Dynamics and biological interactions of phosphorus cycling in central Amazonian forests

Submitted by **Laynara Figueiredo LUGLI** to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Geography, December 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Soil nutrient availability is considered to constrain the productivity of terrestrial ecosystems, with phosphorus (P) considered to be the most limiting nutrient in tropical forests. Due the great importance of Amazon forests in carbon (C) cycling and the fact that the majority of Amazon forests grow in low-fertility soils, understanding how nutrient limitation may affect net primary productivity (NPP) in these ecosystems is crucial to predict C storage in response to future climate. The direct effects of nutrient limitation on above and belowground forest functioning can only be tested through experimentation and up to now, the few large scale fertilisation experiments installed in lowland tropical forests indicate that multiple nutrients may limit different aspects of tropical forests, with inconsistent evidence for P limitation. Since much less is known about the potential effects of nutrient limitation on belowground forest functioning, this research aimed to analyse the main belowground mechanisms involved in P cycling, and how roots and soil microorganisms adapt to different conditions of soil fertility in central Amazon forests. I investigated how root morphological traits, mycorrhizal colonisation as well as enzyme exudation both from roots and soil microbes were expressed in natural low-fertility soils and how these traits responded to the short-term addition of P, nitrogen (N) and cations, as part of the first large-scale soil nutrient manipulation experiment in a central Amazon lowland forest near Manaus, Brazil. I show that in natural low-fertility soils, roots display a range of adaptations to increase P-uptake efficiency and investments in root morphological and physiological adaptations as well as association with fungi symbionts are complementary towards maintaining forest productivity in a central Amazon forest. With nutrient addition, I found support for the hypothesis of P-limitation, since trees were able to rapidly adapt their root morphological traits, reduce investments in enzyme exudation and increase association with mycorrhizal fungi. Such responses were also affected by cation addition, reinforcing the idea that multiple nutrients may control the expression of root traits. The soil microbial community was also affected by the short-term addition of nutrients, with a reduction in enzyme production with the addition of phosphorus, indicating a rapid alleviation of phosphorus limitation, but this reduction was eliminated when cations were also added. My results suggest that plants and soil microorganisms can rapidly respond to changes in soil nutrient

availability by changing their investments in nutrient uptake mechanisms, ultimately impacting plant productivity. These responses are crucial if we are to better understand how these forests function and how they may respond to global change.

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Abbreviations

Al: Aluminium

AFEX: Amazon Fertilisation Experiment

AM: Arbuscular Mycorrhizas

ATP: Adenosine Triphosphate

BG: β-glucosidase enzyme

C: Carbon

CO_2: Carbon Dioxide

DGVM: Dynamic Global Vegetation Model

EucFACE: Eucalyptus Free Air CO_2 Enrichment

FACE: Free Air CO_2 Enrichment

Fe: Iron

GPP: Gross Primary Productivity

IMBALANCE-P: Fertilisation Experiment installed in French Guiana

NAG: N-acetyl β-glucosaminidase enzyme

N: Nitrogen

NPP: Net Primary Productivity

P: Phosphorus

PFT: Plant Functional Type

PHOS: Phosphomonoesterase enzyme

RTD: Root Tissue Density

SMA: Standard Major Axis analysis

SRA: Specific Root Area

SRL: Specific Root Length

Chapter 1 - General introduction

1. Introduction

1.1. Amazon forest

Tropical forests cover just about 10% of the Earth's surface but represent ~50% of the area covered by all forest biomes (Pan et al. 2011). Tropical forests also host over half of the world's known species, including more than 43,000 tree species (Terborgh 1992; Fine and Ree 2006). Among many ecosystem services, tropical forests are an important component of global biogeochemical cycles, since these ecosystems contribute substantially to the global carbon (C) cycling, accounting for about 70% of the global gross C sink in terrestrial ecosystems (2.4 ± 0.4 of 4.0 Pg C $year^{-1}$) (Field et al. 1998; Malhi and Grace 2000; Pan et al. 2011). For instance, tropical forests found in South America contain almost half of all tropical forest biomass (Pan et al. 2011; Saatchi et al. 2011) and the Amazon itself contributes to about 50% of the total C assimilated by tropical forests (Pan et al. 2011). Amazon forests also host about a quarter of all global terrestrial species (Field et al. 1998) and play a crucial role in controlling precipitation regimes in South America (Werth and Avissar 2002). Across the Amazon basin, forests are very diverse in terms of climate, species composition and soil physical and chemical properties (Fyllas et al. 2009; Quesada et al. 2012). Nonetheless, determining what controls tree growth and C assimilation in Amazon forests, and how they will respond to climate change has major implications for predicting future C and water cycles, affecting the lives of millions of people who directly or indirectly depend on the maintenance of this ecosystem (Malhi et al. 2004; Bonan 2008; Pan et al. 2011).

