral pH. No P (C), Struvite (S), and Potassium phosphate (KP).** Plants shown in the picture are a significant representation of the phenotype observed in each treatment.

#### 3.3.2.3 Non-destructive measurements of leaf area in the SCREEN HOUSE-phenotyping station

In experiments 5, leaf area over time was obtained from the projected shoot area measured in the ScreenHouse (2.6.1). The total leaf area was estimated from the projected leaf area using a calibration curve for which 60 plants were measured with the cameras immediately before and with a leaf area meter (Li- 3100, Li-cor, Nebraska, USA) after harvest. The plants used for the calibration were from experiment 5A, with 3 different levels of fertilization high P, low P and no P) (2.5.5). The obtained calibration curve was linear with a $R^2$ of 0.95 (Fig. 20). From hereon we present total leaf area, as estimated based on this calibration.

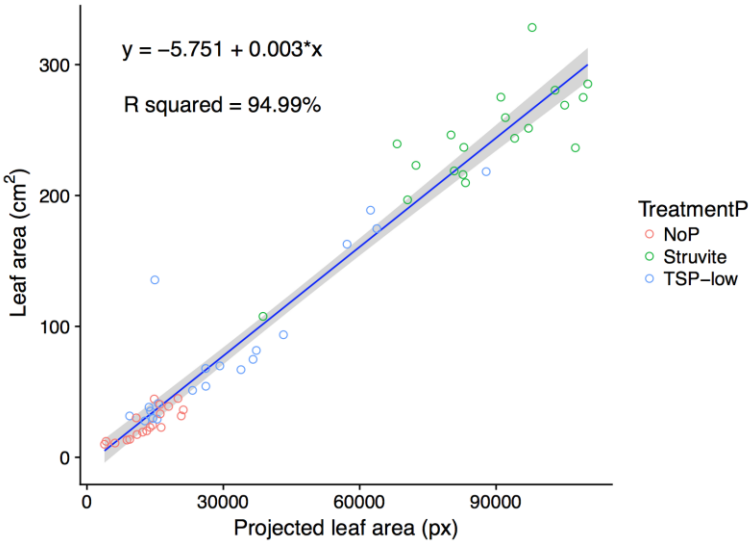


**Fig. 20 Association between images based projected leaf area from ScreenHouse (pixels) and destructively measured leaf area after harvesting in a root scanner ($cm^2$).** Points are individual measurements from images of the experiment 5A, with three different levels of fertilization. The leaf area was measured three times per week with a total of 17 measurements days.

As observed with the biomass (Fig. 18), struvite treated maize plants had greater leaf area at the end of the experiment compared to TSP (Fig. 21 see Maize 40 Days after sowing).

For lupine, there were no significant differences in leaf area between struvite- or TSP- treated plants. Between 9 to 21 DAS, however, lupine plants had greater leaf area when fertilized with TSP compared to struvite. This difference was greatest at 16 DAS when TSP fertilized plants had 14% greater leaf area. From 21 DAS onwards the leaf area of lupine plants treated with TSP did not differ from those treated with struvite (TSP, 263 cm2; struvite, 285 cm2; $p < 0.001$ as an example for 44 DAS).

In maize, a very similar pattern was observed; however, the greater leaf area of TSP fertilized plants during early growth stages was not as strong (1.6 %), and occurred later, at 24 DAS. From 26 DAS leaf area increased more rapidly in the struvite fertilized plants, which had at the end of the experiment 9.5 % more leaf area compared to those fertilized with TSP.

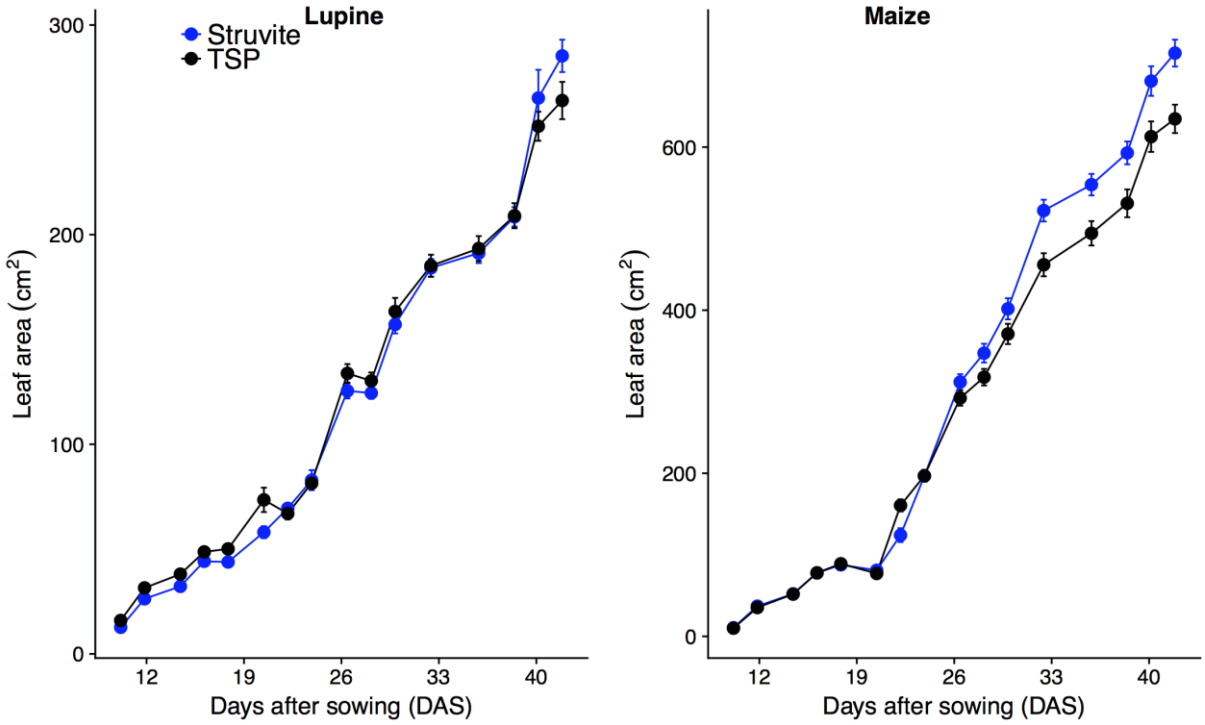


**Fig. 21 Projected leaf area (pixels) of lupine and maize treated with struvite (blue) or TSP (black), calculated every measurement day (MD from 0 to 17).** Struvite treated plants had higher leaf area than TSP at the end of the experiment, being significantly for maize plants (see MD17). The graph shows the typical growth curve for higher plants with an initial slow growth (Lag phase), until MD 7 approximately, then a rapid period of growth (exponential phase) where maximum growth is seen and the last phase where growth will be slow. The plants did not reach a steady phase. Points are average n=10.

### 3.3.3 Struvite fertilizer effect compared with organic fertilizers

In experiment 7 (2.5.6), struvite was evaluated as an ammonium source for tomato and lupine plants. As one of the traits to assess differences between fertilizers, shoot biomass was measured at two time points and compared between struvite and an organic N fertilizer.

The measures at time point 1 (20 DAS) showed no differences in lupine dry weight among any of the N treatments (with an average of 0.14g for struvite and organic treated plants and 0.13g for control plants). Only in the second harvest, at time point 2 (35 DAS), plants treated with organic fertilizer had a higher dry weight (average 0.66g) than struvite or no N (average 0.42g) (Table 14).

**Table 14 Influence of fertilizer type: no fertilizer (NoF), organic (ORG) and struvite (STR) on plant performance of tomato and lupine in organic substrate in function of time**. Values are mean (n=5). Tpt 0= sowing day, tpt1 = time point 1 (harvest 1) and tpt 2= time point 2 (harvest 2). NA = not available. Different letters mean significant differences.

| Variable | Plant | Tpt | Fertilizer | | |
|---|---|---|---|---|---|
| | | | NoF | ORG | STR |
| Leaf area ($cm^2$) | Lupine | 0 | NA | NA | NA |
| | | 1 | 23.9c | 25.7c | 22.4c |
| | | 2 | 52.9bc | 86.9b | 65.6b |
| | Tomato | 0 | NA | NA | NA |
| | | 1 | 6.64c | 182.3b | 102.1b |
| | | 2 | 95.3b | 990.3a | 734.9a |
| Fresh weight (g) | Lupine | 0 | NA | NA | NA |
| | | 1 | 1.11b | 1.25b | 1.24b |
| | | 2 | 2.58b | 4.5b | 3.36b |
| | Tomato | 0 | NA | NA | NA |
| | | 1 | 0.14b | 3.92b | 2.16b |
| | | 2 | 2.46b | 35.18a | 25.22a |
| Dry weight (g) | Lupine | 0 | NA | NA | NA |
| | | 1 | 0.13b | 0.145b | 0.143b |
| | | 2 | 0.408b | 0.667b | 0.423b |
| | Tomato | 0 | NA | NA | NA |
| | | 1 | 0.02b | 0.278b | 0.16b |
| | | 2 | 0.24b | 3.08a | 2.24a |

For tomato plants, with no nodulation, the organic fertilizer led to a higher biomass in the plants harvested at the first time point (average of 0.27g) in comparison with struvite (average of 0.16g), and struvite produced a higher yield in comparison with no N (average of 0.02g). The differences between fertilizers are smaller at time point 2, but significant (Fig. 22). Plant ($P < 0.05$), fertilizer ($P < 0.05$) and time point ($P < 0.05$) had significant effects on leaf area, fresh weight and dry weight (Table 14).

The largest mean leaf area (990.3$cm^2$) was measured with the organic fertilizer treatment. The leaf area of the tomato plants was significantly different from the lupine and the overall average was 351.9$cm^2$ and 46.2$cm^2$, respectively (Table 14). Organic fertilizer application resulted in the also in the highest fresh and dry weight (on either plant), whereas struvite resulted in a decrease of the mean total leaf area, and of fresh and dry weight between 26-28%.

**Fig. 22 Tomato plants growing in rhizotrons at time point 2 (35 Days after sowing).** Left to right: Tomato treated with no nitrogen, tomato treated with organic fertilizer and tomato treated with struvite. 2 plants per rhizotron.

### 3.3.4 Struvite as part of a fertilizer blend effect on biomass.

Biomass of viola plants treated with different fertilizer blends was compared at the end of the experiment 10 (2.5.9) (Fig. 23). Blends 2, 4, 6, 8, 10, 12 had in common the addition of ammonium sulphate. Plants growing in those blends died presumably as consequence of high electrical conductivity of the substrate. Plants growing with ammonium nitrate looked healthy.

Blends 5, 7, 9 and 11 are those with clean recovered struvite (laboratory grade or recovered from the project pilot plant). There were no significant differences in biomass among them. Blend 1 is the positive control made from commercial mineral fertilizers. Blend 3 resulted in lower biomass and a lower concentration of ammonium and phosphorus inside the plants, however the leaf tissue concentrations (further shown at 3.4.4) were not lower than combination 14 (osmocote), which had a higher biomass production.

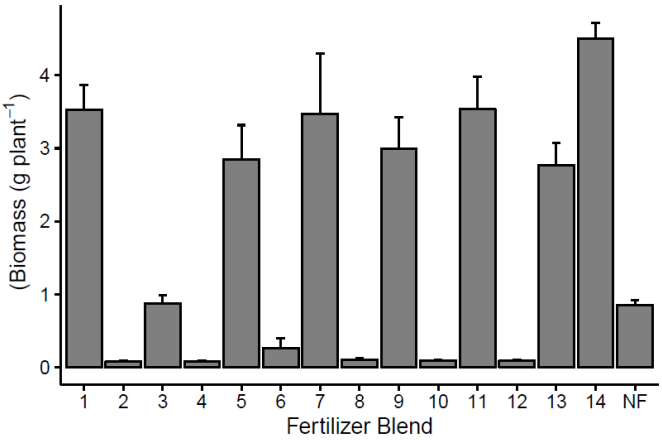


**Fig. 23 Average biomass (g dry weight plant $^{-1}$) (n=7) of viola plants for each fertilizer blend**. Numbers refer to each specific blend that are a combination of different recovered nutrients applied at the same final dose. Blend 1 is the positive control, blend 14 is osmocote, a commercial slow release fertilizer, and NF:no fertilizer.

## 3.4 Plant nutrient uptake

Element composition of plant tissues can be expressed as concentration (mg g$^{-1}$ plant) as concentration in percentage (mg 100mg$^{-1}$ plant) or as content (mg plant$^{-1}$). The analyses of P content in the plant tissues allowed to calculated P recovery as modified from Hammond et al.

(2009): P-recovery= (plant P concentration at P treated plants × DW P treated plants) *100/ amount of P applied. This allows comparing the % of P recovered from specific P sources between plants species and N treatments. Concentration or ranges of the major elements and micronutrients in mature leaf tissue generalized as deficient, sufficient or excessive for various plant species used as reference are taken from Munson and Kalra (1998).

### 3.4.1 Plant P-uptake efficiency from struvite and TSP as affected by N source applied

In experiment 4 (2.5.4), the concentration of P in shoots and roots was analyzed. In this first approach, the concentrations of other micro and macro elements were also analyzed. In the following experiments (Experiment 5A and 5B), only the P concentration in the shoot of plants is shown as P uptake was the main focus. Also, nutritional status of a plant is better reflected in the mineral element content of leaves than of roots (Marschner, 2011).

Table S2 supplementary with the data from experiment 4, shows the F value for the concentration of P and other elements present in shoot and roots of lupine and maize as affected by N source, P source and soil pH when all treatments are analyzed together. Significant different values given after the post-hoc HSD test for the P, K, N and Mg content in shoot and root as affected by P source, N source or soil type are shown in supplementary Table S3.

P and N source applied affected the root P concentration ($p<0.001$) in both species. Shoots P concentration, however, were affected by P and N source in maize plants only. P concentration in maize shoots was higher when fertilized with ammonium (0.074%) instead of nitrate (0.067%). Surprisingly, the initial soil pH did not affect the P concentration in either species. In comparison to nitrate, ammonium fertilized maize plants not only had greater shoot P concentrations, but also greater shoot Mg concentrations. This was not the case for lupine plants. In both species, shoot N concentrations were higher when fertilized with ammonium than with nitrate. Maize P concentrations in shoots and roots were in all treatments in the phosphorus deficient range (<0.16%), with a maximum concentration in shoot of maize plants growing in acidic sand treated with struvite ammonium (0.086%). Lupine P concentration in shoot (average of 0.13%) was nearer to the low range, but still within the deficient range. This shows that the amount of P applied in both sand (0.010g P $plant^{-1}$ and $L^{-1}$ soil) was not sufficient for healthy plant growth even for a short growing period (4 weeks).

In experiment 5A (2.5.5), shoot P content (mg P $plant^{-1}$) was used as an approximate measure of total P uptake by the plant. Although ammonium-struvite fertilized plants did not have greater biomass than nitrate-struvite fertilized plants, I did observe greater P uptake by the ammonium-struvite fertilized plants in experiment 5A, as hypothesized. The ANOVA suggests that the N

treatment effect on P uptake from struvite is independent of the species studied (Table 15). This response to fertilizer N form found in experiment 5A was not observed in experiment 5B. In this experiment, I only observed differences in P uptake between the species. The P uptake was higher in maize than in lupine, as maize is a faster-growing plant that accumulates more biomass and therefore more total P in the shoots (Table 15). In order to compare both species irrespective of plant size, the shoot P concentration (mg P g plant- 1) was analyzed (Table 15).

The species had a significant effect on P concentrations that were on average greater in lupine than in maize in both experiments; however, P and N fertilization had much greater effect in the P concentrations than the species. In experiment 5A, shoot P concentration was most strongly affected by P fertilization, and within the struvite treatments, ammonium fertilization resulted in significantly greater P concentrations compared with nitrate. The same was observed in experiment 5B, where, however, the N effect on P concentration (TSP or struvite) was only observed in lupine.

**Table 15: Influence of P fertilizer and N source applied on shoot P content (P uptake), and P concentration of maize and lupine plants growing in acidic sand for Experiment 5A and 5B**. Values represent the mean of n=10 for Experiment A, and the mean of n=5 for Experiment B. Different letters indicate significant differences at p<0.05.

| | Species | P source | N source | P applied (mg P $kg^{-1}$ soil) | Shoot P content (mg P $plant^{-1}$) | Shoot P concentration (mg P g $plant^{-1}$) |
|---|---|---|---|---|---|---|
| Experiment A | Maize | struvite | $NH_4^+$ | 10 | 5.1 a | 1.5 b |
| | | | $NO_3^-$ | 10 | 4.4 a | 1.3 c |
| | | NoP | $NH_4^+$ | 0 | 0.5 d | 0.8 d |
| | | | $NO_3^-$ | 0 | 0.4 d | 0.9 c |
| | Lupine | struvite | $NH_4^+$ | 10 | 3.5 b | 1.9 a |
| | | | $NO_3^-$ | 10 | 2.5 c | 1.5 bc |
| | | NoP | $NH_4^+$ | 0 | 0.3 d | 0.9 d |
| | | | $NO_3^-$ | 0 | 0.4 d | 0.7 d |
| Experiment B | Maize | struvite | $NH_4^+$ | 10 | 8.1 a | 1.1 c |
| | | | $NO_3^-$ | 10 | 9.3 ab | 1.2 c |
| | | TSP | $NH_4^+$ | 10 | 6.9 ab | 1.2c c |
| | | | $NO_3^-$ | 10 | 6.9 ab | 1.2c c |
| | Lupine | struvite | $NH_4^+$ | 10 | 4.9 b | 2.3 a |
| | | | $NO_3^-$ | 10 | 5.3 b | 1.9 b |
| | | TSP | $NH_4^+$ | 10 | 5.5 b | 2.1 ab |
| | | | $NO_3^-$ | 10 | 4.9 b | 1.8 b |

The P concentration (mg P g plant$^{-1}$) was analyzed in different shoot tissues (Fig. 24). Leaves and stem in both species had different P allocation strategies. Lupine plants accumulate more P in the leaves, contrary to maize that showed no differences with the P concentration in the stems. Therefore, in order to compare between both species, both tissues were analyzed together and considered as shoot P concentration (mg g$^{-1}$ plant) (Table 15).

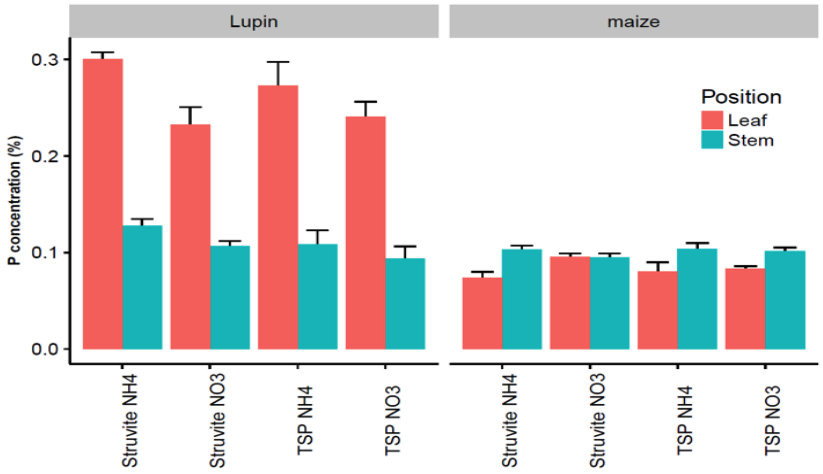


**Fig. 24. P concentration (%, mg P g plant−1) in leaf and stem of lupine and maize plants as affected by the fertilizer added (P and N sources)**. Lupine accumulates higher amounts of P in the leaves, however maize plants showed no differences in P allocation between leaf and stem. Bars mean ± SE n=5.

### 3.4.2 Struvite-P recovery as affected by pH of the rooting medium

In experiment 9 (2.5.8), shoot and root P content (total mg P plant$^{-1}$), shoot and root P concentration (mg P g$^{-1}$ plant) and P recovery (mg P plant$^{-1}$*100/P applied) in lupine was compared among sand pHs (acidic, neutral and alkaline) and P sources (struvite and potassium phosphate, KP, applied at 20 mg plant$^{-1}$).

P content increased in response to the solubility of the P source, with the highest P content in the plants treated with the highest soluble P form (Potassium phosphate-KP), and followed by struvite and no P, as related with the biomass production (Fig. 18). The neutral pH had the highest plant P content along pH treatments as well as the maximum plant growth.

Plant P concentration showed a different pattern (Table 15). Plants treated with KP and struvite had a higher P concentration than control (as observed with the total P content), but with no significant differences. In contrast to what was observed in plant growth, plants growing in alkaline pH had the highest P concentration, followed by acidic and neutral (Table 16). Shoot and root P concentrations were analyzed separately. The highest shoot concentration was found in alkaline

conditions (considered toxic as it was > 0.8 % under all fertilization) followed by acidic and neutral. Struvite had higher concentration than KP and the control (Table 16). Struvite in acidic condition gives a toxic concentration in shoot and root as well; however, no visual toxicity symptoms were observed in the shoot.

P recovery was calculated based on the total plant P content (shoot and root) and the P applied (19.5mg) (Table 17). At neutral pH, KP and struvite were recovered at same percentage. P recovery from struvite was significantly higher than from KP in acidic conditions, and no significant differences were observed in alkaline conditions (Table 17). There were no differences in the P recovery between pHs from the control condition

**Table 16 Shoot and Root P concentration of lupine plants at experiment 9 growing at three different sand pH**. Values are mean (n=5). Different letter means significant differences ($p<0.05$).

| | | P concentration (mg $g^{-1}$ DW) | | |
|---|---|---|---|---|
| | P source | Acidic | Neutral | Alkaline |
| Shoot | KP | 6.61bc | 4.61 bc | 10.16bc |
| | Struvite | 11.77bc | 5.6bc | 22.37a |
| | Control | 8.33bc | 7.98bc | 12.75b |
| Root | KP | 6.11c | 6.74bc | 9.06bc |
| | Struvite | 17.86a | 6.59c | 15.44ab |
| | Control | 9.6abc | 7.37bc | 8.15bc |

**Table 17 Struvite and KP P recovery calculated in lupine plants at experiment 9 growing at three different sand pH**. PUE: mg P $plant^{-1}$*100/P applied. Values are mean (n=5) ±, SE, Different letter means significant differences ($p<0.05$).

| | P recovery (mg P $plant^{-1}$*100/P applied) | | |
|---|---|---|---|
| P source | Acidic | Neutral | Alkaline |
| KP | 16±1.7bc | 25.87±3a | 12.16±2c |
| Struvite | 25.9±3.7a | 30.83±3.3a | 19.20±1.8b |

### 3.4.3 Plant nutrient uptake from struvite and organic fertilizers applied as N source

In experiment 7 (2.5.6), shoot nutrient concentrations of lupine and tomato were compared between struvite and organic fertilizer applied as N sources (Fig. 25).