1.2. The role of phosphorus controlling Amazon forest productivity

Most of our understanding about Amazon forest dynamics comes from studies focusing on aboveground C stocks and fluxes such as photosynthesis, respiration and net primary productivity (NPP) (Malhi et al. 2004; Quesada et al. 2009). Much less is known about belowground stocks and the partitioning of total NPP between above and belowground compartments and the drivers controlling belowground C allocation in Amazon forests (Aragão et al. 2009). Soil development and nutrient availability are intrinsically connected and have strong effects on nutrient limitation in terrestrial

ecosystems. Because of the importance of nitrogen (N) and phosphorus (P) affecting a wide range of physiological and metabolic processes in plants, these two elements are usually considered the most limiting in terrestrial ecosystems, with N generally limiting temperate forests and P limiting tropical forests. This is due the different sources of N and P in soils, with N being derived mainly from atmospheric deposition and N_2 fixation by bacteria in soil, with N therefore accumulating with soil age. The main source of P, on the other hand, is the weathering of parent rock material, but the importance of this source tends to decrease with soil development, explaining why old tropical soils are usually low in P.

Across the Amazon basin, soils in central and eastern portions are highly weathered and therefore characterised by very low availability of rock-derived elements, with particularly low concentrations of P and exchangeable cations. Among different soil chemical properties, P availability appears as the most important factor controlling wood production rates in Amazon forests, with some evidence also pointing to K as playing an important role controlling wood density (Quesada et al. 2010; Quesada et al. 2012). Nonetheless, about 60% of Amazon forests grow in low-fertility soils, suggesting that the majority of lowland Amazon forests is likely to be P or cation-limited (Quesada et al. 2011). More specifically, P plays a key role in an array of plant processes, including intracellular energy transport (ATP), respiration, photosynthesis, N-fixation, nucleic acid synthesis and production of enzymes (Vance et al. 2003; Raghothama and Karthikeyan 2005; Lambers et al. 2006). Calcium is related to membrane stability, maintenance of cell integrity, cell division and elongation (Kirkby and Pilbeam 1984). Potassium is crucial for osmoregulation and stomata movement, while magnesium is a component of chlorophyll, directly impacting photosynthesis and protein synthesis (Maathuis 2009; Hawkesford et al. 2012).

Most of our knowledge about nutrient limitation in terrestrial ecosystems, however, comes from temperate forests that are usually N-limited and much less is known about carbon-nutrient interactions in highly weathered soils. Since any increase in tropical plant growth under atmospheric CO_2 enrichment is hypothesised to be ultimately controlled by the amount of available nutrients in soils (Kimball and Idso 1983; Hungate et al. 2003; Ainsworth and Long 2005; Norby et al. 2005), understanding how plants and microorganisms can acquire and use those nutrients is critical to predict the actual role of tropical forests in a future climate. Moreover, only by investigating

the direct nutrient controls on forest productivity will we be able to develop process-based models to better represent forest responses to environmental change (Goll et al. 2012).

1.2.1. Plant strategies to acquire phosphorus

In low-fertility soils, plants are expected to employ a wide range of above and belowground strategies to overcome potential nutrient limitation by combining an efficient uptake and translocation of nutrients to maintain their normal functioning (Raghothama and Karthikeyan 2005). Plants may be able to alter the expression of root traits, by investing in different root morphological adaptations, increase the secretion of exudates such as enzymes and carboxylates and alter their reliance on mycorrhizal fungi to optimise nutrient acquisition (Raghothama 1999; Richardson and Simpson 2011). Although such belowground plant nutrient uptake mechanisms directly affect tropical forest productivity (Bardgett et al. 2014), little is known about the actual mechanisms involved in nutrient cycling in Amazon forests and how the expression of these different belowground properties changes with soil nutrient availability.