The nutrient concentrations analyses in tomato plants (Fig. 25) showed normal concentrations (mg $100g^{-1}$ DW plant) of N in plant tissue in the first and second harvest (no deficiency or toxicity ranges, as defined by Marschner 2011). There were no significant differences in the N concentration between struvite and organic fertilization at any time point (4.5% N from struvite and 4% N from organic fertilizer in the second harvest). P concentration was significantly higher with the struvite than with organic fertilizer (0.95% versus 0.66% with the organic fertilizer at second harvest) due to the struvite stoichiometry. There were no significant differences between struvite and organic fertilizers in the K concentration, both significantly higher than the control. The Mg concentration was low in all the treatments with no differences between fertilized and unfertilized plants.

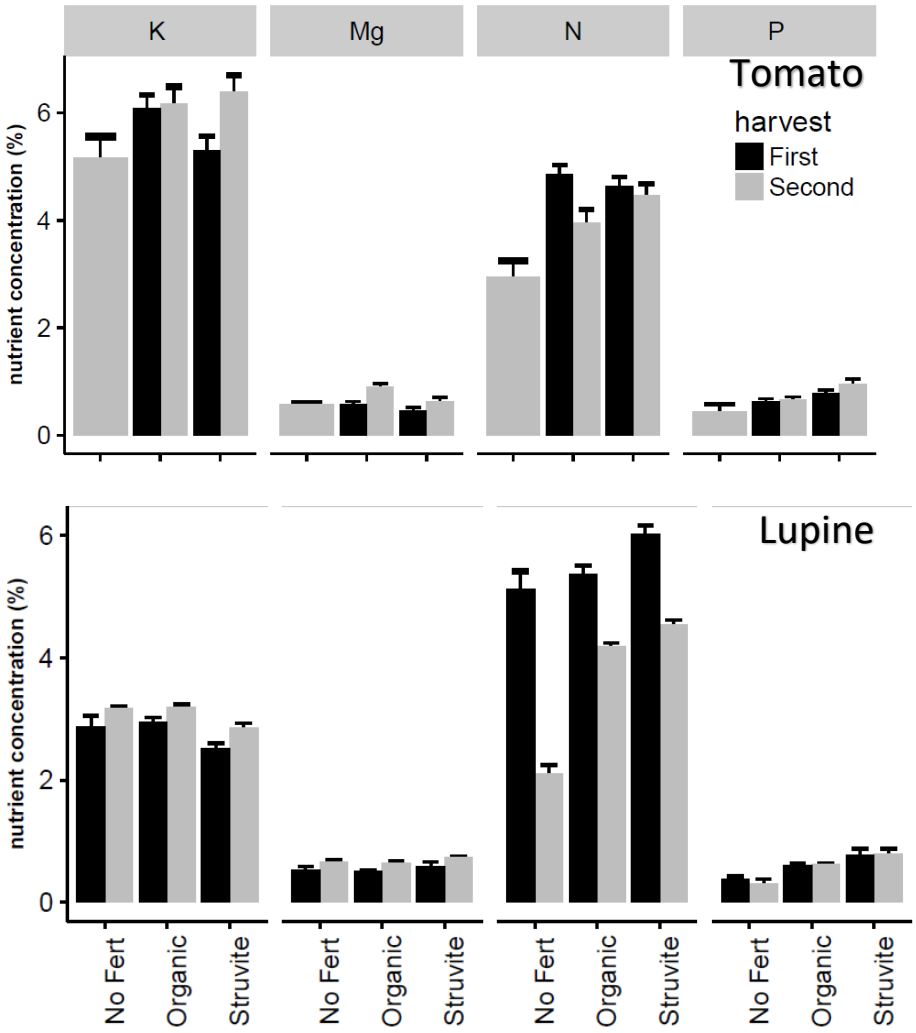


**Fig. 25 Nutrients concentration (mg 100g-1 DW plant) in the shoot of tomato (top) and lupine (bottom) at final harvest as affected by the different fertilizers (No Fert=no nitrogen fertilization, organic and struvite).** Bars mean ± SE n=5.

For lupine (Fig. 25), struvite led to a higher concentration of N (6% versus 5.3% organic in the first harvest and 4.4% struvite and 3.9% organic in the second) that was not statistically significant. P concentration with struvite was significantly higher than with organic fertilizer at both time points (0.79% struvite and 0.62% organic at second harvest). There were no significant differences in the K and Mg shoot concentration between treatments. For the tomato plants, N content (defined as N uptake) and N recovery were also calculated, as the substrate of tomato was further analyzed for in depth studies of nutrient turnover related to the microbial community (3.5.2 and 3.8). To calculate the N recovery, the shoot N uptake (defined as the mg of N in the shoot tissue) was divided to the amount of N applied as described in 2.9.3.

The N uptake in the non-fertilized tomato plants was in average 6.2mg plant-1 for the first harvest. Plants treated with organic had 55% more N uptake than struvite treated plants, in accordance with the biomass (that was almost double with organic than with struvite in the first harvest, Table 14). In the second harvest, as also observed in the biomass, the differences in the N uptake between struvite and organic fertilizers were smaller (in line with the hypothesis that struvite is a slow release fertilizer). N uptake in tomatoes treated with organic is at this point only 19% higher than struvite treated plants.

The N recovery in the first harvest was 2.6% with organic fertilizer and 1.5 with struvite. This percentage increased in the second harvest, with a 24% N recovery with the organic and 20% N recovery for the struvite.

### 3.4.4 Plant nutrient uptake from struvite as part of fertilizer blends

The concentrations (mg $100g^{-1}$) of P, K and Mg were analyzed in plant tissues of viola for the blends that allowed the plant have a healthy growth at the end of experiment 10 (2.5.9). In this case, there were no significant differences in the concentration of P, Mg and K among the different blends.

The N analyses were done in plant tissues harvested following growth on all blends. It showed that combinations with ammonium sulphate (even combinations from 2 to 12) had N concentrations considered excessive or toxic (from 5.8 to 6.8 % DW). The blends with struvite and ammonium nitrate (5, 7, 9 and 11) and the positive controls blends osmocote (14) and mineral fertilizer (1), had a concentration sufficient or normal (from 2.5 to of 3.4% DW). The blend with no fertilizer addition and blend with K struvite (blend 13 and 3) showed deficient concentrations of nitrogen (<2.5%).

### 3.5 Soil chemical analyses

#### 3.5.1 Nutrient content in the sand

In experiment 5B, struvite was compared with TSP (2.5.5). The nutrient analyses in the soil showed that nitrate concentration (µg $ml^{-1}$) was <0.3 and ammonium <0.6 in all the treatments. The concentration of P in the sand (mg $L^{-1}$) showed no differences between struvite and TSP treated with ammonium (0.04 for struvite and 0.03 for TSP), or with nitrate (0.02 for both struvite and TSP) in the lupine pots. The P measured in the sand of maize pots was no higher than 0.02 in any treatment.

#### 3.5.2 Nutrient content in the organic substrate

Ammonium concentrations (mg $L^{-1}$) in the substrates of lupine and tomato setups were analyzed in experiment 7 (2.5.6). At the beginning of the experiment (sowing day: time point 0), the concentration after the addition of struvite was higher for both species than after the addition of organic fertilizer, even though the total N added was similar with both fertilizers (100mg $NH4^+$-N $L^{-1}$). The concentration when no fertilizer was applied was as measured in the initial substrate analyses (1.7 mg $NH4^+$ $L^{-1}$) (Table 18).

At harvest 1 (20 DAS), the ammonium concentration in the substrates with the no fertilizer treatment was similar for lupine and tomato (an increase of around 3 mg ammonium $L^{-1}$ substrate since time point 0). The concentration of ammonium with organic fertilizer was different between species. Lupine had higher concentration than tomato (24mg $L^{-1}$ vs 3.5mg $L^{-1}$), as part of the ammonium in tomato substrate apparently had already been mineralized to nitrate (23mg $L^{-1}$). This is not observed with the struvite treatment, where the ammonium concentration was similar between tomato and lupine (68mg $L^{-1}$ for lupine and 42mg $L^{-1}$ for tomato). In this case the struvite treated substrate of tomato plants had higher ammonium than substrates treated with organic fertilizer, indicating that ammonium from struvite had not been mineralized (Table 18).

At harvest 2, the ammonium concentration in the substrate of lupine plants decreased with both fertilizers, correlating with an increase in nitrate concentration in both conditions. In the case of tomato the ammonium concentration was reduced to the minimum in the case of organic fertilizer (it was mineralized to nitrate) but was at the maximum in the case of struvite (no mineralization at all occurred) (Table 18).

**Table 18 Influence of fertilizer type (no fertilizer - NoFert, organic fertilizer – ORG and struvite-STR) on the nutrient dynamics and pH in non-sterile organic substrate with plants (lupine and tomato) and without plants (no plant, as control) in function of time**. Values are mean (n=5) ± SEM (P<0.05).

| Time point | Species | Fertilizer | $NH_4$ (mg $L^{-1}$) | $NO_3$ (mg $L^{-1}$) | $P0_4$ (mg $L^{-1}$) | pH |
|---|---|---|---|---|---|---|
| Starting exp. | lupine | NoFert | 1.7 ± 0.05 | nd | 17.8 ± 2.5 | 5.5 |
| | | Organic | 17 ± 4.6 | nd | 29.13 ± 7.25 | 5.7 |
| | | Struvite | 48.43 ± 8.9 | nd | 211.7 ±30 | 5.6 |
| Staring exp. | tomato | NoFert | 1.7 ± 0.1 | nd | 18.31 ± 2.6 | 5.5 |
| | | Organic | 20.59± 1.7 | nd | 26.06 ± 30.7 | 5.7 |
| | | Struvite | 36.46± 9.6 | nd | 188.18 ±13 | 5.6 |
| First Harvest | No plant | NoFert | 4.56 ± 0.9 | nd | 14.12 ± 3.5 | 6.2 |
| | | Organic | 20.08 ± 4 | nd | 20.58 ± 2.7 | 6.2 |
| | | Struvite | 41.68 ± 16 | nd | 101.68 ± 35 | 6.1 |
| | Lupine | NoFert | 5.88 ± 2 | nd | 12.40 ± 1 | 6.7 |
| | | Organic | 22.78 ± 5.6 | nd | 25.52 ± 4.4 | 6.3 |
| | | Struvite | 70.8 ± 15 | nd | 224.76 ± 73 | 6.1 |
| | Tomato | NoFert | 4.9± 0.78 | nd | 10.3 ± 0.9 | 5.9 |
| | | Organic | 3.5 ± 2.4 | 23.21 ± 1.7 | 18.19 ± 1.2 | 5.7 |
| | | Struvite | 41.1 ± 8.7 | nd | 126.8 ± 55 | 6 |
| Second harvest | No plant | NoFert | 0.66 ± 1 | nd | 11.88 ±2.1 | 6 |
| | | Organic | 0.22 ± 0.18 | 31.08 ± 4.3 | 20.52 ± 3.11 | 5.6 |
| | | Struvite | 40.8 ± 32 | 29.37 ± 17 | 199.9 ± 107 | 5.6 |
| | Lupine | NoFert | 1.66 ± 1.1 | nd | 29.52 ±23 | 6.1 |
| | | Organic | 3.02 ± 2.8 | 36.38 ± 8.5 | 32.34 ± 5.15 | 5.3 |
| | | Struvite | 48.26 ± 5.6 | 30.52 ± 9.5 | 306.32 ±53 | 5.4 |
| | Tomato | NoFert | 1.9 ± 0.2 | nd | 9.5 ± 0.8 | 5.7 |
| | | Organic | 0.5 ± 0.023 | 3.1 ± 2.2 | 18.3 ± 1.3 | 5.5 |
| | | Struvite | 74.3 ± 16 | nd | 290.7 ± 47 | 5.6 |

Interestingly, in the last harvest the concentration of nitrate in the substrates of tomato plants treated with organic fertilizer is very small, and it was assumed that the nitrate was taken up by the plants (related with the optodes results, see section 3.7.3, and the higher biomass, see 3.3.3). Those results showed that the tomato plant was more effective than lupine in taking up ammonium from organic fertilizer and that the tomato soil conditions allowed a more effective mineralization or the ammonium from than from struvite (related to microbial analyses, results at 3.8). In contrast, lupine plants were more effective than tomato in dissolving the ammonium from the struvite.

Table 18 shows the influence of fertilizer type on the nutrient dynamics in non-sterile organic substrate with and without plants as a function of time. P concentration in the soil (mg $L^{-1}$) was significantly affected by fertilizer application. Also plant species had a significant effect, with a slightly higher concentration in the substrate of lupine plants (23.3 with organic and 331 with struvite) compared with the substrate of tomato (18.3 and 290) ($p<0.001$). The concentration of other nutrients like $K^+$ or $Ca^+$ was slightly higher with organic than with struvite, and concentration of $Mg^+$ was slightly higher with struvite than with organic fertilizer.

To analyze the N balance, the percentage of the combined total amount of N in the substrate and total N uptake by the plant was calculated in relation to the amount applied. In the first harvest, 29% of the applied N in the organic was measured, whether 43% with struvite. In the second harvest, 28% of the organic was measured and 94% of the struvite. It was observed that the plants grew better with the organic, with always a higher precentage of N uptake than with the struvite (Table 19).

**Table 19 Analyses of nitrogen balance**. Total N uptake shows the mg of N measured in total within the two plants per rhizotron. Total N shows the concentration of N (total N, ammonium and nitrate) in the substrate. N recovery indicates the % N recovered by the plant in relation to the N applied and % of N substrate stand for the amount of N applied that is measured at each harvest in the soil (total, ammonium and nitrate). Total % measured indicates the N applied that is measured combining the total N in the soil and the plant tissue for each replicate. Each number is the mean value of n=5

| | Harvest | Total N uptake | Total N | $NH_4^+$ | $NH_3^-$ | N recovery | % N subst. | total % measured |
|---|---|---|---|---|---|---|---|---|
| No Fert | 1 | na | 24.8 | 24.8 | 0 | na | na | na |
| Organic | 1 | 13.4 | 134.2 | 17.8 | 116.39 | 2.6 | 26.8 | 29.5 |
| Struvite | 1 | 7.4 | 210.6 | 210.6 | 0 | 1.4 | 42.1 | 43.6 |
| No Fert | 2 | 6.2 | 9.5 | 9.5 | 0 | 1.2 | 1.9 | na |
| Organic | 2 | 122.8 | 18 | 2.5 | 15.5 | 24.5 | 3.6 | 28.1 |
| Struvite | 2 | 98.9 | 370 | 370 | 0 | 19.7 | 74 | 93.7 |

Those results might indicate that the N from the organic is moving to another system (microbial community) that is not measurable, and in contrast, the N release from the struvite stay immobilize in the soil, as it will be further discussed.

### 3.5.3 Chemical characteristics of different blends

The different recovered nutrients and mineral fertilizer used in experiment 10 (described in section 2.5.9 table 7) were blended at final concentrations described in Table 8, accordingly to the nutritional requirement of the viola plants.

As reflected in the plant performance (Fig. 23), the use of different blends had a significant effect on substrate chemical characteristics. Table 20 shows the concentrations of different nutrients in the substrate after addition of the respective blend and their effects on the electrical conductivity (EC). Blend 14 (Osmocote), and blend 1 (mineral fertilizer) were used as a positive control. Blends with even numbers (2 to 12) contained ammonium sulphate. Odd numbered blends (3 to 11) had ammonium nitrate.

**Table 20 Concentration of different nutrients (mg $L^{-1}$) in the blends in experiment 10 and their effects in the pH and Electrical conductivity (EC).** Values are mean (n=5).

| Blend | pH ($H_2O$) | EC (µS/cm) | $NO_3^-$-N (mg $L^{-1}$) | $NH_4^+$-N (mg $L^{-1}$) | P (mg $L^{-1}$) | K (mg $L^{-1}$) | Ca (mg $L^{-1}$) | Mg (mg $L^{-1}$) | SO4 (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4.9 | 713 | 44 | 61 | 226 | 718 | 900 | 205 | 917 |
| 2 | 5.1 | 1800 | 8 | 690 | 170 | 540 | 1028 | 233 | 1945 |
| 3 | 5.3 | 528 | 0 | 12 | 88 | 530 | 858 | 285 | 902 |
| 4 | 5.3 | 1330 | 0 | 492 | 85 | 428 | 918 | 300 | 1761 |
| 5 | 6.5 | 708 | 44 | 75 | 357 | 928 | 878 | 905 | 997 |
| 6 | 6.5 | 2030 | 8 | 838 | 302 | 723 | 1085 | 845 | 2191 |
| 7 | 5.8 | 652 | 26 | 111 | 256 | 693 | 873 | 398 | 928 |
| 8 | 5.7 | 2200 | 0 | 897 | 236 | 690 | 1013 | 410 | 2215 |
| 9 | 6 | 730 | 17 | 123 | 335 | 745 | 945 | 470 | 965 |
| 10 | 5.8 | 1760 | 0 | 667 | 317 | 748 | 980 | 495 | 1922 |
| 11 | 6.2 | 820 | 26 | 208 | 474 | 858 | 903 | 700 | 956 |
| 12 | 6.2 | 2100 | 7 | 834 | 511 | 933 | 965 | 710 | 2033 |
| 13 | 6.1 | 270 | 0 | 68 | 260 | 193 | 950 | 563 | 269 |
| 14 | 5.1 | 156 | 0 | 7 | 16 | 105 | 773 | 218 | 235 |
| 15 | 5.2 | 137 | 0 | 5 | 10 | 90 | 705 | 200 | 213 |

The use of ammonium-sulphate had a high impact on the electrical conductivity, which increased up to 2,000 µS $cm^{-1}$ and on the ammonium concentration. Ideally the nitrate:ammonium ratio should be 0.6:1 (Marschner 1995) but in all the blends the ammonium concentration related to the nitrate was higher than for the ideal condition . In the blends with struvite plus ammonium nitrate (7, 9 and 11), the NH4:NO3 ratio was 0.2:1 for the struvite derived from waste water treatment plant (blend 7) and around 0.1:1 for both struvites recovered from manure (blend 9 and 11). These, differences are even higher in the blends containing ammonium sulphate, with nitrate concentrations in a ratio lower than 0.01:1 or without nitrate at all.

Blends with potassium struvite (3 and 5) showed lower concentration of ammonium. Combinations with a concentration near 900 mg N $L^{-1}$ showed that the electrical conductivity was unacceptably high. That means that combinations 6, 8 and 12 are not suitable for plant growth. In conclusion, combination 7 (blend of struvite form WWT, ammonium-nitrate and potassium-sulphate) has the best chemical composition followed by combination 13 (blend of potassium-struvite, struvite from WWT, ammonium-sulphate and potassium-sulphate).

### 3.5.4 pH analyses of the rooting media at harvest time

In experiment 4, the pH variation of an acidic and an alkaline sand was analyzed after the addition of different P&N sources (2.5.4). Struvite application significantly increased the pH of the acidic sand with lupine (pH 7.37) in comparison with TSP treatment (pH 6.89) ($p<0.05$) (Table 21), but it had no effect in alkaline sand. A similar increase was observed in the acidic with maize plants, however the differences were not significant (pH 6.97 with struvite and 6.8 with TSP). The effect of the N source in the pH of the sand was not significant. Nevertheless, it was observed with lupine plants that the ammonium application produced a decrease in the sand pH compared with the nitrate.

**Table 21 pH at harvest point of acidic and alkaline sand measured at experiment 4 as affected by N and P sources applied and plant species**. Values are mean (n=5).

| | | Acidic sand | | Alkaline sand | |
|---|---|---|---|---|---|
| | | $NH_4$ | $NO_3$ | $NH_4$ | $NO_3$ |
| lupine | TSP | 6.89 | 6.98 | 7.67 | 7.6 |
| | Struvite | 7.37 | 7.56 | 7.40 | 7.48 |
| | Control | 6.64 | 7.06 | 7.48 | 7.58 |
| Maize | TSP | 6.80 | 6.67 | 7.72 | 7.84 |
| | Struvite | 6.97 | 6.95 | 7.68 | 7.88 |
| | Control | 6.2 | 6.34 | 7.53 | 7.83 |

In Experiment 5 plants were grown in the acidic sand previously described (pH=4.7). The aim of this experiment was to first analyze if the effect of struvite was affected by the plant species and the nitrogen source applied (setup 5A) and then compared this effect with the highly soluble TSP (setup 5B) (2.5.5)

The pH was measured at the end of the experiment in both set-ups. The results show that in 5A, the N source had an effect on the pH only when the plant was able to grow, i.e. when a P source such as struvite was also applied. Nitrate with struvite increased the pH of the sand significantly compared with the initial sand pH (4.7). The ammonium with struvite slightly decreased the pH until 4.4 in the case of maize, however this was not significant (Table 22).

**Table 22 PH measured at harvest of acidic sand at experiment 5A as affected by N and P sources applied and plant species.** Values are mean (n=5). Different letters mean significant differences.

| | | Acidic sand | |
|---|---|---|---|
| | | $NH_4$ | $NO_3$ |
| lupine | Struvite | 4.25c | 5.97a |
| | No P | 4.77bc | 5.12b |
| Maize | Struvite | 4.42c | 5.82a |
| | No P | 4.65c | 4.75c |

The no-P treatments (with ammonium or nitrate) showed no significant variation from the initial pH of the sand (4.7). In 5B the soil pH showed no significant differences between P or N treatment (data not shown).

In experiment 7, lupine and tomato plants were grown in organic substrate in the rhizotrons (2.5.6). Plant species had a bigger effect than the fertilizer applied on the pH of the substrate ($p<0.005$). The pH changes following lupine growth where bigger than following tomato growth, even though the final pH was similar in both treatments. The effect of time had the bigger influence on the modification of the pH ($p<0.001$). Plant species, fertilizer and time point significantly influenced the pH ($H_2O$) of the bulk zone (Table 18). The overall pH ($H_2O$) was 5.6 ± 0.03 at the start, increased to 6.2 ± 0.03 at the second time point and decreased again to 5.7 ± 0.03 at the third time point, in all plants. Organic fertilizer and struvite resulted in similar pH changes in the growing medium.

Abovementioned pH analyses were done in the bulk medium. PH analyses of rhizosphere samples done at experiment 9 are shown in 3.7.2.

## 3.6 Root morphology and physiology analyses

Changes in the external nutrient supply modulate root system architecture (RSA) over time. As described by Postma et al. (2014), the relative availability of the nitrogen and phosphorus will affect the density of the lateral root branching in maize. To understand possible plasticity responses with respect to the N and P source applied, the root morphology was analyzed in experiment 5, 7 and 9.

In experiment 5A and 5B lupine and maize plants were grown in acidic sand and harvested around 6 weeks after transplanting (2.5.5). Roots were collected at harvest for morphological and architectural measurements as described in 2.7.2. Briefly, the roots were partitioned into 11 diameter classes. Data for various root traits, such as total root length, root surface area, average root diameter and Diameter Class Length (DCL, root length within a diameter class) were generated in WinRHIZO from root images for each root.

### 3.6.1 P source applied modifies root morphology modulated by the nitrogen source applied

Taking into account the high mobility of nitrate in the soil, and the restricted amount of ammonium available within a given soil area, the most efficient root architecture may vary depending on the N source applied. That was the motivation to analyze, besides the effect of P source, the effect of the N source applied on root morphology. Total root length, root surface area, average root diameter, and specific root length were analyzed to identify root traits that related to an increase in the P uptake efficiency (Table 23).

In experiment 5A total root length, root surface area and average root diameter of lupine and maize plants treated with struvite and no P were compared when applied with ammonium or nitrate (Table 23). Both lupine and maize plants fertilized with struvite had greater root length and root surface area compared to those that were unfertilized. As may be expected, the larger maize plants had much greater root length and root surface area also in the no P treatments. It was analyzed if the source of P or N might influence root growth. In experiment 5A it was observed an increased root length and root surface area when plants were fertilized with nitrate, although the magnitude of the effect differed among species and P treatments (Table 23). In maize plants, the differences between N treatments in the root length were higher than in lupine. The largest effect was observed in struvite-fertilized maize, which had 78% greater root length when N was applied as nitrate. When no P was applied, nitrate had also higher root length than ammonium, but no significant differences were established in this case. Mean root diameter of maize plants treated with struvite ammonium was not significantly different than struvite with nitrate in any treatment.

**Table 23 Root morphological traits (total root length, root surface area, average root diameter and specific root length) of lupine and maize treated with struvite and affected by the N source applied ($NH_4^+$ and $NO_3^-$) compared with the no P application (control) in lupine and maize in Experiment 5A, and with TSP in lupine in Experiment 5B.** Values are mean (n=10/5) ±SEM. Different letters indicate significant differences.

| | | P source | N source | Total root length (cm) | Root surface area ($cm^2$) | Average root diameter (cm) | Specific root length (cm/mg) |
|---|---|---|---|---|---|---|---|
| Experiment 5A | Lupine | Struvite | $NH_4^+$ | 2167 ± 743a | 462.7 ± 150cd | 0.7 ± 0.04a | 4.6 ± 1.03b |
| | | | $NO_3^-$ | 2688 ± 512a | 611.2 ± 139b | 0.7 ± 0.02a | 5.7 ± 0.7b |
| | | No P | $NH_4^+$ | 553 ± 181b | 123.5 ± 42f | 0.7 ± 0.05a | 5.1 ± 0.9b |
| | | | $NO_3^-$ | 995 ± 248b | 229.8 ± 49ef | 0.7 ± 0.06a | 5.0 ± 0.9b |
| | Maize | Struvite | $NH_4^+$ | 4104± 599d | 492.4 ± 68bc | 0.31 ± 0.04b | 8.1 ± 3.08b |
| | | | $NO_3^-$ | 11430 ± 1371c | 895.8 ± 59a | 0.3 ± 0.03b | 17.5 ± 1.75a |
| | | No P | $NH_4^+$ | 2949± 609e | 259.9 ± 62e | 0.3 ± 0.07b | 14.7 ± 4.1a |
| | | | $NO_3^-$ | 3092 ± 882de | 338.6 ± 59de | 0.3± 0.09b | 18.2 ± 4.6a |
| Experiment 5B | Lupine | Struvite | $NH_4^+$ | 10244.5 ± 3535a | 5721.4 ± 2086a | 1.7 ± 0.06b | 17.2 ± 3.1a |
| | | | $NO_3^-$ | 12217.5 ± 1220a | 7034.3 ± 549 a | 1.8 ± 0.09ab | 16.1 ± 2.6a |
| | | TSP | $NH_4^+$ | 12877.9 ± 3326a | 7562.1 ± 1929a | 1.8 ± 0.1ab | 16.4 ± 1.4a |
| | | | $NO_3^-$ | 10295.2 ± 3240a | 6370 ± 1953a | 1.9 ± 0.03a | 13.3 ± 2.7a |

The goal of the experiment 5B was to compare struvite with the highly soluble P source TSP (2.5.5). Although on average large differences were observed in experiment 5B, they were not significantly different, possibly because of the lower number of replications (five instead of ten). As observed in the previous setup 5A, if struvite was applied with nitrate plants had higher root length than with ammonium. This is in contrast to what observed with TSP treatments. Here, lupine developed higher total root length with ammonium than with nitrate. This is in line with the analyses

of root surface area or average root diameter, however no significant differences could be established between P and N treatments.

The DCL (average length within a specific diameter) in lupine plants of experiment 5B was analyzed. As observed for TRL, plants treated with struvite nitrate had higher DCL for every diameter range than struvite applied with ammonium. In contrast, TSP had higher DCL when applied with ammonium for every diameter class (Fig. 26). Besides that not significant differences between N treatments could be stablished.

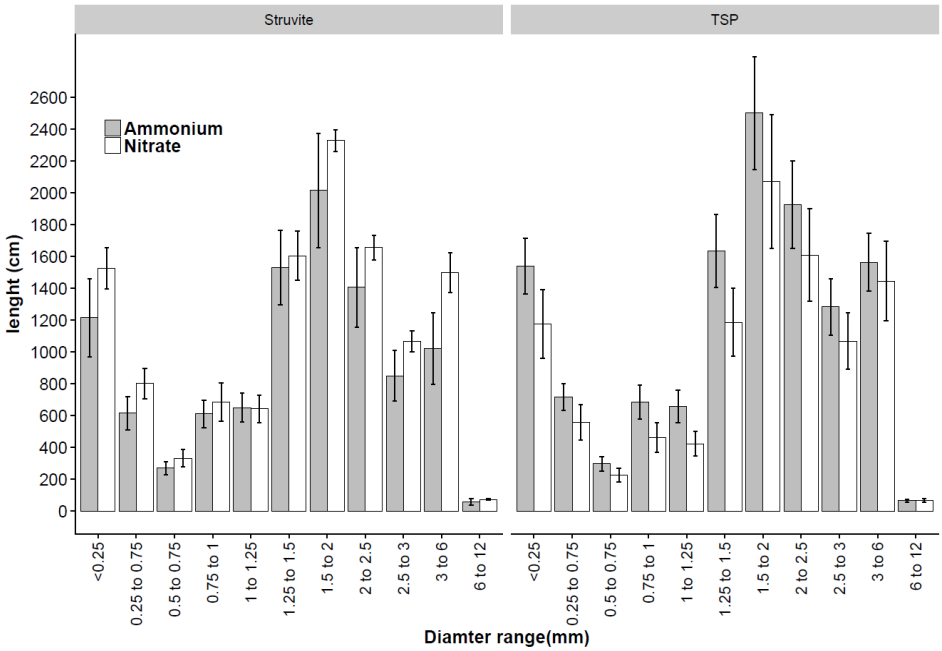


**Fig. 26 Average root length (cm) within specific diameter ranges (mm) of lupine plants treated with struvite or TSP applied with either ammonium of nitrate as N source in experiment 5B**. Bars are mean ± SE, n=5. Lupine treated with struvite had higher root length when treated with nitrate within all the root diameters, contrary than lupine treated with TSP that had a higher root length with the ammonium treatment.

The specific root length (SRL, root length / root dry biomass) indicates if plants are investing more in thin roots (higher SRL) than in thick roots (low SRL). At experiment 5B, lower root biomass, and lower SRL was observed in lupine plants treated with ammonium with both struvite and TSP treatments. By the time of the harvest, plants treated with struvite nitrate had higher total root length than lupine plants treated with struvite ammonium. This was not observed in plants treated with TSP ammonium that also had a higher total root length than the TSP with nitrate. Results might indicate that plants treated with TSP ammonium create thin roots faster than struvite treated with ammonium.

Root length in maize plants was not analyzed in experiment 5B, however results from experiment 5A showed also higher root length when struvite was applied with nitrate as well the SRL was higher with ammonium. In conclusion, struvite modified root morphology compared with no P application resulting in increased total root length. In both cases (struvite and no P treatments) nitrate led to a higher root length than ammonium. It was shown that the N source applied to plants modified the root morphology depending on the P source present. Lupine treated with ammonium increased root length if the P source is highly soluble, such as TSP, however root length increased in plants treated with nitrate if the P source is struvite.

### 3.6.2 P source effect on root morphology is modulated by the sand pH

There are other factors that can influence as well the effect of P on root morphology, comparable to that of the N source (as shown in 3.6.1). In experiment 9, the effect of three different pH conditions (pH 4.5, pH 6.5 and pH 7.8) on the roots of lupine plants treated with struvite and a highly soluble P source, potassium phosphate (KP), was analyzed (2.5.8).

Total root length, average root diameter, diameter class length (DCL) and SRL modification were also analyzed in response to the pH, besides that in response to the nutrient source applied (Table 24).

**Table 24 Total root length, average root diameter and root surface area of lupine plants growing in sand at modified pH values in experiment 9.** Values are mean (n=5). Different letters mean significant differences ($p<0.001$).

| | | Total root length (cm) | Average root diam. (mm) | Root surface area ($cm^2$) |
|---|---|---|---|---|
| Acidic | KP | 1025b | 1.65b | 272.7ab |
| | Struvite | 538.4bc | 0.79c | 142.9bc |
| | Control | 404.1bc | 0.86c | 110.5c |
| Neutral | KP | 1218a | 2.5a | 343.6a |
| | Struvite | 1327a | 2.64a | 403.7a |
| | Control | 279.8bc | 0.91c | 82.13c |
| Alkaline | KP | 164.6bc | 1.06c | 55.22c |
| | Struvite | 89.84c | 1.11bc | 30.16c |
| | Control | 104.2bc | 1.21bc | 39.16c |

The root system in general was dominated by the taproot and primary lateral roots (first-order branches), however significant differences in the rooting pattern and branching type were observed among pHs. The lupine plants showed great variation in total root length and root surface area in response to pH. When plants grew in neutral conditions, total root length was highest with

struvite, followed by KP with no significant differences, and lowest in control conditions (Table 24). Lupine plants growing in acidic conditions had higher total root length when KP was applied, almost double than with struvite. There were not significant differences with no P application in the acidic or alkaline conditions. In alkaline pH (6.5), lupine roots did not elongate and surface area was reduced by 90% compared with roots growing at pH 4.5. Lupine plats treated with struvite in the neutral pH had also the highest root surface area, however it was not significantly different than those treated with potassium phosphate at any pH.

There were no significant differences observed between struvite and KP on root length within individual pHs. The average root diameter in acidic conditions with struvite was significantly lower than with KP. For every diameter range, the relative diameter class length was calculated for the specific pH (acidic, neutral and alkaline).The percentage values shown Table 25 represent the % of the average total length for this individual pH.

**Table 25 Average DCL, (root length within a diameter class) and relative diameter class length (rDCL) = DCL/ root length (%).** N=5, different letters mean significant differences ($p<0.001$). At acidic pH thin roots (<1mm) account almost 80% of total root length, compared with alkaline (57%) and neutral (71%). In alkaline the percentage of thick roots (>2) increased until almost 9%, significant higher than for acidic (2.6%) or neutral (3.5%).

| Root Diameter (mm) | | DCL | | | rDCL(%) |
|---|---|---|---|---|---|
| | | KP | Struvite | Control | |
| <0.5 | Acidic | 216.3a | 127.9a | 77.23bc | 21.31 |
| | Neutral | 188.5a | 206.3a | 57.36bc | 17.17 |
| | Alkaline | 20.15a | 19.55a | 13.98c | 15.81 |
| 0.5-1 | Acidic | 599.8a | 286.5a | 255.3c | 58.29 |
| | Neutral | 710.5a | 703.2a | 141.1b | 53.91 |
| | Alkaline | 80.62b | 34.9b | 36.5b | 40.96 |
| 1-1.5 | Acidic | 146.7bc | 142.9bc | 69.94cd | 16.08 |
| | Neutral | 234.4ab | 286.1a | 50.4cd | 19.6 |
| | Alkaline | 34.93cd | 16.49d | 27.38cd | 21.95 |
| 1.5-2 | Acidic | 39.46bc | 22.24bc | 18.91bc | 4.21 |
| | Neutral | 54.02ab | 84.45a | 18.52bc | 5.8 |
| | Alkaline | 16.99bc | 10.7c | 15.37bc | 12.32 |
| >2 | Acidic | 23.09ab | 12.2b | 14.24b | 2.68 |
| | Neutral | 30.48ab | 46.9a | 12.45b | 3.49 |
| | Alkaline | 11.89b | 8.19b | 10.9b | 8.93 |

The results show that at acidic pH thin roots (<1mm) account for almost 80% of total root length, compared with alkaline (57%) and neutral (71%). This is in line with what was observed in the lower average root diameter in acidic condition with struvite. In alkaline conditions the percentage of thick roots (>2) increased up to 9%, significantly higher than the % calculated for neutral (3.5%) or acidic (2.7%) conditions.

Lupine plants investigated in experiment 9 showed great variation in SRL in response to pH. When plants grew in acidic conditions, SRL was highest with KP (4.77m $g^{-1}$ DW), followed by struvite (4.1m $g^{-1}$ DW), and lowest in control treatments (4.03m $g^{-1}$ DW). There were no significant differences between P treatments. SRL for neutral pH ranged from 3.1 to 3.15m $g^{-1}$ DW. SRL reached its minimum when soil was alkaline being reduced from 2.4 with KP to 2.03 with no P addition. In acidic pH, with the highest SRL, the roots were significantly thinner than in the other pHs when treated with struvite.

### 3.6.3 Root mass ratio & allometric analyses

The differences in root architecture between pHs, just described in the previous section, were further analyzed. The shoot biomass of lupine plants growing in neutral pH were the highest, however the root analyses showed that plants growing in acidic pH had the highest SRL. Therefore the root mass ratio (root DW:total DW) was calculated (Table 26), showing as well a higher root mass ratio in acidic than in neutral conditions.

**Table 26 Root mass ratio (root dry mass/total dry mass) of lupine at different sand pH as affected by the P source applied.** Values are mean (n=5). Different letters mean significant differences (p<0.001).

| | Fertilizer | Acidic | Neutral | Alkaline |
|---|---|---|---|---|
| Root mass ratio | KP | 0.41a | 0.37a | 0.24a |
| | Struvite | 0.42a | 0.39a | 0.23a |
| | Control | 0.39a | 0.32a | 0.28a |

The allometric analyses offer a visual description of root and shoot biomass distribution, which is not confounded by plant size (2.9.2) Fig. 27 shows the differences between P treatments of root:shoot relationship with significant differences in slope, *k* (p<0.01).

Similar analyses were also done regarding pH, and in general it confirms that the slope of alkaline is similar to no P treatment (result not shown). This allocation strategy shows plants investing more in roots when no P applied or the pH of the sand it is not optimum for growth.

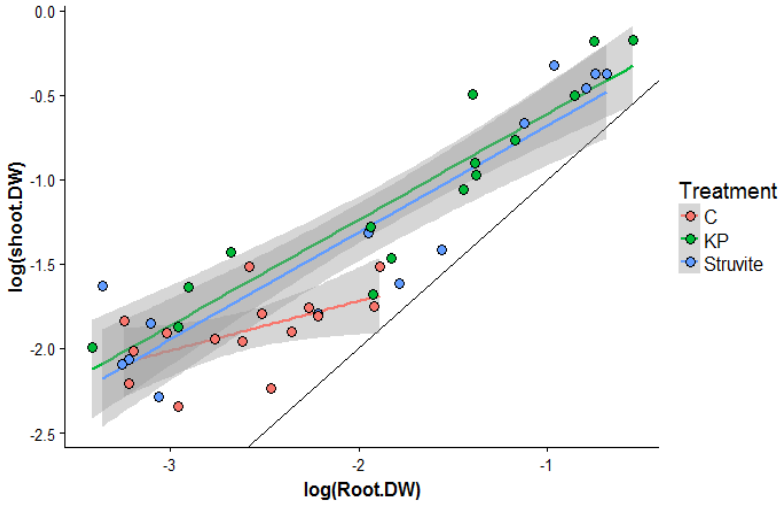


**Fig. 27 Allometric analyses of shoot:root biomass distribution (log transformed shoot:root distribution of the dry weight) as affected by the P source applied (No P (C), Potassium phosphate (KP) and struvite).** Plants were grown in neutral pH. Different slope of the control treatment compared with the P treatments indicated lupine plants with no P are investing more in root than in shoot.

### 3.6.4 P uptake efficiency (PUE): P uptake per unit of root length

In experiment 5, the P recovery and the P concentration were compared between P and N fertilizers in lupine and maize (Table 15) (3.4.1). After the root analyses (Table 23) (3.6.1), combination of both parameters allowed to analyze differences between treatments in the P uptake efficiency (PUE: Shoot P content, taken as a proxy of total P uptake, normalized for the total root length, mg P $cm^{-1}$ root) (Fig 28). For these analyses, the total P uptake (shoot and root) was combined.

The P uptake efficiency was affected by the N and P source applied. In experiment 5A, PUE from struvite was 2 (lupine) and 3 (maize) times higher ($p<0.05$) when combined with ammonium than with nitrate (Fig. 28A). It was observed a similar trend in experiment 5B for struvite (Fig. 28B), although the effect was smaller and statistically not significant. For TSP there was no effect of N source on PUE. Results show that P applied as struvite will be used more efficiently by plants when delivered together with ammonium, however for other P sources such as the highly soluble TSP, it will make no differences.

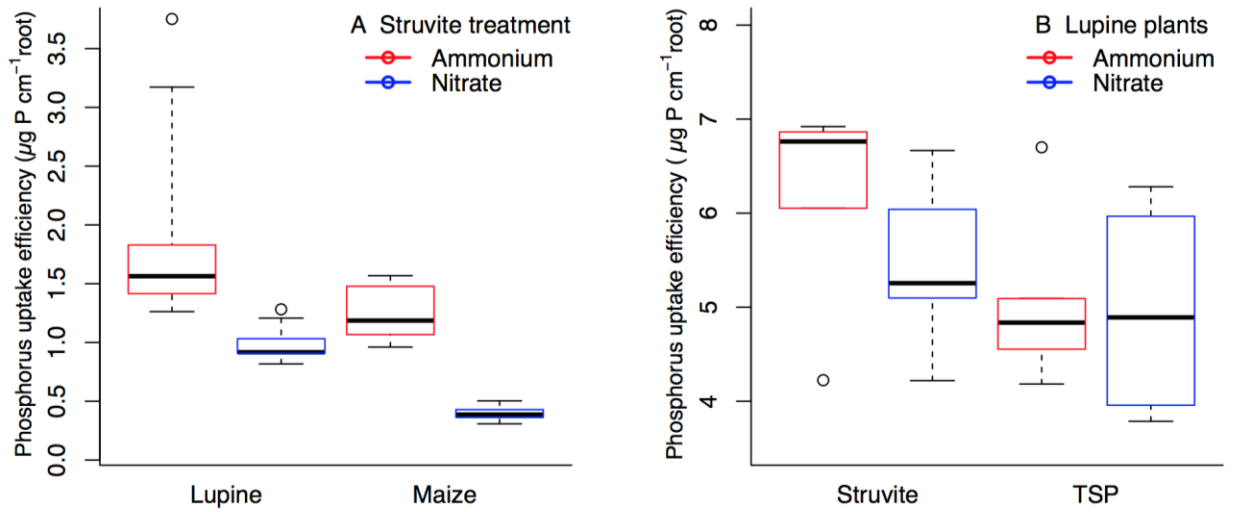


**Fig 28 Phosphorus uptake efficiency (µg P applied as struvite recovered per cm root) in lupine and maize plants in experiment 5A (A), and phosphorus uptake efficiency (µg P applied as struvite or TSP recovered per cm root) in lupine plants in experiment 5B (B) as affected by the N sources applied (ammonium or nitrate).** The positive effect of ammonium applied together with the struvite in the efficiency of the P uptake, as observed in Experiment A and B in both species, is not observed with the TSP treatment in Experiment B. Bars represent mean ± SE n=10 for Experiment A and n=5 for Experiment B.

### 3.6.5 Root morphological analyses with non-destructive measurements along the experiment

Compared with aboveground plant parts, roots are not easily accessible by non-invasive analyses and research is still largely based on destructive methods at harvest. Plants grown in soil-filled rhizotrons (up to a volume of ~2 L), as done for experiment 7 (2.5.6), allow quantitative measurement of root architecture parameters in 2D and shoot biomass evaluation at the same time (as described in 2.6.2).

Root growth analyses over time permit to observe changes in root architecture at different stages of root development. The percentage of visible roots (~20%) decreases with increasing average root diameter of the plant species studied and depends, to some extent, on environmental conditions (Nagel et al, 2012). That might explain the better results for tomato than for lupine plants (with higher root diameter). For lupine plants, higher root length was observed when no fertilizer was applied and similar distribution of primary-thicker roots, and secondary-thinner roots between organic and struvite fertilizers (Fig. 29).

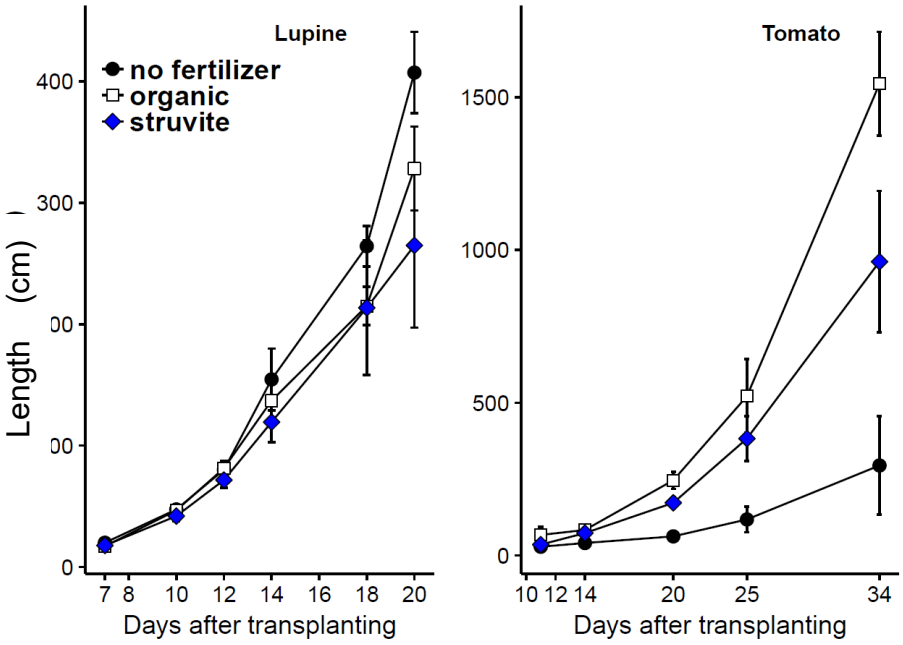


**Fig. 29 Total root length (cm) of lupine and tomato growing in rhizotrons filled with organic substrate as affected by fertilizer applied (no fertilizer, organic or struvite). Non-invasive measurements were done at different time points indicated in the X-axe as days after transplanting.** N=7 +SE. For lupine plants the highest root length was measured when no nitrogen was applied. For tomato plants, the organic fertilizer, followed by the struvite had significantly higher root length than the no nitrogen application. The differences are not observable until 22 days after transplanting for lupine (final harvest), however for tomato the differences are already visible 14 days after transplanting.