1.2.2. Phosphorus limitation of soil microbes

Soil nutrient availability not only affects plants, but also the soil microbial community responsible for organic matter decomposition and nutrient transformations in soils, making soil microorganisms a fundamental compartment affecting ecosystem maintenance (Chen et al. 2013). Since soil microorganisms can be a sink or a source of nutrients in soils (Richardson 2001; Oberson and Joner 2005; Richardson and Simpson 2011), identifying the extent to which nutrients limit soil microbial community function is crucial if we are to understand plant-soil feedbacks in Amazon forests. This becomes particularly important under elevated atmospheric CO_2, since changes in plant biomass accumulation will depend on the balance between microbial nutrient immobilisation and mineralisation via organic matter decomposition (Blagodatskaya et al. 2010).

1.3. Nutrient manipulation experiments in the tropics

Nutrient limitation in terrestrial ecosystems refers to a constraint on primary production or other ecosystem processes by low rates of nutrient supply (Chapin et al. 1986; Vitousek and Farrington 1997). One of the most robust ways to directly quantify nutrient limitation in complex ecosystems is through experimentation (Vitousek and Farrington 1997). Up to now, however, nutrient manipulation experiments have not been able to detect direct evidence for P limitation in species-rich tropical forests, and generally point to multiple nutrients limiting different ecosystem processes (Mirmanto et al. 1999; Wright et al. 2011; Alvarez-Clare et al. 2013). The majority of large-scale fertilisation experiments in lowland tropical forests added nutrients for less than five years, which could also explain the absence or the wide range of responses captured by such experiments. A recent meta-analysis indicated that weaker tree growth responses after fertilisation were found in old growth tropical forests, whilst stronger responses were commonly found in secondary forests with faster tree growth (Wright et al. 2018). Accordingly, in the longest on-going fertilisation experiment in lowland forests in Panama, the addition of N and K, but not P, stimulated growth of small diameter trees only (Wright et al. 2011).

Nonetheless, plant tissues of faster turnover, such as leaves and roots were found to be affected by the addition of multiple nutrients in different fertilisation experiments. For instance, litterfall production and fine root biomass were among the processes commonly influenced by the addition of N, P and K in Borneo and Panama (Mirmanto et al. 1999; Wright et al. 2011; Sayer et al. 2012). Litterfall production was stimulated after four years of N and P addition in Borneo (Mirmanto et al. 1999), as well as in Panama after 11 years of P addition (Wright et al. 2011). Fine root biomass, however, increased after 4 years of P addition (Yavitt et al. 2011) but decreased after 11 years of K addition in Panamanian forests (Wright et al. 2011). Even after almost 15 years of nutrient addition in lowland forests in Panama, no clear evidence for P affecting the expression of root morphological traits were found, although it increased root length colonised by arbuscular mycorrhizal fungi (Wurzburger and Wright 2015). The majority of results from Panama, however, points to K as playing a critical role controlling plant functioning, similarly to the evidence that K also appears to affect tree traits in Amazon forests (Quesada et al. 2012). The strongest evidence for P limiting such forests in Panama comes from the microbial community, in which after 10 years of P addition,

investments in phosphatase enzymes were significantly reduced, pointing to an alleviation of P-limitation by the soil microbes (Turner and Wright 2014).

Currently, the nutrient manipulation experiment installed in Panama is the longest experiment in duration (almost 20 years) and despite the importance of results derived from this experiment, strong support for the P-paradigm in tropical forests is still lacking. Nonetheless, total soil P-levels in Panamanian forests are about 4 and 9 times greater than total P and the sum of bases found in the majority of soils across the Amazon basin (Quesada et al. 2010; Wright et al. 2010), emphasising the importance of conducting similar experiments in Amazon forests before any generalisation about P limitation could be made. Despite the assumption that the majority of tropical forests are P-limited, evidence for P limitation in Amazon forests can only now start to be directly tested with the establishment of large-scale nutrient manipulation experiments.