In contrast, in tomato plants, organic fertilizers exerted great influences on the root morphology. In general, the total root length, the root surface area and the root volume were slightly decreased with struvite, but significantly decreased by no N addition, in comparison with the roots of plant treated with the organic fertilizer. In particular, struvite total root length was only about 50% of that observed following organic fertilization. These data revealed not only the difference in modifying the root system architecture between plant species, but also the differential effects on these parameters exerted by a particular fertilizer treatment (Fig. 29).

## 3.7 Rhizosphere dynamics

### 3.7.1 pH dependent P solubilization via carboxylates release

In experiment 9 (setup at 2.5.8), the amounts of carboxylic acids that lupine released into the rhizosphere, expressed as total concentration and concentration per root dry mass, were compared between the two P treatments (struvite and potassium phosphate, both applied at 15µg P $g^{-1}$). The differences in the carboxylate exudation between P sources was analyzed at three different sand pH conditions (methodology for carboxylates collection at 2.7.5). It was observed that the total amount of carboxylates (mainly citric acid and malic acid) measured in the rhizosphere of lupine plants treated with struvite in neutral pH increased in comparison with KP, which is more soluble at this pH (Fig. 30 for citric acid).

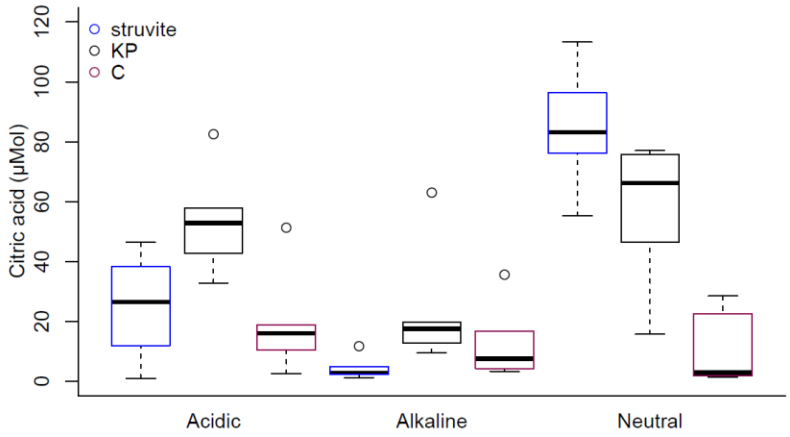


**Fig. 30 Citric acid concentration in rhizosphere of lupine at different pH conditions as affected by the P source applied.** Bars represent mean ± SE n=5. Lupine plants treated with struvite increase the exudation of citric acid at neutral pH, condition where the struvite is less available, in comparison with the KP treatment. This is not observed in acidic conditions.

Under acidic conditions, exudation of carboxylates was less, with no significant differences between P sources as struvite and KP are highly soluble at this pH. In this pH, the exudation of carboxylates was even slightly higher with the KP compared with the struvite. The root growth at alkaline pH was highly inhibited; therefore the total amount of carboxylates exudation at this pH was very small (<5µM, without significant differences between treatments). Treatment, pH, and the interaction had a significant effect on the total concentration of citric acid (µMol) ($p<0.001$).

At alkaline pH, the root growth was strongly inhibited and the total concentration of carboxylates (µmol per plant) was highly reduced (2.6 at Alkaline, 4.5 at Acid and 11.1 at Neutral pH).

Nevertheless, the concentration per unit root dry weight (µmol g $^{-1}$ root) was still high (Fig. 31). It was shown that lupine roots modified the exudation of carboxylates (mainly citric acid) in response to the availability of the P source applied. In alkaline pH, with no P available, plants increased exudation as a primary response to high pH/low available P. In neutral pH, with struvite being less available than KP, lupine increased the exudation to mobilize the P from struvite.

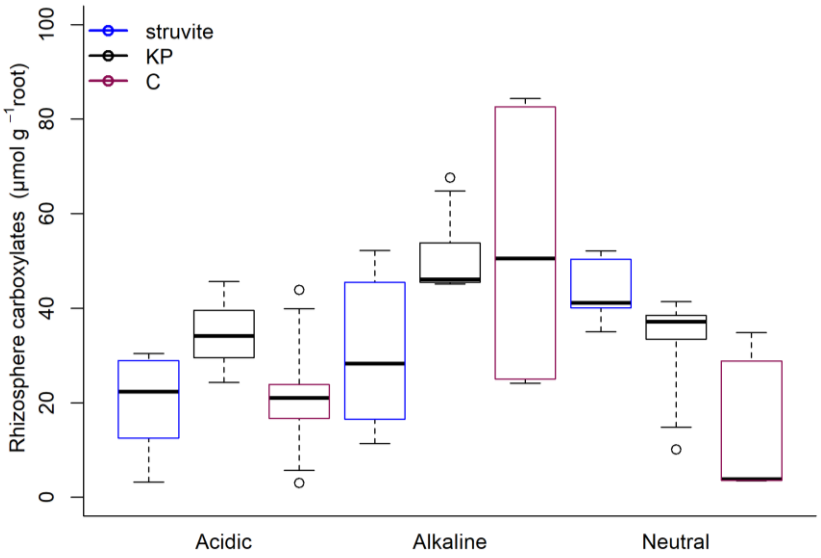


**Fig. 31 Carboxylates concentration per unit of root dry weight in rhizosphere of lupine at different pH conditions as affected by the P source applied (struvite, potassium phosphate as KP and Control, with no P application.** Bars represent mean ± SE n=5. Root growth of lupine plants growing in alkaline condition were significantly reduce, but mon-significant differences between pHs were observed in the carboxylates exudation per g of root.

### 3.7.2 Rhizosphere pH analyses

In previous section 3.4.4 it was shown the pH measurements of the bulk soil. In experiment 9, it was analyzed the pH of the rhizosphere solutions (extracted with 0.2 mM $CaCl_2$). The rhizosphere of control plants (without P) had a pH higher than that of struvite and KP treated plants in all pH conditions. Struvite treatment seemed to increase pH slightly in comparison with KP treatments in acidic soils, however there were no differences with KP in neutral soils, possibly due to higher exudation rates shown in Fig. 30, 31.

### 3.7.3 Measurements of pH dynamics in the rhizosphere with planar optodes

In experiment 7 lupine and tomato plants were grown in rhizotrons, allowing the installation of optodes (2.5.6), the present state-of-the-art technology to monitor the spatial and temporal pH

dynamics (Neumann et al., 2009). Non-invasive quantitative imaging of the proton dynamics in the rhizosphere were translated into continuous pH measurements (Fig. 32) (methods in 2.6.3)

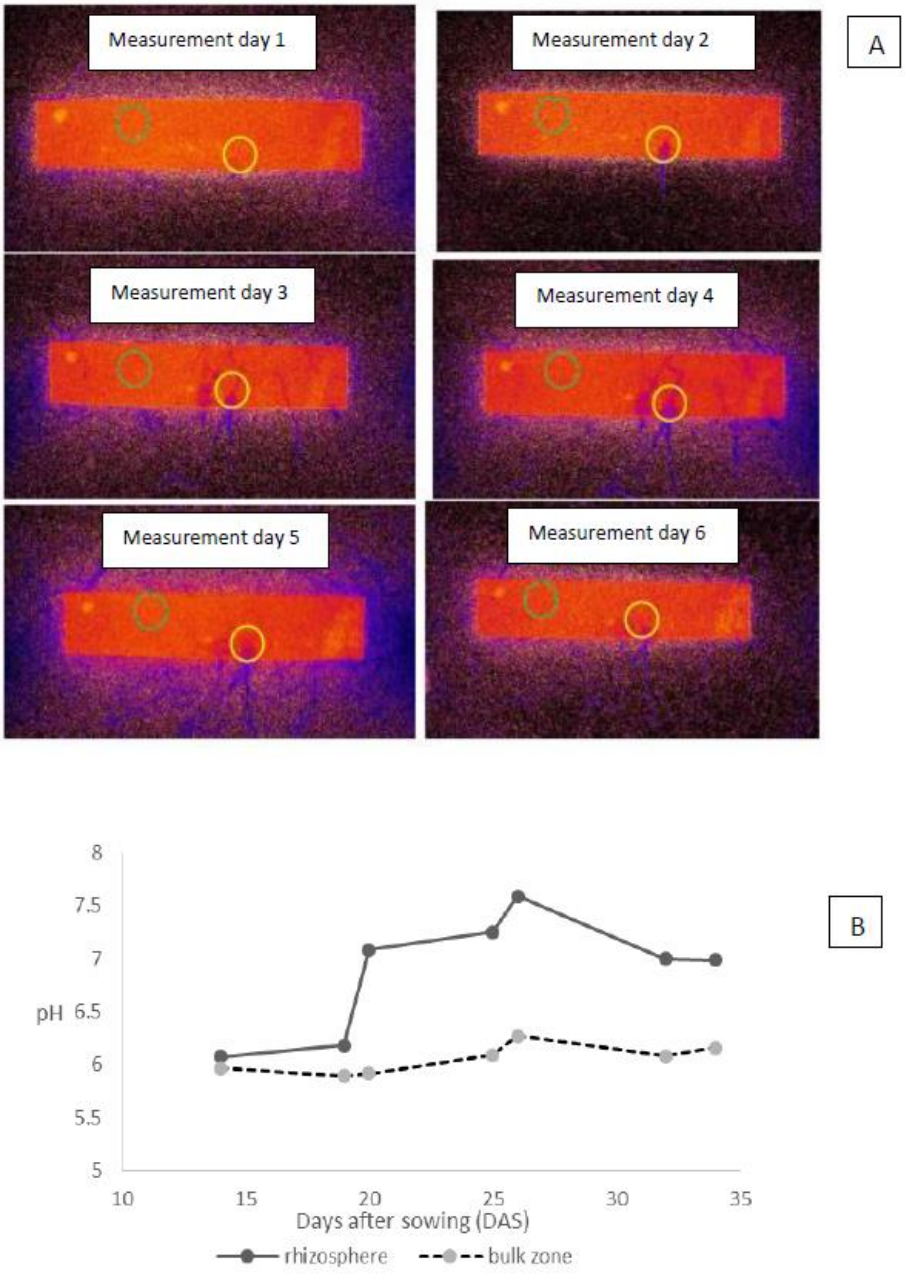


**Fig. 32 (A) from left to right and top to bottom sequence of pH change in the rhizosphere of the tomato plants measured with the upper pH optodes under the organic fertilizer treatment**. Green circles shown measurements for the "bulk soil", and yellow circles shown measurements for rhizosphere **(B) Evolution of the pH value in the rhizosphere and bulk zone** obtained extrapolating the image value in the calibration curve**.**

The effect of struvite and organic fertilizer on the pH was monitored in the rhizosphere and the bulk zone via optodes. It revealed that tomato plants modified rhizosphere pH only with the organic fertilizer and not in combination with struvite or no fertilizer treatments. The lupine plants did not show any detectable pH changes. In general, in the rhizosphere, there is an increase of the pH when the root is crossing the optode, showing a change from 6.2 to 7.6. This effect was decreasing over time, meaning that the pH in the rhizosphere is decreasing again to pH 6.9 measured at harvest. The pH of the bulk zone increased slightly to a value of 6.2, which matched with the value measured in the growing medium with a pH ($H_2O$) meter.

### 3.8 Struvite effect in the microbial community structure

#### 3.8.1 Analyses of rhizosphere and bulk soil together

Multi factor analyses (MFA) were performed to indicate the significance of each covariate (time, fertilizer and location) on the microbial community. Three aspects were taken into account to measure the microbial community structure: species richness (the total number of species in the community), species relative abundance (evenness: refers to how common or rare a species is relative to other species in a given community) and species diversity (pool of species). The analysis confirmed that plant and time point significantly contributed to the differences in the relative abundances of the bacteria genera ($P < 0.05$). Evenness and richness were higher when no fertilizer was supplied, followed by the organic fertilizer treatment and then by struvite ($P < 0.05$).

With respect to species richness, tomato showed a significant higher amount of species ($p<0.005$) than lupine and no effect of fertilizer and location was found. However, it was observed that no fertilizer treatment had a higher amount of species compared to organic fertilizer and struvite, as well as the bulk zone had a higher amount of species than the rhizosphere. The microbial community in combination with the no fertilizer treatment and the treatment with struvite were more even compared to the organic fertilizer treatment. Fertilizer and plant significantly influenced biodiversity ($p<0.005$). The no fertilizer treatment showed a higher diversity index than the organic fertilizer treatment followed by the struvite. The diversity index was significantly higher in combination with tomato compared with lupine. MFA showed the differences in the relative abundance of the microbial community in the bulk substrate and the rhizosphere combined of tomato at time point 2 (dimension 2) and lupine at time point 2 (dimension 3).

Results suggest that the overall “young” microbial communities of lupine and tomato will be independent and also that they are not impacted by the use of the fertilizer. MFA shows that he

plant effect is determinant on the differences in the relative abundances of the microbial communities (Fig. 33) that is starting to separate between species. To summarize, a specific microbial community colonizes each plant species. The microbial community associated with the organic substrate blended with recovered nutrients differed between plants species (based on the relative abundances of the bacterial genera) rather than between fertilizers applied.

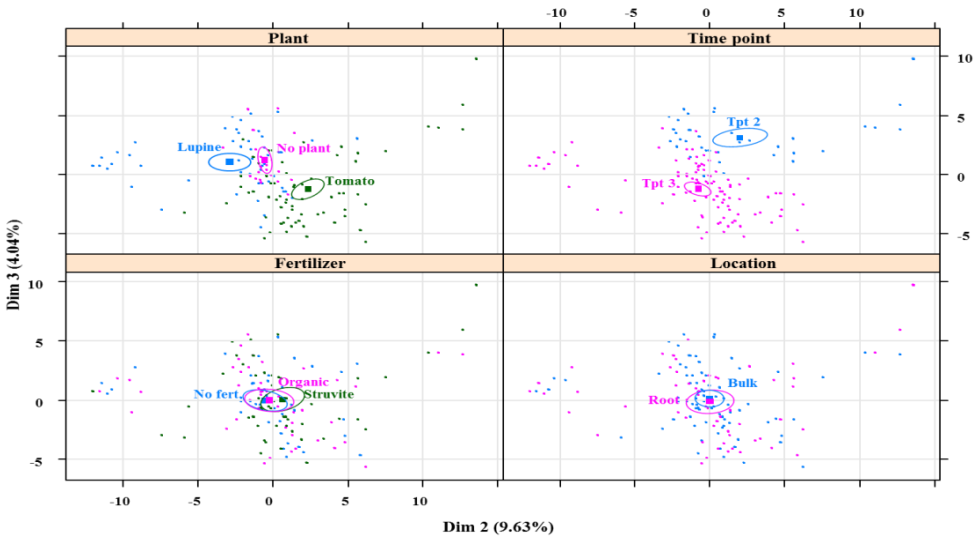


**Fig. 33 Microbial community shifts of the bulk growing media and the rhizosphere of tomato at tpt 2 (dimension 2) and lupine at tpt 2 (dimension 3).**

### 3.8.2 Plants influence the microbial community composition in the rhizosphere

Previous analyses were done focus in the overall microbial community. As expected, MFA for the microbial community only in the rhizosphere showed differences between plant species. Differences in the relative abundances of the bacterial genera suggested the presence of distinctive and stable microbial communities associated with the rhizosphere, which differed between plants. Time point also had significant influence on the microbial community (Fig. 34).

Ellipses show confidence Intervals (CI) of 95% for each sample type. The second dimension of the MFA described the growing medium harboring tomato plants, while the fourth dimension was constructed by the relative abundances of the bacteria associated with the growing medium harboring lupine. As a result, these two dimensions were projected in the map and variations in the bacterial relative abundances over time, and in response to plant and fertilizer were detected.

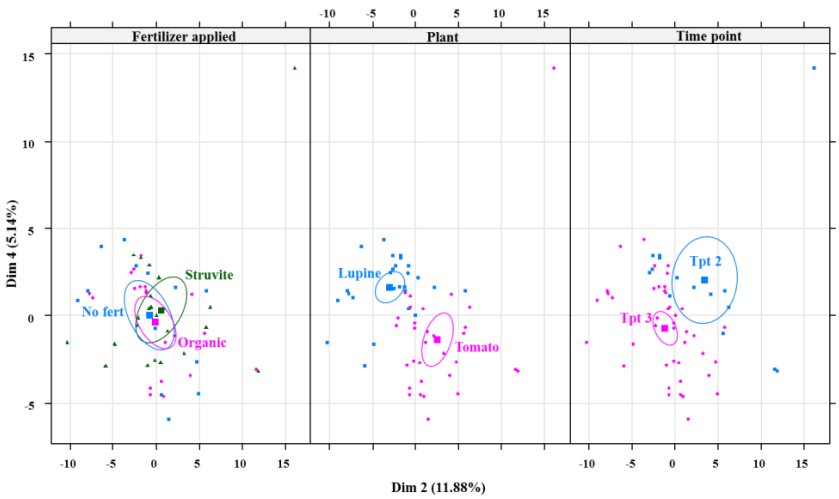


**Fig. 34 Microbial community shifts of rhizosphere in growing media harboring two different plants, supplemented with fertilizer over time.**

### 3.8.3 Is the microbial community in the bulk soil also affected by the plant species?

Surprisingly, the Multiple Factor Analysis (MFA) done only for the bulk substrate also showed differences between tomato and lupine microbial community abundance (Fig. 35). The first dimension describes the growing medium supplemented with struvite and harboring lupine and the third dimension describes the growing medium supplemented with organic fertilizer and harboring tomato plants.

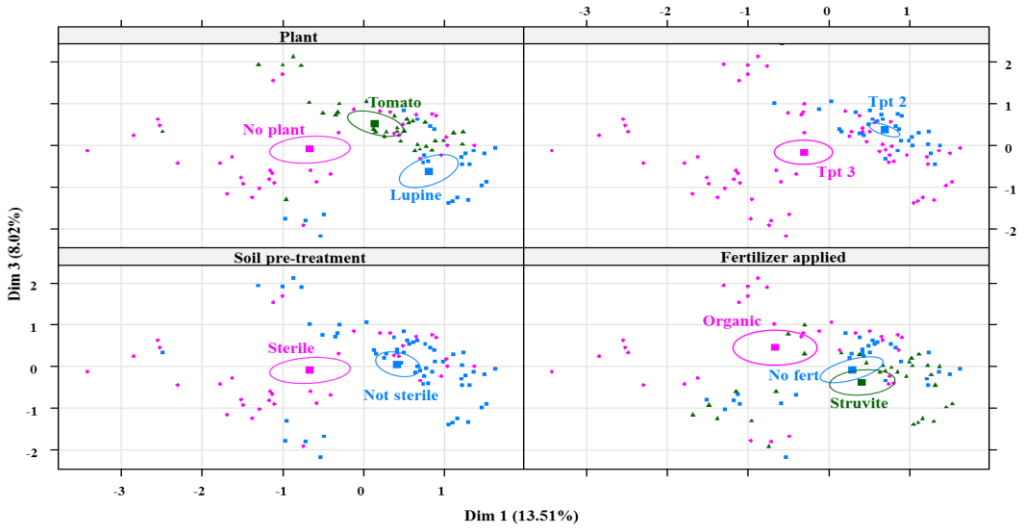


**Fig. 35 Microbial community shifts of pre-treated bulk zone harboring two different plants, supplemented with fertilizer and followed over time.**

Combination analyses of bulk and rhizosphere microbial community showed that the growing medium in combination with tomato had a higher richness and diversity than with lupine. As suggested by Baudoin et al. (2002) time is also an important factor and mentioned study indicates

that the differences between bulk and rhizosphere soil responses are more pronounced after 4 weeks (Day 30) compared to 2 weeks old plants.

To summarize section 3.8, results showed that plants drive microbial community composition in the organic substrate. The rhizosphere community is more diverse and differs from the bulk soil. Plants affect microbial community also in the “bulk zone” and not only in the area near the root.

# 4. Discussion

The experiments carried out in this dissertation proved that struvite is an effective phosphorus fertilizer for different plant species. Nevertheless, struvite-P availability was highly influenced by soil conditions, mainly by soil pH. Other essential factors that affected fertilizer efficiency were the nutrients added jointly with the struvite and plant-induced changes in the rhizosphere. In the presence of struvite plant-associated microbe communities were modified, raising the question how this in turn influences nutrient availability from struvite.

It was shown that to improve the utilization efficiency of the recovered products using them as plant fertilizers, it is necessary to preform individual case studies of which plant-rhizosphere-soil factors have a major effect on its availability. In this respect, we agree with the reports from FAO (2015) that to become less dependent on rock phosphorus reserves and fuel-derived fertilizers, not only do nutrients from waste have to be completely recovered, but also, in order to close the nutrient balance, those nutrients need to be recycled more efficiently. Having an integrated approach considering: first, the development of new recovery technologies to waste management systems such as ManureEcoMine, and secondly, having a better understanding of all specific factors increasing fertilizer efficiency, will enhance the value of products such as struvite and will consequently further the development of its market. In this context, struvite availability is discussed in the following sections in regard to i) the effect on biomass yield and P utilization in maize, lupine, tomato, and viola ii) the variation in root architecture and carboxylate exudation, iii) the effect of soil pH and rhizosphere pH on P availability, and iv) the effect of recovered products on microbial community distribution

## 4.1 Effectiveness of struvite

My first hypothesis formulated that P content (mg P plant$^{-1}$), as an approximate measure of total P uptake by the plant, would be comparable to the P uptake of conventional P fertilizers derived from rock phosphate producing similar plant biomass yields. This P uptake efficiency will not differ between fertilizers; however, it would be specific for each plant species, due to differences in root morphology and physiological characteristics. In this section, the effectiveness of struvite is discussed regarding biomass, P (and N) uptake and the effect on germination. In addition, to explain the differences in the struvite- P acquisition efficiency between species in more detail, the effect of rooting medium pH on plant growth and struvite availability will be discussed.

### 4.1.1 Struvite has the same fertilizer efficiency as mineral P sources regarding plant biomass and P uptake but delayed seed germination

The initial approach to test the first hypothesis was to compare the biomass production of struvite-treated plants with plants treated with highly soluble fertilizer in various plant species. After that, the elemental concentration of plant tissues was measured, as in most cases the element concentration of leaves reflects better the nutritional status of a plant (Marschner 1995).

Biomass results suggest that struvite has the potential to replace highly soluble P sources like the commercial TSP. There are several studies that confirm struvite as a good candidate to be used as a P source for crops or potted plants (Gonzalez-Ponce et al., 2009; Plaza et al., 2007). These studies agree with the results of this dissertation where struvite effects on aboveground biomass equalled or surpassed that of TSP fertilized plants (Fig 17).

Struvite was previously reported to produce significantly more biomass than TSP, however not for maize but for other crops such as lettuce in loamy sands (Gonzalez-Ponce et al., 2009) or garden rocket on acidic soils (Yetilmezsoy et al., 2013). So far, the use of struvite as a P fertilizer for maize plants was reported to produce only similar biomass production and P uptake than TSP (Thompson 2013). As it will be further discussed, the finding of Thompson might be explained by the slow release of nutrients from struvite that requires longer time to surpass the quick-soluble P fertilizers.

Gonzalez-Ponce and Garcıa-Lopez-de-Sa (2008) showed that the P provided by struvite was recovered by a lupine species (Lupinus albus) in the same amount as from TSP, even though the latter is much more soluble in water. Likewise, in experiment 5 there were no differences in the P uptake between both P sources, neither for lupine nor maize (Table 15). Thus, the higher biomass of maize plants when treated with struvite in comparison to TSP cannot only be explained by a higher P uptake. This higher maize biomass production with struvite compared with TSP was probably related to the extra magnesium that was released together with struvite. Gonzalez-Ponce et al. (2009) attributed the better agronomic performance of struvite-treated lettuce to the larger amount of Mg incorporated with struvite, avoiding the $Mg^{2+}$ deficiency that might occurred with the TSP treatment. Leaf interveinal chlorosis is the typical $Mg^{2+}$ deficiency symptom due degradation of chlorophyll, since $Mg^{2+}$ acts as central atom in the chlorophyll molecule. Nevertheless, it is a late visible symptom that is often hardly to diagnose in the early stages of the plant (Cakmak and Yazici 2010), explaining why not deficiency symptoms were observed in my experiments. As an earlier response to $Mg^{2+}$ deficiency, decreases in plant yield (as observed in plants not treated with struvite) can be expected due to restricted supply of carbohydrates to the roots (Cakmak et al., 1994).

As also hypothesized, the P uptake was plant species specific. Shoot biomass and P uptake were higher in maize compared to lupine, as expected due to inherent differences in biomass and growth rate among the two species (Table 15); this might be explained by the better maintenance of leaf production that monocots normally have under P stress compared with dicots (Halsted and Lynch 1996). Contrastingly, results showed that lupine had a significantly higher P concentration in leaves and stem than maize plants (Table 15) and that the P concentration in lupine was significantly greater if treated with struvite ammonium than with any other treatment.

Initially, it can be stated that struvite fertilization was more successful in maize than in lupine, as struvite increased biomass significantly compared with TSP only in maize. Lupine showed no differences in biomass between treatments, but surprisingly the use of ammonium incremented the P uptake from struvite, resulting in a higher P concentration in the plant compared with TSP. The effect of nitrogen source on struvite availability was further investigated by analyzing root morphology and soil pH changes in the following sections (4.1.2 for pH, 4.2.2 for roots, and 4.4.1 for P uptake efficiency analyses).

Biomass might be a good indicator if the interest is the yield of the plant, but for deeper knowledge with the aim of increasing the fertilizer use efficiency, biomass analyses are not enough. As stated above, struvite treatment created more biomass than TSP in maize, but the analyses of maize tissues showed that both P treatments resulted in similar P concentrations in plant tissues. The P concentrations were lower than the average concentration stated as sufficient for adequate growth falling in the deficient range (<1.6, as described in Marschner et al., 2011 for soybean) for all the treatments except for lupine treated with struvite ammonium.

The deficient P levels in lupine and maize plants might be explained by the fact that the dose-response curve on which the amount of P applied was based (Fig. 12), was done with highly available mineral nutrients in shorter time (28 days growth) and showed healthy plant growth, with no nutrient deficient symptoms, at a dose of 12 mg plant $^{-1}$ (Table 6). In experiment 5, an application of 36 mg plant $^{-1}$ of struvite or TSP was not sufficient for 40 days growth (as shown by the deficient level of P in most of the plants in Table 15). It was concluded that it is important to setup a dose-response curve preceding the full-scale research using the experimental rooting medium, and the nutrients in the same source that will be later applied.

To analyze the effectiveness of struvite, as well as other similarly recovered products, it is also necessary to take into account the effect on seed germination. This knowledge will ensure the implementation of the recovered product in the soil in the right time. In experiment 2, the germination of lupine and maize seeds as affected by struvite and nitrogen (N) application was analyzed. Results showed a germination delay of seeds treated with struvite in acidic conditions

in comparison to those treated with DI water (Fig. 13), with no adverse effect of solely N addition as ammonium nitrate (shown as no P treatment). The N release from struvite as ammonium-N might explain this effect. Seed germination and seedling establishment can be inhibited by $NH4^+$ toxicity (Barker et al., 1970, Westwood and Foy 1999). Possible mechanisms described are i) penetration of gaseous ammonia into the seed, which would block respiration temporarily (Openshaw 1970) or ii) high pH and high osmotic values which would slow down the hydration of the seed (Hegarty 1978). The concentration of gaseous ammonia depends on the concentration of $NH_4^+$-N (6.64 ± 0.17 % in the struvite) via the equilibrium $NH_4^+$- and $NH_3 + H^+$ and the volatilization of $NH_3$ (Bennett & Adams, 1970). A concentration of NH3 of 13 mM has been previously proven to be toxic (Bennett & Adams 1970). However, the concentrations in our experimental soil were below this value (3.2mM as calculated from the $NH_4^+$ concentration). Alternatively, high osmotic values caused not only by $NH_4^+$ but by all ions, might explain the delay as it would slow down the hydration of the seed and therefore avoid germination. Further investigations are needed to elucidate why struvite delayed germination. For example by measuring seed hydration using MRI that will allow to observe if there is a lower hydration in struvite-treated seeds, validating the hypothesis of high osmotic values.

To summarize, the effectiveness of struvite was comparable to conventional P fertilizers. Acquisition of P from struvite was modified by the nutrients applied jointly (ammonium increased PUE compared with nitrate) and depended on the plant species (maize has higher biomass but lupine higher P concentration). No adverse effects in terms of TSP in plant performance or nutrient recovery were observed; in contrast, it was noticed that struvite application delays seed germination. Based on these results, pre-germination of seeds in water is recommended to avoid adverse effects of struvite and ensure uniform germination between treatments. Alternatively, seeds could be germinated in a thin layer of soil not treated with the fertilizer directly in the pots, without contacting the soil/fertilizer mixture below. Investigation on the potential application of struvite clearly needs to be accompanied with different trials for specific crops adapted to certain soil conditions.

### 4.1.2 Rooting medium pH: main factor affecting struvite availability and plant growth

The physical and chemical nature of struvite makes it highly insoluble in alkaline conditions, sparingly soluble under neutral conditions, but highly soluble in acidic conditions. Consequently, it is expected that the pH of the rooting medium (from here on: any kind of soil, substrate or sand in which plants were grown in during this thesis) affect struvite solubility. Besides affecting the the solubility of the fertilizer applied, in natural conditions, chemical properties of the soil also determine the growth of plants that are adapted to particular soil conditions (Kinzel 1983). Plant

adaptation to different soils is mainly related to tolerance to certain elements, the availability of which is directly related to the soil pH (Marschner 1995). Therefore, the pH of the rooting media will directly affect the plant growth.

In experiments 4 and 6, the effect of rooting medium pH (acidic pH 5 or alkaline pH 7.8) was analyzed with respect to i) lupine and maize performance, and ii) struvite- and TSP-P uptake efficiency. At the same time, comparison of plant performance and fertilizer use efficiency between both experiments allowed an analysis of the effect of physical structure and fertilityof the rooting media (poor sand, in experiment 4 or field soils in experiment 6).

The ANOVA test performed shows that the percentage of biomass variability in the plants explained by each factor (P source or soil pH) was different for each plant species (Table 11 and 12). Maize will grow better in those soils with higher fertility (high concentration of available nutrients like P, independently of the pH); but in contrast, the main factor constraining lupine growth will be the pH rather than the nutrient concentration in the soil. This was concluded as lupine performed better both acidic conditions even if in experiment 6 the acidic soil had lower available P content than the alkaline. Maize plants showed a higher tolerance to high pH than narrow-leafed lupine that is known to grow poorly on neutral to alkaline soils compared to other lupine species such as Lupinus pilosus (White 1990; Tang et al., 1992).

In poor sand (experiment 4, Table S1), significantly higher biomass accumulation was observed in acidic conditions for both lupine and maize plants. As the optimum pH for struvite dissolution is acidic, it was expected that not only biomass but also the concentration of P in the leaves would be greater than in alkaline conditions. Interestingly, sand pH had no significant effect on the concentration of P neither in lupine nor maize, but it had a significant effect on the concentration of some other nutrients such as Mg, Ca or Al that were higher in acidic pH (Table S2). The hypothesis is that higher concentration of Mg in this case, directly related with the availability at certain pH, might have a stimulating effect, as described in the previous section for experiment 5, giving the plant a better overall performance.

It has been shown that pH affects each species differently. Accordingly, the effectiveness of struvite at various pH will be species dependent. To further investigate how struvite availability is modulated by the plant at different pHs, another experiment was performed focusing on lupine. Lupine is tolerant to low pH (the pH where the struvite is more soluble) but also can acidify the rhizosphere at higher pH (Lambers et al., 2008), potenitally increasing the dissolution of struvite-P. In experiment 9, lupine growth and struvite availability were analyzed in washed sand adjusted to three different acidic, neutral and alkaline pH values. Struvite was compared with potassium phosphate (KP), a fully water-soluble phosphate fertilizer and highly efficient source of phosphorus

and potassium for plants. In this section, the effect of sand pH on plant growth and struvite-P recovery will be described. Further mechanisms of lupine to increase P uptake efficiency at different pHs will be discussed later (see 4.2.2 and 4.2.3).

The response of lupine plants to P application showed substantial differences in biomass among pH conditions, but no differences among P sources. As expected, all plants grew relatively poorly without the addition of P in all pH conditions. Likewise, all plants grew poorly at alkaline conditions, with no significant differences on biomass between P treatments. The chlorosis of old leaves at alkaline pH or when no P was supplied was observed in most of the plants, possibly reflecting the process of translocation of P and other nutrients to young leaves. Both conditions of high pH and low P were not optimal for plant growth; however, the high content of mineral nutrients in large-seeded species like lupine, and their translocation from cotyledons during early growth possibly enabled the seedling growth under stress conditions for a period of two weeks (Milberg et al., 1998).

At neutral and acidic pH, the effectiveness of struvite and KP was similar. Both P treatments resulted in higher total biomass than no P, with no significant differences between them ($P<0.05$) (Fig. 18). The biomass yield of lupine was 80% lower at pH 7.8 compared to pH 6.5, and 65% lower at pH 4.5 compared to pH 6.5 (Fig. 18). It was previously reported that an optimal pH for lupine growth is between 5.0 and 5.5 (Tang et al., 1992); similar to the rhizosheath pH found in the present study at neutral condition. As for the biomass, it was expected that nutrient content of lupine would decrease at high pH, as shown by Jessop and Mahoney (1982) for lupine grown at a pH above 6. However, this is in disagreement with results in experiment 9, where lupine grown in alkaline pH exhibited significantly higher P content (mg $g^{-1}$ DW) compared with acidic and neutral conditions (Table 17). To understand these values, it is necessary to take into account nutritional effects associated with growth responses, such as dilution and concentration effects or nutrient allocation and translocation (Imo and Timmer 1992). The correlation between nutrient concentration and plant biomass may depend on the plant developmental stage due to the dilution effect. If the uptake of a nutrient occurs in an early plant developmental stage and the nutrient is later metabolized or translocated to other plant parts, its concentration in the plant or old leaf will decrease with age. Consequently, the concentration of such nutrients would be negatively correlated with plant biomass.

Struvite-treated plants growing in acidic pH showed a very high concentration of P in the shoot (>0.8%, considered as toxic according to Marschner 2011) (Table 17). Plants were slightly smaller than those treated with KP (Fig. 19B), but no visual sign of phosphorus (or any other nutrient) toxicity was observed (perhaps due to the short term of the experiment). The high P concentration

in lupine shoots is clearly related to the high solubility of struvite at acidic conditions. Struvite was applied at 20mg P per plant in acidic sand, for a growth period of five weeks. For lupine species like L. micranthus, high sensitivity to phosphate toxicity has been reported, showing significant reduction of dry weight at 10mg P (Abdolzadeh, 2010), and suggesting poor regulation of P uptake, which might also be occurring in L. angustifolius.

In light of these results, we determine that: i) a lower dose of struvite should be applied on lupine plants (<20mg P plant for six weeks growing in acidic pH <4.5), and ii) further analyses on plant nutrient concentration and nutrient imbalance must be calculated. In conclusion, these results show that for plants that are adapted to grow in neutral to acidic conditions like L. angustifolious, no pH implications can be concluded concerning the agronomic value of struvite, as there were no differences between KP and struvite at any pH tested. Lower applications of struvite than KP in acidic conditions might be recommended, as the P concentration observed with struvite was close to the levels considered to be toxic even though no toxicity symptoms were observed.

### 4.1.3 Slow-release properties of struvite compared with TSP

Besides the final biomass production, it was also examined the fertilizer effect on leaf area development during the full growth period, in order to monitor the possible advantageous nature of slow rate of nutrient release from struvite (Rahman et al., 2014). In experiment 5, phosphorus availability from struvite in acidic sand and subsequently dry matter accumulation in lupine and maize shoot were analyzed using a shoot-imaging platform (Screen-House) that captures shoot traits via image analysis over a defined growth period (Nakhforoosh et al., 2016). Non-invasive plant phenotyping provides relevant information to analyze the nutrient status of a plant at different developmental stages, and therefore observation of an effect of delayed nutrient release is possible.

Initially, the phenotyping of plant shoots in the early plant growth phase revealed significantly higher leaf areas in the TSP treatment compared with struvite treatment (Fig. 21). After the first 20 days of growth, there were no significant differences between P treatments until the time of the harvest, when maize plants fertilized with struvite showed a higher leaf area and higher biomass than those with TSP. These differences were not a result of different germination rates as we pre-germinated the seeds in water in light of experiment 2 (Fig. 13) and seedlings were transplanted to the already fertilized soils. The mechanism that may explain this is related to the higher solubility of the commercial P fertilizer TSP in comparison with the struvite. Slow- release causes a delay in nutrient availability for plants after application, consequently resulting in a delay in the growth response compared to plants fertilized with quickly available nutrients. So far it was unclear whether the slow release of nutrients can meet crop demand (Tilman et al., 2002), unless they

have specific characteristics like coated-fertilizers that are designed to release the nutrients according to precise time intervals (Guan et al., 2014). For the analyzed experimental soil and plant parameters, measurements indicated that the slow nutrient release from struvite could ensure a steady nutrient supply according to the needs of the plants improving fertilizer efficiency after three weeks and even producing higher biomass and leaf area production at the end of the experiment.

The slow-release of nutrients can be considered advantageous compared with highly soluble fertilizers that are faster in their nutrient release to the rooting medium. Slow-release can contribute to having a more sustainable P fertilization by i) reducing soil P immobilization processes (Talboys et al., 2016) ii) improving fertilizer P recovery, and iii) lessening the risk of fertilizer P loss (Hart et al., 2004; Withers et al., 2014). Additionally, in environments such as those in e.g. Western Australia with low P retention in the acidic sands (Summers et al., 1993), fertilizers like struvite with a slow release of P can potentially be used as a nutrient management strategy.

Not only for P, but also for some nutrients like nitrogen (also present in the struvite), delay in the fertilizer release can reduce the risk of loss to leaching or volatilization. Therefore, if struvite is used to fertilize horticultural plants, where a considerable amount of irrigation water is drained, and the soil or growth media has a poor buffering capacity, a slow fertilizer release can be beneficial.

Struvite was previously described as slow-release fertilizer; however, the use of automatic phenotyping platforms allowed showing the exact time (three weeks of plant growth in acidic condition) where it starts to be as efficient (or more) as TSP. This provides useful information about biomass yield that can be used as indicators for harvest in the field. Further measurements of nutrient leaching are recommended, to verify the reduction of nutrient loss after applying struvite.

### 4.1.4 Recovery of struvite-derived phosphorus was greater than that of ammonium

In the three previous sections, the effectiveness of struvite as a P fertilizer was discussed. Aside from P, struvite also contains magnesium ($Mg^{+2}$: 17.5%) and $NH_4^+$ (6.5%), the effect of which should also be analyzed in order to define the general efficiency of struvite as a fertilizer. Mg+2 can have a significant effect on plant performance (as discussed in section 4.1.1). Likewise, the N cycle (dominated mainly by microbial processes), has a great impact on soil chemistry and consequently soil fertility (Jetten 2008). Therefore, in experiment 7, the fertilizer effect of struvite as N source was analyzed on tomato and lupine performance and on soil chemistry, as well as on soil microbial community (further discussed in section 4.3.4).

Struvite was compared with an organic fertilizer made of amino acids, whose application as a fertilizer has been demonstrated to have a beneficial effect on leaf mineral status (Garcia et al., 2011). It was hypothesize that struvite would have a benefitial effect as N source, as it delivers the ammonium directly in the rooting medium. In the other hand, the organic fertilizers need a supplementrary conversion firtst from the organic N to ammonium to be plant available. Surprasingly, in experiment 7, leaf area, shoot fresh, and dry weight in both tomato and lupine were lower when fertilized with struvite compared with the organic fertilizer (Fig. 22, Table 14).

To explain those reults, calculation of N balance in the plant-substrate were performed. At the beginning of experiment 7, the ammonium concentration in the soil samples was analyzed immediately after the fertilizer addition, resulting in higher N concentration with the struvite than with the of organic fertilizer even though the initial amounts of total N added by both fertilizers were the same. The ammonium from the struvite seems to be more quickly exchangeable than the ammonium from the organic fertilizer. The significantly higher amount delivered by struvite than by the organic fertilizer could have impacted negatively soil chemistry or microbial community, and therefore plant performance resulting in lower growth rates, leaf area and stunted growth in high ammonium sensitive plants such as legumes (Britto and Kronzucker, 2002). Surprisingly, this was not reflected in the N concentrations in the plants, which were within normal range.

With the struvite treatments, the ammonium concentration increased continuously in the substrate of tomato plants while it reached a peak already at the time of the first harvest in the substrate of lupine (Table 18, first harvest). This was observed also in the N concentration of lupine, with higher amounts in first harvest than in the second. In contrast, even though the ammonium concentration continuously increased in the substrate of tomato, N concentration of tomato biomass was reduced at the second compared to the first harvest (Fig. 25). When organic fertilizer was provided both soils reached a peak in ammonium concentration in the first harvest. The ammonium concentration in the substrate of tomato plants was smaller than in that of lupine since part of the ammonium had already been transformed to nitrate (Table 18, first harvest tomato). In the second harvest, the ammonium concentration in the substrate of both species was reduced with the organic fertilizer. At this time point, also in the substrate of lupine, part of the ammonium was transformed into nitrate. Accordingly, N concentrations of both plants were higher in the first than in the second harvest. Interestingly, for tomato plants, the concentration of nitrate in the substrates measured in the second harvest was strongly reduced. This might indicate that the nitrate was taken up by the plants as also suggested by the pH changes in the rhizosphere observed with optodes (this will be further discussed in 4.3.3) and the higher biomass observed in tomato plants treated with organic fertilizer (Table 14).

We hypothesize that in the tomato substrate, the ammonium delivered by the struvite was not able to be transformed into nitrate; since the amount of ammonium measured in the substrate increased continuously. Additionally, lupine plants also did not take up the nitrogen delivered from the struvite. However, in the substrate of lupine plants, the ammonium from struvite could be transformed into nitrate, as assumed by the decrease of ammonium in the substrate and increase of nitrate. Contrastingly, the ammonium delivered by the organic fertilizer was transformed into nitrate in the substrate of both species, but only tomato plants were able to take it up (Table 19). One explanation can be that lupine plants were able to fix nitrogen successfully and might did not need extra nitrogen

So far, the effect of ammonium from struvite has been tested mainly as multi-nutrient P&N fertilizer (Ganrot et al., 2007), showing an optimal amount of plant available P for plant growth, but an N concentration in the deficient range. To our knowledge, only one report in the literature has analyzed before the effect of struvite as the only N source increasing the application until fulfilling plant N requirements (Li and Zhao 2003). In that study, the struvite application dose was increased to reach 70mg per kg of soil (i.e. an addition of 150mg of P per kg soil) resulting in P toxicity. Similarly, the struvite dose in experiment 7 was increased to fulfill N requirements of tomato and lupine, respectively. The analyses of nutrient content in the plant material showed adequate concentrations of N (%) for tomato and lupine (neither deficient nor toxic levels) with no differences between struvite and organic fertilization. However, P levels at the end of the experiment were higher under struvite treatments, approaching toxic levels (Fig. 25).This can be explained because struvite has a low N-content (6%) and a low N/P ratio (1:2.5), which is suboptimal for plant growth (Marschner 2011).

In agriculture, the required amount of N is usually higher than that of P, with an average N:P ratio in shoot dry matter for adequate growth of 1000:60 µM g-1 DW. In experiment 7, the dose of struvite applied added a higher amount of P than of N to the substrate (Table 18 time point 0). This was reflected in the high concentration of phosphorus in tomato and lupine leaves treated with struvite in comparison with organic fertilizer (Fig. 25). This might be another explanation for the lower biomass compared with the organic fertilizer (Table 14).

The results presented in this section show that ammonium availability from a fertilizer might be related not only to specific plant nutrient turnover strategies (nitrogen fixer or not) or to the form that the nitrogen is delivered in (organic or inorganic). Also, other factors like the microbial activity associated to each species (as will be further discussed (section 4.3.4) that might prefer one source of ammonium over another, affecting the mineralization of ammonium different regarding the N source.

### 4.1.5 Struvite is a good alternative to mineral fertilizers as part of a fertilizer blend

As mentioned in the section before, the ratio of N:P:K of struvite can cause an imbalance of nutrients concerning specific plant requirements. Therefore, struvite must be combined with other nutrients to produce fertilizer blends suitable for agriculture or horticulture. Struvite is already used by fertilizer companies as an additive or to substitute raw material in standard fertilizer production technology (Li and Zhao, 2002). Nevertheless, the additional use of salts of ammonium and potassium to formulate a balanced NPK fertilizer together with struvite is necessary.

In order for struvite to be part of a fertilizer blend more suitable for green horticulture or sustainable agriculture, it needs to be combined with other nutrients, ideally recovered from waste streams. The ManureEcoMine technology allows the recovery not only of struvite but also of other nutrients such as ammonium sulfate, ammonium nitrate, and potassium struvite.

In experiment 10, the effect of struvite as part of different blends on the biomass of viola plants was determined. The 15 types of blends applied to the substrate had different effects on plant dry weights (Fig. 23). Plants treated with blends containing the recovered ammonium nitrate showed a healthy growth and resulted in optimal concentrations of N in mature leaf tissue (described in 3.4.4). In contrast, combinations using the recovered ammonium sulphate resulted in an unacceptable increase in electrical conductivity (Table 20). High electrical conductivity can produce osmotic stress, resulting in decreased water uptake, which could cause chemical burning of the roots. I assume that the high electrical conductivity observed when ammonium sulphate was included in the blends (which increased up to 2,000 µS/cm) might be caused by the high ammonium concentrations rather than the sulphate concentrations. Blends with ammonium sulphate had an ammonium concentration near 900 g $N/m^3$; however, blends with ammonium nitrate had an ammonium concentration around 100 g $N/m^3$.

Besides the positive controls (the rapidly available mineral fertilizers and the commercial slow release organic fertilizer - osmocote,), it seems that blend 7 (combination of struvite from waste water, recovered ammonium-nitrate and potassium sulphate) has the best chemical composition (Table 20) and non-significantly different biomass from the positive controls. Fertilizer blends with recovered products (P as struvite and N as ammonium nitrate) can substitute the use of mineral fertilizer blends for the growth of ornamental plant species such as viola. Still, the preparation of green fertilizer blends requires an exhaustive control and knowledge of every single product that is added. The effect of struvite as a P source to add into a fertilizer blend needs to be further evaluated to enable a successful combination with other recovered materials. Specific combinations will significantly affect soil chemical properties and therefore plant growth, and those results might be species dependent.

## 4.2 Root morphological and physiological plasticity in response to pH and fertilizer treatments

My second hypothesis stated that the different mechanisms of variation in root diameter, total root length and the capacity to produce carboxylates are different between lupine and maize, and that this would have an effect on the phosphorus uptake efficiency of struvite-P. It was expected that lupine has a greater PUE of struvite P due to its high capability to accumulate carboxylates (Pang et al., 2010).

It is known that the availability of plants to take up P is related to several root characteristics including morphological traits such as length and surface area (Williamson et al., 2001), or root architecture (Lynch 1995). In field studies, some legumes show higher P-acquisition efficiency than other crops related to a higher physiological plasticity, for example due to the release of carboxylates (Boland et al., 1987). Therefore, by analyzing specific root morphological and physiological adaptations of the plants to the recovered products, it would be possible to increase fertilizer use efficiency.

### 4.2.1 Allometric responses to low P availability.

The first strategy to increase nutrient uptake includes the allocation of a significant portion of their biomass to the root system. As described by Hermans et al. (2006), this reaction is a consequence of metabolic changes in the shoot and an adjustment of carbohydrate transport to the root that will modify the shoot: root biomass ratio.

Root:mass ratio (root DW: total DW) showed no significant differences between fertilizers (Table 26). More precise analyses were performed through allometric studies of shoot:root relations (log transformed shoot: root dry weight relation), which are common in plant nutrition analyses as they are independent of plant size (Imo and Timmer 1992). In experiment 9, lupine was treated either with struvite or TSP growing at three different pHs. Allometric analyses for both factors (P source and pH) showed similar patterns: those plants with no P and/or alkaline conditions showed an allocation strategy of investing more in root biomass than in shoots. No differences were observed between struvite and the easily available P source ($p<0.05$) (Fig. 27).

With both fertilizers, the strategy to adapt to adverse conditions was investing more in roots. Further analyses, as will be shown in the next sections, were necessary to elucidate the specific adaptations of root morphology but also root physiology of struvite-treated plants in comparison with plants treated with commercial mineral P sources.

### 4.2.2 Root morphology changes between fertilizers and pH

I asked if the application of struvite would affect root morphology compared to no P and to TSP treatments. Both species (lupine and maize) modified their root morphology in response to P and also to N fertilization.

It is known that mineral nutrient supply has a main effect on root morphology (as reviewed by Forde and Lorenzo, 2001). Experiment 5B provided evidences that struvite behaves as a good slow release P fertilizer, and consequently struvite treatment might limit the P availability during the initial period of plant growth, triggering a distinct root growth response in comparison to the quick release P fertilizers like TSP. Unexpectedly, the P source applied (struvite or TSP) did not show a significant effect on the root morphology (Table 23). Results in the literature about the effect of P on roots show a range of different outcomes that are often species-dependent. Limited P is reported to inhibit primary root growth and increase lateral root elongation and density in some species such as Arabidopsis (Williamson et al., 2001). For maize, unlike in Arabidopsis plants, some studies have found no reduction in root elongation, no effects in lateral root density but negative effects on emergence of new axile roots (Mollier and Pellerin 1999). The decrease in the total root length (TRL) observed in Experiment A for maize treated with no P might be due to a reduction in the emergence of new roots (Table 23). Expansion of root surface area by the prolific development of root hairs has been previously described as an adaptation of different species to low P (Vance et al., 2003); however, no effect was observed in the root surface area between P treatments in Experiment B.

Under P stress a reduction in root diameter, root mass density, and therefore an increase in SRL has been described as a common strategy for some species (Hill et al., 2006). Reduced lateral root diameter has also been observed under low P in maize (Zhu and Lynch 2004). In this study P starvation had no effect on lateral root density in maize, causing that the SRL remained similar to the SRL of struvite-treated plants. Under P starvation, lupine modified the root morphology by increasing the primary root elongation, similar to what was observed previously by Wang et al., (2008) who also described a large number of first-order lateral roots with probably large amounts of root hairs developed in lupine grown under low P conditions. This would explain why in our study the SRL in no P treated lupines increased in comparison with struvite treated lupines.

In experiment 5A, the root length of maize and lupine was compared when treated with struvite ammonium or struvite nitrate. In experiment 5B the effect of N source was observed not only in struvite treated plants but also in TSP treated ones. The N-source-dependent changes in the root morphology (total root length, root surface area, root mean diameter, SRL) were stronger than in reaction to P form and similar for both plant species (Table 23). Struvite-fertilized lupine and maize

increased the total root length and root surface area when they were grown under $NO_3^-$-N compared to $NH_4^+$-N. This is contrary to what we expected as it has been reported that when ammonium is applied, the N availability is reduced, stimulating lateral root growth. However, it might be explained by the negative effects that nitrate has on lateral root development described by Nibau et al., (2008). On the other hand, nitrate application has been reported to increase primary root growth, which can be highly correlated in some species with increased total root length (Gruber et al., 2013; López-Bucio et al., 2003). I expect this might be the case in lupine, as it has a typical legume root system consisting of a dominant taproot with a relatively large number of primary lateral roots and few secondary roots (Clements et al., 1993)

It is known that an increase in root diameter does not correlate with an increase in the uptake capacity of NO3- or NH4+ (Garnett et al., 2009). In this study, I did not observe changes in the SRL of lupine treated with ammonium in comparison with nitrate. Contrastingly, nitrate treated maize in Experiment B increased the SRL in comparison with ammonium. This effect might be explained by an increase in the number and the average length of lateral roots after nitrate application, as reported previously for maize plants (Schortemeyer et al., 1993). Plants treated with struvite plus ammonium probably invested more in thin roots than with nitrate, even if it was not translated into an increased total root length.

A number of previous studies have shown that the availability of different nutrients can distinctively affect different root morphological parameters. Postma et al., (2014), concluded that there is a root architectural trade-off for the acquisition of nitrate and phosphorus, suggesting that roots might modify the morphology depending on the relative availability of both nutrients. In this study the P form did not fundamentally alter the responses of root morphology; however, the N form had a large effect. Furthermore, the level of the effect was plant species-specific. Maize showed greater root morphological plasticity than lupine. This is in line with our hypothesis relating to lupine possibly relying more on the release of carboxylates than maize, with maize relying more on morphological changes.

In experiment 7, root morphology changes of lupine and tomato to the presence of two different N sources was analyzed. In this case, the focus was put on the form that the ammonium was delivered to the substrate: in form of struvite, i.e. as an inorganic form of N or in the form of amino acids as a representative of the major form of soluble organic N. Root morphology was analyzed in plants growing in rhizotrons filled with organic substrate and the total root length was measured continuously in both plant species. In previous experiments similar growth of plants treated with amino acids or ammonium was shown (Chapin et al. 1993). Contrastingly, some amino acids have been also reported to inhibit root growth (Forde and Walch-Liu 2009).

Results of experiment 7 showed that he use of an amino acid (organic N) in the substrate resulted not only in higher total biomass but also that root surface area was significantly enhanced in tomato plants in comparison with struvite (inorganic N) (Fig. 29). Similar results were observed in previous studies with herbaceous species supplied with glutamine as N source, where plants had proportionally more roots than if treated with inorganic N (Cambui et al., 2011).

Experiment 7 also shows that different plant species had distinct mechanisms for sensing N. In terms of their effects on root architecture, in tomato plants, the struvite and organic N stimulate secondary root growth and root branching. In contrast, in lupine, the non-fertilized plants had a higher total root length than the fertilized plants independently of the N source (Fig.29). This might be explained by the nutrient contents in the seed. White and Veneklaas (2012) hypothesized that a delay in P acquisition by roots of maize might be explained if the plant has sufficient seed P for growth. I hypothesized that for lupine plants, the seed P concentration was sufficient for the establishment of the seedling, but that this was not the case of the tomato; it is also possible that lupine did not need much of the extra N added by the struvite or the organic fertilizers as successful nodulation was observed in both P treatments. Therefore, as discussed before for plants with sufficient P and N levels, lupine plants increased primary root growth and a decrease the lateral root density.

At the end of the experiment, tomato plants treated with struvite reduce root length by 50% compared with organic fertilizer, however shoot biomass was reduced only by 27% (Table 14, Fig. 22). The observation that organic N promotes root growth without a clear effect in the shoot, is consistent with previous observations of organic N increasing root:shoot ratio (Paungfoo-Lonhienne 2012). Lupine plants had higher biomass with organic N than struvite and no N even thoug there was no effect observed in the roots.

In experiment 8, root morphology of lupine plants grown at three different pH conditions was analyzed. The morphology of *L. angustifolius* was also markedly altered by pH of the growing medium. High pH caused the disintegration of the root surface, inhibiting root elongation with a reduction of up to 90% in the surface area between lupine growing at pH 6.5 compared with lupine growing at pH 4.5. A reduction of root surface area due to pH increase was also observed by Tang et al. (1992). Lupine plants treated with struvite under neutral pH had the highest root surface area. However, this was not significantly different from those treated with potassium phosphate at any pH (Table 24).

Plants growing in acidic pH increased their SRL compared with plants growing in alkaline or neutral pH. A known plant strategy to increase SRL and therefore increase nutrient uptake is reducing the length while increasing the thickness of the primary root and increasing the length of

lateral thin roots (Hammond et al., 2004). In lupine growing in acidic pH, thin roots (0.1-1mm) accounted for almost 80% of total root length, compared with alkaline (57%) and neutral (71%) (Table 25, rDCL %). This reduction in mean root diameter observed in lupine at acidic pH is in line with previous studies) for relative diameter class length of lupine (Chen et al., 2012), where approximately 70% of total root length was in the diameter classes between 0.1 and 1.0 mm. We hypothesize that the relatively significant increases in SRL occurred in acidic condition as a consequence of decreasing root diameter might be a response of species adaptation to low pH. In contrast to what was observed in acidic pH, alkaline conditions modified root morphology increasing the proportion of thick roots. Normally, thicker roots (>2mm) account for approximately 4% of total root length (Chen et al., 2012), a similar proportion to what was measured at acidic and neutral pH. However, at alkaline pH, this increased to 9% (Table 25, rDCL %). The explanation might be not related with the nutrient availability but with the high pH. Under nutrient stress it would be expected an increase of SRL as mentioned before, however plants growing in alkaline pH with no nutrients available showed that the root elongation was totally inhibited, avoiding the development of lateral and secondary roots. Plants growing with no P at alkaline pH had thicker roots mostly belonging to the top root part near the shoot, without any further root elongation.

In contrast to the effect of pH, SRL did not vary between P applications within specific pH conditions. Control plants showed no significant differences in the SRL in comparison to the P-treated plants. However, in acidic and neutral pH, a small increase in the mean root diameter in response to P application compared with no P was observed. In alkaline pH, in contrast, the average of thicker roots (>1.2mm) was higher in the no P control than in the P treatments. Indeed, a decrease in root diameter is not a universal response to a low P availability (Schroeder and Janos 2005).

To summarize, the N source applied had a higher effect on root morphology than the P source applied. Nitrate increased the root biomass due to an increase of the number of thick primary roots compared with ammonium. However, ammonium might increased number of thin roots. Soil pH had a significant effect on root morphology. Lupine species, adapted to low pH, showed at this pH specific root morphological changes that increased nutrient uptake (like the reduction of MRD, related to an increase in the SRL). No significant differences in root morphology were observed between P sources, only between P and no P application.

These results demonstrate that the effect of a particular fertilizer on root architecture depends on plant species. To expand existing knowledge, further studies with ON and IN are necessary. In section 4.3.4, it will be analyzed the effect of plants on microbial community of the substrate, and the possible interactions with the N source applied, that will be specific for each plant species.

### 4.2.3 Does the high physiological plasticity of lupine improve struvite P availability?

Besides the morphological root changes described in sections 4.2.1 and 4.2.2, in some species like lupine, a common strategy to increase nutrient solubility is to modify root physiological processes, such as carboxylate exudation (Hinsinger 2001). The plant P uptake depends on the P concentration gradient and diffusion in the soil. The carboxylate exudation will have a strong effect on nutrient availability and solubility due to i) dynamic changes in the rhizosphere pH, specifically acidification of the rhizosphere that would results in an increase of the availability and uptake of sparingly soluble P (Thomson et al., 1993) and ii) the displace the phosphate from the matrix by ligand exchange (Lambers et al., 2006) that will also increase the P availability.

The mobilization of P from struvite via the addition of different citrate concentrations was analyzed in experiment 8 and 9. In experiment 8, the mobilization of P via carboxylates was simulated using external addition of citrate to the sand. High and low amounts of struvite were added to acidic or alkaline sand. Then, each one was flushed with citrate and the leachate was collected. The amount of soluble P measured in the leachate was compared with controls that were flushed with water. Increased struvite P solubilization in alkaline sand was observed when a high concentration of citrate was used (Fig. 16). Calculations showed that citrate is able to mobilize and solubilize around 60% of the P; a value that was calculated as 30% if the sand was flushed with water. This percentage agrees with that found by Wei et al. (2010) that found that citric acid, in comparison with water, will enhanced the solubilization of organic P at similar percentages that what observed in experiment 8.

Analyses made in experiment 8 were followed up in experiment 9. Here, the carboxylate exudation of lupine roots growing at three different pH conditions and treated with two P sources (struvite and KP) was analyzed. It was noted that lupine was able to release a higher amount of organic acids at neutral conditions when the struvite was applied, in comparison with the easily available $K_2PO_4$ (Fig. 30 and 31). The increase in organic acid concentration, especially citric acid, made the P from the struvite more available. Nevertheless, it could not be concluded that the effect was due to a decreased of the rhizosphere pH (see results in 3.7.2). Consequently it was hypothesized that the reason why struvite-treated plants in neutral conditions created the same biomass than the KP-treated plants, even though KP is much more soluble at this pH (Fig. 18) was an increased availability of struvite-P produced by the cation exchange with struvite. It was shown that the exudation is dependent not only on the P source applied but on the initial soil pH. The higher exudation was observed at alkaline conditions without differences between P treatments. The total concentration, as expected, was very low as the lupine root growth was highly affected. Interestingly, even though the total carboxylates concentration was lower in the

alkaline condition, the concentration per unit of root dry weight was higher in alkaline conditions, showing that this might be one of the first strategies of lupine to mobilize nutrients.

These results are in agreement with Talboys (2016), who affirms that struvite's effectiveness as a P fertilizer may be enhanced for crop species that exude organic acids in large quantities. The use of high exudate species might be a good strategy for alkaline soils where struvite is not initially soluble, as observed in experiment 8. However, to verify that, the use of other species less sensitive to pH than *L. angustifolius* is recommended.

## 4.3 Interactions between struvite, pH, and soil microbial community

In section 4.1.2 the pH of the rooting medium as the main factor modifying struvite availability and plant growth was discussed. It was shown that pH changes will not only alters nutrient availability but also will inherently modifies plant growth through changes in root morphology as described in section 4.2.2. So far the different pH values were due to differences in the initial rooting media pH. My third and fourth hypothesis stated that changes in soil pH can also be induced by the addition of other nutrients (such as ammonium versus nitrate) and that different nutrients sources such as organic fertilizer and struvite would influence differently the microbial community composition in the rhizosphere associated with the substrate and plant species analyzed. Therefore, in this section it will be discussed the causes that drive physical-chemical changes in soil properties, analyzing not only the pH changes but also the activity and structure of the microbial community, as microbial community will also strongly affect soil nutrient availability through mineralization and/or competition (Van Der Heijden et al., 2008).

### 4.3.1 Effect of P and N sources applied and root-induced changes on soil pH

Plant roots can modify rhizosphere pH mostly from the release of $H^+$ or $OH^-$ that occur due to the uptake of cations and anions (Hinsinger 2001). Another root induced pH change can be explained by exudation of organic acids. However, as shown by other authors (Haynes, 1990) the organic acids are rather exudate as anions than acids. Therefore the acidification in the rhizosphere will not be a direct effect, but a consequence of $H^+$ release when the roots uptake the anions, as described before. This in line with the results from experiment 9, discussed in section 4.2.3, where it was shown that lupine was able to increase exudation of organic acid and thus mobilize P from the struvite but probably not due to decrease of pH (as not significant pH changes were observed) but due to cation exchanges between organic acids and struvite. In results section 3.5.4, the pH of the different rooting media for experiment 4 and 5 was described; lupine and maize were grown on acidic or alkaline sand under different fertilization regimes. Even though in experiment 5 the

pH of the lupine sand had a trend of being lower than the pH of maize sand, no significant effect of plant species on the pH of the rooting medium (Table 21) was observed. That might be explained by the low exudation rate of lupine when the external conditions are already acidic, and that in highly alkaline conditions root growth was inhibited (as observed in experiment 4).

In section 4.2.2, the effect of N source on root morphology with ammonium increasing the amount of thin roots and the SRL in comparison with nitrate was discussed. Likewise, the nitrogen source applied also has an effect on the pH of the rooting medium. It was hypothesized fertilizing with ammonium can produce rhizosphere acidification (Gahoonia et al., 1992). Consequently, ammonium fertilization might result in greater P uptake per unit root length (effect that will be further discussed in section 4.4.1

The pH was monitored in experiment 5. In experiment 5A it was observed an increase of the pH when nitrate was applied with the struvite, and a slight decrease in pH with the ammonium application, consistent with our hypothesis. Unpredictably, in experiment 5B, I did not observe acidification.

The pH change caused by the N source applied it is explained by the mechanisms of transport for the main two ions (ammonium or nitrate) into the roots. $NO_3$ uptake is relate with OH- exudation and will produce alkalinization, and $NH_4^+$ uptake will be balance by H+ exudation and will produceacidification (Raven and Smith, 1976; Marschner et al., 1991; Britto and Kronzucker, 2002). In experiment 5 it was observed that nitrate with struvite increased the pH of the sand significantly compared with the initial sand pH. The ammonium with struvite slightly decreased the pH in the case of maize, however this was not significant (Table 22).

Not only plants but also fertilizers can modify the pH of the rooting medium. Johnston and Richards (2003) noted that struvite can increase soil pH and it was therefore ruled more suitable for acidic soils. Rahman et al. (2011) found a tendency of pH value to increasein struvite-treated soils, and to decrease in commercial N and P fertilizer treated soils. This was also observed in experiment 4 in acidic pH conditions where struvite application significantly increased the pH in comparison to TSP. Contrary to that, the P source applied did not have a major effect on the soil pH in experiment 5 (Table 21). This might be related with the volume of sand, as in both experiments soil was analyzed after 40 days of plant growth, however in experiment 4 pots were 1L volume, compared with 3L in experiment 5. In experiment 9, struvite treatment seems to increase the pH slightly in comparison with KP treatments in acidic conditions. However, it is lower than KP in neutral, probably related to the higher exudation rate. Triple superphosphate (TSP) adds P to the soil in the form of the $H_2PO_4^-$, which can acidify soil with a pH greater than 7.2 but has no effect

on acidic soils. Struvite, however, adds P to the rooting medium in the form of $PO_4^3$, as the H+ that will be present for example in the Mono-ammonium phosphate (MAP) is substituted by $Mg^{2+}$ (Fig. 15). This is the reason why struvite increases the pH of the soil, as it will form a conjugate acid by the reception of a proton ($H^+$).

To summarize, struvite increased the pH when it was applied to the acidic sand; however, the effect was not sustained in all the repetitions. Likewise, lupine and maize plants did not have major effects on the sand pH when they grew in acidic substrates and the effect of ammonium decreasing the medium pH was small in our experiments probably as this is a rhizosphere localized effect and not measurable in the bulk soil measured in this experiment. Those results might indicate that the abovementioned hypothesis that plants species as well as fertilizer applied have an effect on the soil pH might be significant as far as the initial pH condition is neutral.

### 4.3.2 Plant species and fertilizer treatments modified bulk soil and rhizosphere pH differentially in the organic substrate

As described in the previous section, plant species and fertilizer applied in acidic sand did not significantly influence pH value. In contrast, in experiment 7 where lupine and tomato plants were grown in an organic substrate, the pH of the bulk zone was significantly influenced by plant fertilizer and time point (Table 18).

Plant species had a bigger effect than fertilizer on the pH of the substrate. Surprisingly, lupine increased the pH of the substrate in the absence of a fertilizer to the slightly alkaline range (5.5 to 6.7) compared to tomato where pH increase was less than half between the first measurement and the first harvest (Table 18). Interestingly, in the substrate of the control treatments (no plants), the pH in the first harvest increased even more than in substrate containing tomato plants (pH 5.5 to 6.2). This might suggest that tomato plant did not increase but decrease the pH. Tomato is known for having a high exudation rate, which might explain the observed acidification of the substrate. Even though lupine is known to exudate large amounts of organic acids, root growth of lupine was mainly reduced to primary and secondary roots in this experiment – reducing, therefore, the exudation rate.

The pH monitoring in the rhizosphere zone via optodes revealed that the investigated tomato plants with the organic fertilizer increased rhizosphere pH at certain time point (Fig. 32), to later decrease it. This was not observed in combination with struvite or the no fertilizer treatment. As hypothesized in section 4.1.4, the low nitrate concentration measured in the substrate of tomato treated with organic fertilizer in the second harvest, could be explained by the plant nitrate uptake. The increase of rhizosphere pH observed with the optodes is consistent with the hypothesis of

nitrate uptake by the tomato, as nitrate uptake will co-uptake protons from the substrate. Also the pH measured in the second harvest time with the optodes, match with the pH of the substrate measured chemically.

This hypothesis needs to be carefully reviewed as the pH increase in the rhizosphere with the optodes was measured in very few replicates due to technical limitations of the system, and are contrasting with the values measured in the bulk zone.

To conclude, it was shown that to monitor pH changes in the rooting medium during plant growth is important. The pH increase observed at certain points in the rhizosphere and that can be of short time duration, could be related with the N concentration in the soil. Those analyses would be useful for future experiments to explain results such as i) changes in the availability of other nutrients, like the increase of P availability associated with a predominant $NH_4$–N uptake compared to $NO_3$–N due to acidification (Riley and Barber 1971; Ruan et al., 2000), or ii) inhibition of nitrification rates by soil bacteria as the rhizosphere acidifies (Haynes and Goh 1978; Falkengren-Grerup 1995).

### 4.3.3 Nodulation of lupine was affected by pH and not by struvite addition

The effect of pH on plant performance and nutrient mobilization was previously discussed, as well as the effect of plant species and fertilizer application on the pH. The effect of pH on root-microbial interactions such was analyzed initially by studying the nodulation. It was observed that pH had a clear effect on nodulation of lupine plants (Fig. 14).

In experiment 9, lupine plants were all inoculated with the same amount of rhizobia. It is known that pH above 6.0 specifically reduces nodulation in some lupine species (Tang and Robson, 1996). The nodulation inhibition was also observed in the roots of plants growing in alkaline conditions in experiment 9. Likewise, in experiment 3 nodulation was observed in the acidic sand but not in the alkaline. The mechanism by which high pH impairs nodulation in lupine is unknown. There are several possibilities, as described by Tang et al. (1992). First, high pH may limit bradyrhizobial growth in the rooting medium. Second, the morphological changes of roots of L. angustifolius grown at high pH as previously discussed may affect bradyrhizobial recognition. Other authors (Romheld and Marschner, 1986) have hypothesized that high pH might induce iron deficiency as iron is directly involved in nodulation in L. angustifolius (Tang et al., 1992).

Besides the pH, it is well known that mineral nitrogen application i.e. ammonium and nitrate can depress $N_2$ fixation (Marschner, 2011; Peoples and Baldock, 2001). However, experiment 3 contradicts those studies since nodulation was not inhibited by the addition of nitrogen as struvite. Additionally, in experiment 7, most lupine plants growing in rhizotrons with a high concentration of

ammonium in the substrate had an effective nodulation. These results are related to the effectiveness of struvite as an N source in lupine plants. As described in section 4.1.4, results suggest that lupine did not need the ammonium from struvite or organic fertilizer as it was able to fix nitrogen. Ingestad (1982) noted how important it is in terms of nodulation whether nutrients are added once or sequentially over an entire period matching plant demands. The slow-release properties of struvite might circumvent the inhibiting effect of nitrogen in the nodulation.

To summarize, pH effect was greater than the effect of struvite on nodulation of lupine since struvite addition did not have any effect on nodulation. This needs to be taken into account when the N fertilizer value of a recovered product is analyzed in plants that can fix nitrogen.

### 4.3.4 Plants rather than fertilizer modifies the microbial community in the rhizosphere

So far, the physiological and morphological changes in the root to increase nutrient uptake have been discussed. Another important strategy used by plants to increase mineralization and solubility of nutrients is the manipulation of the microbial community in the area near the root (Pierzynski and Logan, 1993; Ulen et al., 2010; Balemi and Negisho 2012). When roots start to grow, they immediately encounter the microbial community associated with the substrate, resulting in the establishment of a microbial rhizosphere community closely interacting with the plants and a microbial community distinct from the bulk soil. Although it is known that additional N supply affects soil microbial community structure (Ai et al., 2012), limited information is available on how N fertilization influences the plant-associated microbiome in the rhizosphere and the bulk zone.

The microbial community associated with the substrate blended with recovered nutrients was analyzed in experiment 7 for tomato and lupine plants. The goal was to identify which factors (plant species or fertilizers) would have a higher influence on the microbial community structure and activity. It was hypothesized that different nutrients sources such as organic fertilizer and struvite would influence in a different way the microbial community composition in the rhizosphere associated with the substrate and plant species analyzed. It was found that plants rather than fertilizer were able to drive changes in the microbial community (Fig. 33). Possibly the microbial community in the rhizosphere benefitted from the surplus of easily degradable carbon sources provided by the plants and from the release of several compounds into the substrate. Due to this close plant-microbe interaction, plants are able to select a certain rhizosphere microbial community potentially through their root exudates (Gschwendtner et al., 2016), (Girvan et al. 2003).

The beta diversity measurements were significantly different between rhizosphere and bulk zone. This means that there are differences in species composition between the two locations, meaning that not all the same species are present in both environments. This might indicate that the number of species is the same, but not the way they are distributed within time points, within fertilizers and within plants. This was previously observed by Samala et al., (2001) that found an increased relative abundance of some populations in the vicinity of the roots for all three plants species compared to the bulk zone.

Surprisingly, the microbial community in what is considered bulk soil (normally defined as area not influenced by the plant) was also influenced by plant species (Fig. 35). This analysis confirms the dissimilarity in what is define as “rhizosphere” in literature, indicating that the bulk zone of our analyses might be still consider rhizosphere.

Microbial processes in the soil are crucial for plant nutrient supply, given their role in nutrient dynamics. A change in microbial community structure does not always involve change in microbial community function or increase in nutrient availability and plant productivity. Alternately, an increase in the microbial community can reduce nutrient availability due to inmobilization. The results of the chemical (Table 18) and microbial analyses (Fig. 34 and 35) performed in experiment 7 suggest that due to distinct microbial community, tomato plants were able to mobilize ammonium to nitrate from the organic fertilizer and not from the struvite. However, in the substrate of lupine plants, the ammonium from struvite was able to be mobilized to nitrate. It was observed that bacterial abundances in the rhizosphere were significantly different between species regardless of fertilizer supplementation, especially during the early development of the plant (Fig. 34). Moreover, the microbial community in the rhizosphere became more even over time, as indicated by the decrease in variation of the bacterial relative abundances. The abundance of specific groups, however, was not affected by fertilizer application.

Struvite application will not modify the microbial community as much as the plant species. Consequently, different microbial communities will associate to specific plants, which will result in struvite being more available to some species than others due to the microbial community associated to that species. Also for the organic fertilizers, it has been previously shown that amino acids are rapidly uptake by the microbial community (Jones 1999). Competition for amino acids between roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition was analyzed by Owen and Jones (2001). They concluded that “the rapid turnover of amino acids by soil microorganisms, and the poor competitive ability of plant roots to capture amino acids from the soil solution might limit the use of organic fertilizers against inorganic fertilizers”. This is contradictory to what observed in experiment 7, where it was shown that organic fertilizer was

higly efficient for tomato plants. Therefore for future studies it is necessary analyzing which plants, with their specific microbial community, are more suitable to be fertilized with organic or inorganic sources of recycled N.

In conclusion, nutrient-efficient crop systems have to integrate appropriate soil microbiota to contribute to an efficient use of the fertilizers. The information on the contribution of recovered products to crop microbial community in the soil need to be further studied (Paungfoo-Lonhienne 2012).

## 4.4 Bringing concepts together: P uptake efficiency of struvite as influenced by nitrogen source, phosphorus fertilizer and plant species

The last hypothesis stated that changes in soil pH induced by soil chemical properties or by the addition of other nutrients (such as ammonium versus nitrate) would affect struvite P availability differently compared to TSP.

### 4.4.1 Struvite availability is modulated by the nitrogen source applied

As a compilation of the main parameters analyzed in previous sections, the P uptake efficiency of struvite (PUE, P uptaked per unit root length) was calculated for each plant species. As hypothesized, lupine and maize plants had a significantly higher PUE from struvite when combined with ammonium than with nitrate (Fig. 28) despite a reduced total root length. Rhizosphere acidification can be produced by fertilizing with ammonium. Consequently, ammonium fertilization would enhance Struvite-P availability resulting in greater P uptake per unit root length. These results are in agreement with previous reports on maize that stated an increase in the P uptake efficiency when P was applied together with ammonium (Jing et al., 2010)

Although P uptake efficiency was higher in lupine grown with struvite and ammonium, the positive effect of applying ammonium together with struvite did not translate into higher biomass. This result is analogous to findings of Temperton et al. in relation to N uptake as a consequence of N facilitation in grassland species growing with legume neighbours (Temperton et al., 2007). Here a grass species (*Festuca pratensis*) managed to translate higher leaf N when growing near legumes into higher biomass, whereas a forb (*Plantago lanceolata*) did not. This highlights how species-specific the parameter-dependent effects can be.

In contrast to what observed with struvite, in other highly soluble P sources such as TSP, the PUE was not affected by the N source in the acidic conditions (Fig. 28). Enhanced availability of soil P due to root induced acidification in the rhizosphere for plants treated with ammonium was

previously reported (Gahoonia et al., 1992), however as discussed before, the acidification was not measured in all the cases. This could mean that besides the acidification other mechanisms might play a role, such as stimulating effects of ammonium on the formation of root hairs. In agreement with previous studies (Ma et al., 2013) we proposed that the enhanced P uptake by maize when banding P and ammonium could be explained by modifying root spatial distribution and not only acidification mechanism related to the N application can help to mobilize struvite-P.

The specific root length (SRL, root length / root dry biomass) indicates if plants are investing more in thin roots (higher SRL) than in thick roots (low SRL). At experiment 5B, lower root biomass, but contrastingly lower SRL was observed in lupine plants treated with ammonium with both struvite and TSP treatments. Likewise, plants treated with struvite nitrate had higher diameter than struvite applied with ammonium. In contrast, TSP had higher diameter when applied with ammonium (Fig. 26). Those roots modifications indicates with ammonium plants invested more in thin roots than when treated with nitrate, and that this might be an advantage for the uptake of P from struvite, however the application of ammonium with highly soluble TSP did not show any advantage in the TSP-P mobilization. As a practical application, the use of struvite together with ammonium is recommended to increase the physiological use efficiency of the recycled phosphorus. This might not be related to an increase in the biomass production in the first stage of the plants growth, however the increase in the use efficiency will equal the plant yields in a later state compared with mineral fertilizers, at the same time that will reduce the application of fertilizers and leaching.

### 4.4.2 Plant species did not differ in their uptake of struvite derived P per unit root length.

We had expected that lupine would have greater uptake per unit of root surface area than maize, as it is known to actively exude organic acids in relatively high amounts (Pang et al., 2010) that would mobilize the P from the struvite and therefore make it available for the plant. Indeed, in experiment 4 it was observed that uptake of lupine was slightly higher than maize, although not significantly higher. It was observed that the effectiveness of a particular plant species to uptake applied P would depend, to a large extent, on the pH where the plant was growing. This was observed with maize being able to be more efficient at alkaline pH than lupine, but lupine being able to increase root exudation at neutral pH to mobilize struvite.

### 4.4.3 P uptake per unit root length can be higher in alkaline soils

Throughout this thesis, the influence of pH and soil chemical composition on the effectiveness of struvite as a fertilizer was analyzed. Several rooting media were employed (poor sand, organic substrates, and field soils), with pH ranging from acidic to alkaline, and various plant species with

different nutrient mobilization strategies. Lupine plants, which are nitrogen fixers, are more adapted to acidic pH and with the ability to exudate organic acids showed significant differences with maize plants, which are less sensitive to pH and grow more quickly. It was observed that in alkaline conditions, lupine plants had a high exudation of carboxylates per unit or root length, increasing the amount of P uptake. This was not observed in the biomass as the growth of the root and shoot was totally inhibited by the high pH.

# 5. Conclusions

## 5.1 The effectiveness of struvite, a slow-release phosphorus fertilizer, is modulated by the nitrogen source applied

The shoot and root responses to struvite fertilization were investigated under different conditions for different plant species. In general, struvite has the same P fertilizer efficiency as mineral sources regarding biomass production, P uptake efficiency, and allometric studies of root–shoot relations.

Analysis with the automatic Screenhouse phenotyping method enables investigation of plant response to struvite slow nutrient release at different growth stages. Compared to triple super phosphate (TSP), struvite-fertilized plants had lower leaf area initially, but later similar (for lupine) or greater (for maize) biomass. This is the first time that the slow-release properties of struvite have been analyzed with respect to the growth of individual plants.

The use of slow-release P fertilizers like struvite can contribute to having a more sustainable P fertilization by i) reducing soil P immobilization processes (Talboys et al., 2016), ii) improving fertilizer PUE, and iii) lessening the risk of fertilizer P loss (Hart et al. 2004; Withers et al. 2014). The slower rate of P release from struvite than from highly soluble fertilizers may improve the efficiency of plant root system P uptake (Sutton et al. 1983; Massey et al. 2009), as demonstrated for maize plants, which presented higher biomass production.

Struvite needs to be applied with other nutrients to totally fulfill a crop´s nutrient demand. This dissertation shows that the plant phosphorus uptake efficiency of struvite-derived phosphate will be higher when applied together with ammonium than with nitrate. This was not observed for other highly soluble P sources like TSP, where the plant physiological use efficiency was not affected by the N source.

The higher phosphorus uptake efficiency observed with the ammonium can be explained by the well-known acidification induced by the ionic balance (Hinsinger 1998), but also by the

modifications that occurred in the root morphology. Nitrate increased the root biomass due to a greater number of thick primary roots, while ammonium application was associated with a decrease in root diameter, a well-known strategy to enhance P acquisition (Lambers et al., 2006), that might indicate an increase in the number of root hairs. Therefore, the application of struvite together with ammonium is recommended to increase the phosphorus use efficiency of the recycled phosphorus.

5.2 Struvite's effectiveness is enhanced for crop species that exude organic acids

Each plant showed specific strategies to adapt to stress conditions like high pH or low P. Maize plants are less sensitive to high pH; however, lupine plants are more adapted to acidic conditions. Maize responds to low P by altering root morphology, rather than increasing root exudation (Wen et al., 2017), contrasting with lupine that will exudate high amount of carboxylates (Pang et al., 2010).

Lupine was able to release a higher amount of organic acids at neutral conditions when struvite was applied, in contrast to what was observed when the readily available $K_2PO_4$ was used. Large quantities of malate and citrate were measured in the rhizosphere of lupine reaching concentrations in the low mM range. This validates the hypothesis that lupine plants are able to increase struvite P solubilization actively. This did not happen at all tested pH conditions. Low carboxylate exudation with struvite was observed at acidic and alkaline conditions. Interestingly, even though total exudate concentration was low in the alkaline state, where lupine root growth was profoundly affected, the amount of carboxylates per unit of root dry weight was similar in alkaline as in neutral conditions, showing that this might be one of the first lupine strategies to mobilize nutrients.

A slow-release fertilizer that actively responds to the presence of a crop root system with specific strategies to mobilize P has the potential to be a more spatially precise and efficient method of fertilizing plants with P than the application of conventional highly soluble P fertilizers.

5.3 Struvite applied alone as N fertilizer was not efficient, however mixing it with other recovered sources can have positive results on plant performance

The efficiency of struvite was demonstrated as a P fertilizer. However, ammonium recovery from struvite was lower than from organic fertilizer based on amino acids, and therefore produced less biomass. Low N:P ratio from struvite makes it not suitable as an N fertilizer alone, as to fulfill the N demands of the majority of crops struvite needs to be applied at concentrations that deliver higher to toxic levels of phosphorus.

Mixtures of struvite and readily soluble fertilizers have the potential to be beneficial at different levels. Struvite, as shown before, is a slow-release fertilizer, therefore applying it with readily soluble P fertilizers could increase the early P uptake levels that may be necessary for some species with low P reserves in the seeds. Struvite might also increase the pH of the soil, as shown in some experiments. Therefore, another advantage might be the chemical equilibrium in the soil when it is applied as a blend with other nutrients.

### 5.4 Summarizing the effect of pH

Our study confirmed the expected results that the initial solubility of struvite increases by a reduction in pH (Bhuiyan et al. 2007; Massey et al. 2009) due to physical properties of struvite. Achat et al. (2014) found that soil pH, besides affecting struvite solubility, did not influence the effectiveness of struvite. The results of this dissertation contradict Achat et al., results, since it showed that struvite effectiveness, besides being modulated by the nitrogen source applied, also varied across different soil types depending on soil pH and buffering capacity.

For plants that are adapted to grow in neutral to acidic conditions like *L. angustifolius*, no pH implications can be concluded regarding the agronomic value of struvite as there were no differences between KP and struvite at any pH tested. This does not mean that the effectiveness of struvite did not change, but it did in the same way than other P sources such as KP. In fact, lower applications of struvite might be recommended in acidic conditions due to its high solubility, since the P concentration could reach toxic levels.

The increases of lupine SRL observed in acidic conditions, as well as the decrease in root diameter, might be adaptation responses to low pH as showed for similar species by Hill et al. (2006); Increase SRL in acidic conditions could be developed as a strategy to uptake more nutrients reducing the length and thickening of the primary root, and increasing the length of lateral roots (Hammond et al., 2004).

It was also observed that plant species are influenced by pH and P source at different percentages. In this thesis, it was shown that maize is less sensitive to higher alkalinity than lupine species, and therefore the effect of P application at high pH will be only observed in maize.

PH monitoring in the rhizosphere and the bulk zone via planar optodes showed that the investigated tomato plants with the organic fertilizer increased tomato rhizosphere pH, while no changes were observed with the struvite. The applied fertilizer will affect the pH not only by its chemical properties but also by resulting differences in nutrient turnover. These new techniques, which allow on-time visualization of rhizosphere pH changes, combined with other root

phenotyping techniques such as MRI and spectral analysis, might help to unravel rhizosphere processes that have been difficult to analyze so far.

### 5.5 Plants rather than fertilizer modifies microbial community in the rhizosphere

Plant species, rather than fertilizer, alter the microbial community in the rhizosphere. It was observed that bacterial abundances in the rhizosphere were significantly different between species regardless of fertilizer supplementation, especially during the early development of the plant. Moreover, the microbial community becomes more specific over time; this can be concluded by the decreased bacterial relative abundances measured in the rhizosphere. Surprisingly, the microbial community in what is considered bulk soil was also influenced by plant species, calling into question the definition of rhizosphere. The specific community in each species, might explain why tomato plants were able to mineralize higher amounts of ammonium from organic fertilizer compared to struvite that was more available for lupine plants.

### 5.6 Outlook

The elucidation of plant-rhizosphere-soil interactions is not a trivial task, and it is necessary for understanding and improving fertilizer efficiency. During this dissertation, it became apparent that the use of the recovered product struvite needs to be accompanied by specific practices to increase plant use efficiency and therefore yields. Thus, the environmental impact of untreated waste application in the fields and the mineral nutrient overuse can also be reduced. The target plants were economically relevant crops such as maize or lupine. This point is important, as the aim of the research is not only to improve the yield in low-input systems but to promote the recycling of recovered products to a great extent.

The results obtained in this dissertation can foster the implementation of recovery technologies in the waste treatment industry. It was shown that the products recovered can substitute the use of mineral fertilizers and therefore have a commercial value. The price of those recovered products needs to be similar to mineral fertilizers on the market, and this should be promoted by governmental regulations that must take into account waste reduction.

This study’s results could also be of significant assistance for farmers. It was shown that it is possible to reduce the use of P fertilizer application, improving fertilizer use efficiency without losing plant yield. As an example, application of ammonium in combination with struvite, as well as the use of high exudative plants in neutral-alkaline soils, is recommended following the results obtained in this study.

6. List of tables

mean (n=5). Tpt 0= sowing day, tpt1 = time point 1 (harvest 1) and tpt 2= time point 2 (harvest 2). NA = not available. Different letters mean significant differences.

**Table 15 Influence of P fertilizer and N source applied on shoot P content, P concentration and P recovery of maize and lupine plants growing in acidic sand for experiment 5A and 5B.** P recovery: (percentage of the P applied that is recovered by the plant)**.** Struvite was compared with no P application and with half dose of P applied as TSP. Values are mean (n=10). Different letters mean significant differences $p<0.005$.

**Table 16 Shoot and Root P concentration of lupine plants at experiment 9 growing at three different sand pH**. Values are mean (n=5). Different letter means significant differences ($p<0.05$).

**Table 17 Struvite and KP P-recovery calculated in lupine plants at experiment 9 growing at three different sand pH**. P recovery: mg P plant$^{-1}$*100/P applied. Values are mean (n=5).±, SE, Different letter means significant differences ($p<0.05$).

**Table 18 Influence of fertilizer type (no fertilizer - NoFert, organic fertilizer – ORG and struvite-STR) on the nutrient dynamics and pH in non-sterile organic growing media with plants (Lupine and tomato) and without plants (No plant, as control) in function of time**. Values are mean (n=5) ± SEM ($P<0.05$).

**Table 19 Analyses of nitrogen balance.** Total N uptake shows the mg of N measured in total within the two plants per rhizotron. Total N shows the concentration of N (total N, ammonium and nitrate) in the substrate. N recovery indicates the % N recovered by the plant in relation to the N applied and % of N substrate stand for the amount of N applied that is measured at each harvest in the soil (total, ammonium and nitrate). Total % measured indicates the N applied that is measured combining the total N in the soil and the plant tissue for each replicate. Each number is the mean value of n=5

**Table 20 Concentration of different nutrients (mg L$^{-1}$) in the numbered blends and it effects in the pH and Electrical conductivity (EC).** Values are mean (n=5).

**Table 21 pH at harvest point of acidic and alkaline sand measured at experiment 4 as affected by N and P sources applied and plant species**. Values are mean (n=5).

**Table 22 pH at harvest point of acidic sand measured at experiment 5A as affected by N and P sources applied and plant species.** Values are mean (n=5). Different letters mean significant differences.

**Table 23: Root morphological traits (total root length, root surface area, average root diameter and specific root length) of lupine and maize treated with struvite and affected by the N source applied ($NH_4^+$ and $NO_3^-$) compared with the no P application (control) in lupine and maize in Experiment 5A, and with TSP in lupine in Experiment 5B.** Values are mean (n=10/5) ±SEM. Different letters indicate significant differences.

**Table 24 Total root length, average root diameter and root surface area of lupine plants growing in sand at modified pH values in experiment 9.** Values are mean (n=5). Different letters mean significant differences ($p<0.001$).

**Table 25 Average DCL, (root length within a diameter class) and Relative diameter class length (rDCL) = DCL/ root length (%).**Values are mean (n=5). Different letters mean significant differences ($p<0.001$). At acidic pH thin roots (<1mm) account almost 80% of total root length, compared with alkaline (57%) and neutral (71%). In alkaline the percentage of thick roots (>2) increased until almost 9%, significant higher than for acidic (2.6%) or neutral (3.5%).

**Table 26 Root mass ratio (root dry mass/total dry mass) of lupine at different sand pH as affected by the P source applied.** Values are mean (n=5). Different letters mean significant differences ($p<0.001$).

7. List of figures

optodes (blue arrow) were located, indicating the sampling area. Black arrow shows the vials in which samples were collected.

**Fig. 11 Tomato plants growing under increasing nutrient doses.** From left to right 0-25-50-75-100-200% modified Hoagland solution.

**Fig. 12 Dose response curve of tomato and maize plants.** Biomass (g dry weight) measured 28 DAS for the different nutrient dose applications (% of modified Hoagland solution). Values represent the mean of five biological replicates and error bars represent the standard error of the mean.

**Fig. 13 Germination rate of lupine and maize seeds (n=70 seeds/ species).** Germination rate (%) counted 3 days after sowing (3) in all the treatments, showing a delay in the germination when struvite is applied and 6 days after sowing (6) with no significant differences in the final germination rate.

**Fig. 14 (A) Representative sample of roots of lupine plants 24 days after sowing under different treatments.** From left to right, Acidic pH with Struvite + N, Acidic pH Struvite with no N, Alkaline pH Struvite + N, Alkaline pH Struvite with no N. **(B) Score of crown nodule.**

**Fig. 15 Concentration of struvite-P speciation forms at different fixed simulated pH values using MinTeQ.** Concentrations are obtained with a simulation of 1.5mM struvite added to a pH buffered water solution. At pH> 7 not all struvite dissolved, this effect will be stronger at higher struvite doses.

**Fig. 16 Concentration of phosphate (µg ml $^{-1}$) in the leachate after flushing 10g alkaline sand containing 60 mg kg$^{-1}$ P in struvite form with 50 ml of different citrate concentrations (10, 1, 0.1 mM and water (0mM)).** Higher amount of phosphate in the lecheate is measured after flushing with 10mM citrate. Concentration is analyzed using a Two-Way ANOVA and Tukey, $P<0.05$. Mean ± SE, n=4. Different letters mean to significant differences ($p<0.05$).

**Fig. 17 Plant biomass of lupine and maize (g plant $^{-1}$) under the different P (struvite and TSP) and N sources ($NH_4$ and $NO_3$) at experiment 5B.** Biomass is analyzed by a Three-Way ANOVA, $P<0.05$. Mean ± SE, n=5. Different letters mean significant differences.

**Fig. 18 Plant biomass of lupine (g plant $^{-1}$) under the different P sources: struvite, potassium phosphate (KP) and Control with no P(C) applied at different adpated sand pH (acidic 4.5, neutral 6.5 and alkaline 7.8).** Biomass is analyzed by a Two-Way ANOVA. Mean ± SE, n=5.

**Fig. 19 (A) Effect of pH on lupine growth under struvite (S) treatment in the acidic (-), neutral (±), and alkaline (+) sand. (B) Effect of P source on lupine growth in neutral pH. No P (C),**

**Struvite (S), and Potassium phosphate (KP).** Plants shown in the picture are a significant representation of the phenotype observed in each treatment.

**Fig. 20 Association between images based projected leaf area from ScreenHouse (pixels) and destructively measured leaf area after harvesting in a root scanner ($cm^2$).** Points are individual measurements from images of the experiment 5A, with three different levels of fertilization. The leaf area was measured three times per week with a total of 17 measurements days.

**Fig. 21 Projected leaf area (pixels) of lupine and maize treated with struvite (blue) or TSP (black), calculated every measurement day (MD from 0 to 17).** Struvite treated plants had higher leaf area than TSP at the end of the experiment, being significantly for maize plants (see MD17). The graph shows the typical growth curve for higher plants with an initial slow growth (Lag phase), until MD 7 approximately, then a rapid period of growth (exponential phase) where maximum growth is seen and the last phase where growth will be slow. The plants did not reach a steady phase. Points are average n=10.

**Fig. 22 Tomato plants growing in rhizotrons at time point 2 (35 Days after sowing).** Left to right: Tomato treated with no nitrogen, tomato treated with organic fertilizer and tomato treated with struvite. 2 plants per rhizotron.

**Fig. 23 Average biomass (g dry weight plant $^{-1}$) (n=7) of viola plants for each fertilizer blend**. Numbers refer to each specific blend that are a combination of different recovered nutrients applied at the same final dose. Blend 1 is the positive control, blend 14 is osmocote, a commercial slow release fertilizer, and NF:no fertilizer.

**Fig. 24 P concentration (% from total dry weight) in leaf and stem of lupine and maize plants as affected by the fertilizer added (P and N sources)**. Lupine accumulates higher amounts of P in the leaves, however maize plants showed no differences in P allocation between leaf and stem. Bars mean ± SE n=5.

**Fig. 25 Nutrients concentration (% dry weight) in the shoot of tomato (up) and lupine (down) at final harvest as affected by the different fertilizers (No Fert=no nitrogen fertilization, organic and struvite).** Bars mean ± SE n=5

**Fig. 26 Average root length (cm) within specific diameter ranges (mm) of lupine plants treated with struvite or TSP applied with either ammonium of nitrate as N source in experiment 5B**. Bars are mean ± SE, n=5. Lupine treated with struvite had higher root length when treated with nitrate within all the root diameters, contrary than lupine treated with TSP that had a higher root length with the ammonium treatment.

**Fig. 27 Allometric analyses of shoot:root biomass distribution (log transformed shoot:root distribution of the dry weight) as affected by the P source applied (No P, Potassium phosphate and struvite).** Plants were grown in neutral pH. Slope of control treatment different from the P treatments indicated lupine plants investing more in root than in shoot.

**Fig. 28 Phosphorus uptake efficiency (µg P applied as struvite recovered per cm root) in lupine and maize plants in experiment 5A (A), and phosphorus uptake efficiency (µg P applied as struvite or TSP recovered per cm root) in lupine plants in experiment 5B (B) as affected by the N sources applied (ammonium or nitrate).** The positive effect of ammonium applied together with the struvite in the efficiency of the P uptake, as observed in Experiment A and B in both species, is not observed with the TSP treatment in Experiment B. Bars represent mean ± SE n=10 for Experiment A and n=5 for Experiment B.

**Fig. 29 Total root length (cm) of lupine and tomato growing in rhizotrons filled with organic substrate as affected by fertilizer applied (no fertilizer, organic or struvite). Non-invasive measurements were done at different time points indicated in the X-axe as days after transplanting.** N=7 +SE. For lupine plants the highest root length was measured when no nitrogen was applied. For tomato plants, the organic fertilizer, followed by the struvite had significantly higher root length than the no nitrogen application. The differences are not observable until 22 days after transplanting for lupine (final harvest), however for tomato the differences are already visible 14 days after transplanting.

**Fig. 30 Citric acid concentration in rhizosphere of lupine at different pH conditions as affected by the P source applied.** Bars represent mean ± SE n=5. Lupine plants treated with struvite increase the exudation of citric acid at neutral pH, condition where the struvite is less available, in comparison with the KP treatment. This is not observed in the acidic.

**Fig. 31 Carboxylates concentration per unit of root dry weight in rhizosphere of lupine at different pH conditions as affected by the P source applied (struvite, potassium phosphate as KP and Control, with no P application.** Bars represent mean ± SE n=5.

**Fig. 32 (A) from left to right and top to bottom sequence of pH change in the rhizosphere of the tomato plants measured with the upper pH optodes under the organic fertilizer treatment.** Green circles shown measurements for the “bulk soil”, and yellow circles shown measurements for rhizosphere **(B) Evolution of the pH value in the rhizosphere and bulk zone** obtained extrapolating the image value in the calibration curve**.**

**Fig. 33 Microbial community shifts of the bulk growing media and the rhizosphere analyzed together. Dimension 2 describes the microbial community of tomato at tpt 2 and dimension**

**3 describes the microbial community of lupine at tpt 2.** The microbial community from tomato and lupine are significantly different, as well as the community at Tpt 1 and Tpt 2. There are no significant differences between fertilizers. Bulk and rhizosphere appear not different as they are analyzed together.

**Fig. 34 Microbial community shifts of rhizosphere in growing media harboring two different plants, supplemented with fertilizer over time.** The second dimension describes the growing medium harboring tomato plants, while the fourth dimension describes the relative abundances of the bacteria associated with the growing medium harboring lupine**.**

**Fig. 35 Microbial community shifts of pre-treated bulk zone harbouring lupine and tomato plants, supplemented with fertilizer and followed over time. Multiple Factor Analysis revealed variations in the relative bacterial abundances and ellipses show confidence Intervals (CI) of 95% for each sample type.** The first dimension of the MFA describes the growing medium supplemented with struvite and harboring lupine and the third dimension describes the growing medium supplemented with organic fertilizer and harboring tomato plants.

8. Publications and conference contributions

Research paper

**Ana A Robles Aguilar, Jiayin Pang, Johannes J. Postma, Silvia D. Schrey, Hans Lambers and Nicolai D. Jablonowski** (2018). The effects of pH on root morphology and physiology of narrow-leaf lupin, grown with a recycled phosphorus source. Plant and Soil

**Ana A. Robles Aguilar, Silvia Schrey, Johannes Postma, Vicky M. Temperton and Nicolai David Jablonowski** (2018). Plant uptake of slowly released phosphorus from struvite is modulated by the nitrogen form applied. Submitted in Frontiers in Plant Sciences.

Proceedings articles

**Ana A. Robles Aguilar, Thomas Bodewein, Silvia Schrey, Johannes Postma, Stephan Blossfeld, Vicky M. Temperton and Nicolai David Jablonowski**. (2016) Effectiveness of Recycled Phosphorus as Struvite is Modulated by the Nitrogen Source Applied WEF/IWA Nutrient Removal and Recovery Conference. Denver, Colorado.

**Ana A Robles Aguilar, Jiayin Pang, Johannes J. Postma, Silvia D. Schrey, Hans Lambers and Nicolai D. Jablonowski** (2017). The effects of pH on root morphology and physiology of narrow-leaf lupin, grown with a recycled phosphorus source. Proceedings Book of the XVIII International Plant Nutrition Colloquium with Boron and Manganese Satellite Meetings (2017). University of Copenhagen, Denmark. ISBN 978-87-99 6274-0-0

Oral presentations

**Ana A. Robles Aguilar, Thomas Bodewein, Silvia Schrey, Johannes Postma, Stephan Blossfeld, Vicky M. Temperton and Nicolai David Jablonowski**. (2016) Effectiveness of Recycled Phosphorus as Struvite is Modulated by the Nitrogen Source Applied WEF/IWA Nutrient Removal and Recovery Conference. Denver, Colorado**.**

**Oliver Grunert, Ana Alejandra Robles Aguilar, Emma Hernandez-Sanabria, Tom Vandekerckhove, Dirk Reheul, Marie- Christine Van Labeke, Siegfried Vlaeminck, Nico Boon, Nicolai David Jablonowski** (2016) Struvite and Organic Fertilizer Impacting the Rhizosphere Microbial Community, Nutrient Turnover and Plant Growth Performance. WEF/IWA Nutrient Removal and Recovery Conference. Denver, Colorado.

**Robles Aguilar, A.A; Temperton, V.M.; Blossfeld, S.; Jablonowski, N.D.** (2015). A more efficient and sustainable fertilization through recycling manure-derived phosphorus. 2° International Conference on manure management and valorization: ManuResource Gent, Belgium

**Ana A Robles Aguilar, Jiayin Pang, Johannes J. Postma, Silvia D. Schrey , Hans Lambers and Nicolai D. Jablonowski** (2017) The effects of pH on root morphological and physiological adaptations of narrow leaf lupine, under recycled P source. International Plant Nutrition Colloquium, Copenhagen, Denmark.

Posters

**Ana Alejandra Robles Aguilar, Oliver Grunert, Silvia Diane Schrey, Johannes Postma, , Vicky Temperton, Stephan Blossfeld, Emma Hernandez-Sanabria, Nico Boon, Nicolai David Jablonowski.** (2016) Response of tomato and narrow-leaved lupine root system architecture and rhizosphere dynamics to nitrogen source EcoSummit Ecological Sustainability: Engineering Change

**Ana A. Robles Aguilar, Oliver Grunert, Silvia Schrey, Johannes Postma, Vicky Temperton, Stephan Blossfeld, Nico Boon and Nicolai D. Jablonowski**. (2016) Variation of root architecture and rhizosphere bacterial community in response to recycled nitrogen DGP international conference, Stuttgart-Hohenheim, Germany

**Ana A Robles Aguilar, Bodewein, T. Johannes J. Postma, Silvia D. Schrey and Nicolai D. Jablonowski (2016)** A more efficient and sustainable fertilization through recycling phosphorus as struvite. International Phosphorus workshop. Awarded as best poster presentation.

**Ana A. Robles Aguilar, Vicky M. Temperton, Stephan Blossfeld, Nicolai David Jablonowski**. (2015) Effect of nitrogen form, pH and plant species in the mobilization and acquisition of P from a recycled phosphorus fertilizer. Rhizosphere 4, 21 – 25 June 2015, Maastricht – Netherlands.

**Ana Alejandra Robles Aguilar, Oliver Grunert, Nico Boon, Vicky Temperton, Stephan Blossfeld, Emma Hernandez-Sanabria, Dirk Reheul, Nicolai Jablonowski** (2015). Microbial community structure in the rhizosphere of narrow-leafed lupine and tomato as related to nitrogen form provided. Rhizosphere 4, 21 – 25 June 2015, Maastricht – Netherlands.

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10. Attachment

Table S1 supplementary: Table 1 shows all the data from the ANOVA including factors: Soil pH, Part of the plant, P and N source and the interactions

| Plant part | Soil pH | Nitrogen | Phosphorus source | | | ANOVA | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P0 | Struvite | TSP | N | P | Soil | Pxsoil | PXN | SoilXN | PXNXSoil |
| Lupine | | | | | | | | | | | | |
| Shoot | acidic | $NH4^+$ | 0.35 | 0.63 | 0.55 | 0.056 | 6.20E-05 | 8.34E-01 | 0.002422 | 0.542 | 0.256 | 0.467 |
| | | $NO3^-$ | 0.35 | 0.71 | 0.57 | | *** | *** | ** | | | |
| | alkaline | $NH4^+$ | 0.19 | 0.20 | 0.24 | | | | | | | |
| | | $NO3^-$ | 0.23 | 0.27 | 0.46 | | | | | | | |
| Root | Acidic | $NH4^+$ | 0.24 | 0.52 | 0.24 | 0.987 | 0.001701 | 5.52E-09 | 9.78E-05 | 0.089 | 0.368 | 0.724 |
| | | $NO3^-$ | 0.19 | 0.42 | 0.31 | | ** | *** | *** | | | |
| | Alkaline | $NH4^+$ | 0.06 | 0.09 | 0.15 | | | | | | | |
| | | $NO3^-$ | 0.08 | 0.09 | 0.24 | | | | | | | |
| Maize | | | | | | | | | | | | |
| shoot | acidic | $NH4^+$ | 0.54 | 2.57 | 1.13 | 0.856 | 3.21E-12 | 1.12E-09 | 9.84E-08 | 0.960 | 0.210 | 0.150 |
| | | $NO3^-$ | 0.67 | 2.21 | 1.00 | | *** | *** | *** | | | |
| | alkaline | $NH4^+$ | 0.66 | 0.77 | 0.50 | | | | | | | |
| | | $NO3^-$ | 0.56 | 1.05 | 0.59 | | | | | | | |
| Root | Acidic | $NH4^+$ | 0.31 | 1.06 | 0.61 | 0.835 | 0.000583 | 2.21E-05 | 2.72E-05 | 0.081 | 0.019 | 0.019 |
| | | $NO3^-$ | 0.32 | 0.66 | 0.65 | | *** | *** | *** | | | |
| | alkaline | $NH4^+$ | 0.37 | 0.30 | 0.27 | | | | | | | |
| | | $NO3^-$ | 0.44 | 0.40 | 0.44 | | | | | | | |

Table S2 supplementary: Table 1 shows all the data from the ANOVA including factors: Soil pH, Part of the plant, P and N source and the interactions

| ***maize*** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Part plant | Factor | ANOVA Pr (>F) | | | | | | |
| | | Ca | Mg | P | Al | K | C | N |
| Shoot | P | ns | 2.945e-05 *** | 1.565e-06 *** | | 7.066e-05 *** | 2.677e-09 *** | 0.0014360 ** |
| | N | ns | 2.175e-06 *** | 0.004388 ** | 0.0009551 *** | ns | na | 0.0001257 *** |
| | Soil | ns | 6.010e-13 *** | ns | 7.676e-05 *** | ns | na | 0.0001964 *** |
| | PXN | ns | ns | 0.014405 * | ns | ns | na | |
| | PXSoil | ns | 3.610e-07 *** | ns | ns | 0.008434 ** | 1.810e-06 *** | 2.271e-05 *** |
| | NXSoil | ns | 7.756e-07 *** | ns | ns | ns | na | 1.470e-05 *** |
| | PXNXSoil | ns | 0.0119 * | ns | ns | ns | na | |
| | | | | | | ns | na | |
| Root | P | 0.0003041 *** | 0.02034 * | 1.704e-08 *** | ns | 0.0003133 *** | 0.0004651 *** | 0.007632 ** |
| | N | ns | 0.00155 ** | 0.0022296 ** | ns | ns | ns | 0.005987 ** |
| | Soil | < 2.2e-16 *** | 1.683e-10 *** | ns | 2.779e-09 *** | ns | ns | ns |
| | PXN | 0.569701 | 0.03789 * | ns | ns | ns | ns | ns |
| | PXSoil | 9.627e-05 *** | 2.281e-06 *** | 0.0063059 ** | ns | ns | ns | ns |
| | NXSoil | ns | 0.02255 * | 0.0003737 *** | ns | ns | ns | ns |
| | PXNXSoil | ns | ns | ns | ns | ns | ns | ns |
| ***Lupine*** | | ns | ns | ns | ns | ns | ns | ns |
| Shoot | P | ns | ns | 1.565e-06 *** | ns | ns | ns | ns |
| | N | ns | ns | 0.004388 ** | ns | ns | ns | ns |
| | Soil | 1.577e-07 *** | 6.065e-08 *** | ns | ns | 0.0009679 *** | 4.132e-10 *** | ns |
| | PXN | 0.0007813 *** | 0.009275 ** | ns | ns | ns | ns | ns |
| | PXSoil | ns | ns | ns | ns | ns | ns | ns |
| | NXSoil | ns | 0.008463 ** | ns | ns | ns | ns | ns |
| | PXNXSoil | ns | ns | ns | ns | ns | ns | ns |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | P | 0.0004767 *** | 9.094e-07 *** | 0.007951 ** | ns | | ns | ns |
| | N | 9.529e-05 *** | 4.794e-12 *** | ns | 0.0001917 *** | 4.677e-05 *** | ns | 0.005816 ** |
| | Soil | < 2.2e-16 *** | ns | ns | 1.095e-11 *** | | 0.0006423 *** | 0.006976 ** |
| Root | PXN | 0.0052600 ** | ns | ns | ns | | ns | ns |
| | PXSoil | 1.072e-11 *** | 0.0090205 ** | ns | ns | | 0.0001101 *** | 0.001928 ** |
| | NXSoil | ns | 0.0004517 *** | ns | ns | | ns | ns |
| | PXNXSoil | ns | ns | ns | ns | | ns | ns |

Table S3 supplementary: Post-Hoc analyses of nutrient content in the plant at experiment 4

***maize***

| Part plant | Mean value, Tukey (alpha = 0.05) | | | | |
|---|---|---|---|---|---|
| | Factor | Mg | P | N | K |
| Shoot | Struvite | 0.47b | 0.075a | 1.92b | 2.69b |
| | TSP | 0.59a | 0.07b | 2.3a | 3.2b |
| | NoP | 0.54a | 0.063c | 2.314a | 3.77a |
| | Ammonium | 2.37a | 0.074a | 2.37a | ns |
| | nitrate | 1.98b | 0.067b | 1.98b | ns |
| | acidic | 0.44b | ns | 2.35a | ns |
| | alkaline | 0.63a | ns | 1.98b | ns |
| root | Struvite | 0.28ab | 0.0484a | 0.95a | 0.52a |
| | TSP | 0.287a | 0.032b | 0.80b | 0.34b |
| | NoP | 0.22b | 0.024c | 0.77b | 0.32b |
| | Ammonium | 0.29a | 0.040a | 0.91a | ns |
| | nitrate | 0.23b | 0.031b | 0.78b | ns |
| | acidic | 0.18b | ns | ns | ns |
| | alkaline | 0.34a | ns | ns | ns |

***lupine***

| Part plant | Mean value, Tukey (alpha = 0.05) | | | | |
|---|---|---|---|---|---|
| | Factor | Mg | P | N | K |
| Shoot | Struvite | 0.806a | ns | ns | ns |
| | TSP | 0.646b | ns | ns | ns |
| | NoP | 0.66ab | ns | ns | ns |
| | Ammonium | 0.64b | 0.13a | 2.37a | ns |
| | nitrate | 0.77a | 0.12b | 1.98b | ns |
| | acidic | 0.54b | ns | ns | 2.11b |
| | alkaline | 0.88a | ns | ns | 2.57a |
| root | Struvite | 0.76b | 0.058ab | ns | ns |
| | TSP | 1.11a | 0.073a | ns | ns |
| | NoP | 0.53c | 0.043b | ns | ns |
| | Ammonium | 0.37a | ns | ns | 0.81a |
| | nitrate | 1.22a | ns | ns | 1.53b |
| | acidic | 0.75b | ns | 1.56b | ns |
| | alkaline | 1.014a | ns | 1.77a | ns |

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