1.3.1. Nutrient manipulation in the Amazon

To address the importance of soil nutrient limitation on Amazon forests, a fertilisation experiment was recently installed in lowland forests in French Guiana (IMBALANCE-P) aiming to test the effects of N and P addition on a range of ecosystem processes along a topographical gradient in low-fertility soils. Also of great importance, the Amazon Fertilisation Experiment (AFEX) was installed in a lowland central Amazon forest near Manaus, Brazil, growing in low fertility soils representative of 60% of the basin (Quesada et al. 2011). AFEX aims to investigate the effects of N, P but also cations (Ca, Mg and K) on the productivity of one of the most important biomes on the planet, using a fully replicated, factorial design. By investigating the responses of all key components of the C cycle to nutrient manipulation, this experiment will enhance our understanding about the role of specific nutrients in controlling the functioning of Amazon forests growing on low-fertility soils. Accordingly, my research fits within the bigger scope of the AFEX project, by analysing how belowground resource allocation towards different P-uptake mechanisms is affected by soil nutrient availability in this central Amazon forest.

1.4. Aim of the research

The general goal of my research is to deliver an understanding of the key belowground processes involved in phosphorus cycling in central Amazon forests and how such processes change with nutrient addition.

1.5. Specific objectives of the research

1) To present an overview of the mechanisms related to phosphorus cycling in tropical ecosystems, with emphasis in Amazon soils;
2) To describe root strategies used by trees to overcome phosphorus limitation in natural low-fertility soils in central Amazon;
3) To determine if the addition of phosphorus, nitrogen and cations affects the nutrient acquisition mechanisms adopted by trees in a central Amazon forest;
4) To determine how nutrient addition affects soil microbial community function and especially investment in different nutrient mineralising enzymes.

1.6. Thesis structure

The research presented here is divided into six chapters (Figure 1.1.). This chapter, **Chapter 1,** presents the general context of phosphorus limitation in Amazon forests, including the motivation for this research and also the aims and specific objectives targeted here. **Chapter 2** presents an overview of current understanding regarding the mechanisms related to phosphorus cycling in tropical ecosystems, with a focus on Amazon ecosystems. In this chapter I address the consequences of soil development in the light of P availability in tropical soils, the main belowground strategies adopted by trees to overcome nutrient limitation, detailed results from nutrient manipulation experiments and CO_2 enrichment experiments and finally, how such mechanisms are currently represented in dynamic vegetation models (DGVMs), including identifying main gaps and recommendations for enhancing our understanding of P cycling under future climate scenarios.

Based on the scientific gaps that I identify in Chapter 2 regarding the role of belowground mechanisms in nutrient cycling in Amazon forests, **Chapters 3 to 5** present the main results from my empirical research which was carried out as part of the AFEX project. Each of these are written in manuscript format, i.e. including

abstract, introduction, materials and methods, results, discussion and conclusions. **Chapter 3** focuses on the mechanisms that plants adopt to overcome soil nutrient limitation in natural low-fertility soils in a central Amazon forest. My hypothesis is that under similar environmental conditions, a trade-off between root morphological traits and P-uptake strategies would occur, with different strategies being associated with roots of different morphologies. To test for possible trade-offs between alternative strategies, I analyse fine root traits related to P-acquisition: root morphology, enzyme exudation and mycorrhizal colonisation.

After describing the range of root adaptations used to increase P-uptake efficiency in natural low-fertility soils in central Amazon, **Chapter 4** aims to test for potential changes in root traits related to P-acquisition following nutrient addition and consequently capture evidence for alleviation or exacerbation of P-limitation in a central Amazon forest. My hypotheses are that P limitation should be manifested by a decrease in investment in root P-uptake strategies after P addition, also resulting in changes in root productivity, and morphology shifting from acquisitive to more conservative traits. I also hypothesise that the addition of other nutrients will exacerbate P limitation, resulting in increased investment in P-uptake mechanisms. To test those hypotheses, I sampled fine roots after seven months of nutrient addition, analysing for changes in root morphological traits, phosphatase enzyme activity and the degree of mycorrhizal colonisation. Results from this chapter discuss if and how plants in central Amazon are able to adapt their belowground investments in face of changes in nutrient availability.

Furthermore, since soil organic matter decomposition is a process mediated by microorganisms, in **Chapter 5** I aim to detect the responses of the soil microbial community to nutrient addition in a central Amazon forest, determining the nutrient status of this belowground compartment. I hypothesise that the soil microbial community will be mainly limited by P and that the addition of P will decrease the investment in enzyme production, whilst the added N will be allocated to overcome P limitation by increasing investments in enzyme exudation. I also hypothesise that the addition of cations will exacerbate P limitation resulting in greater investments in enzyme activity. I use soil samples that were collected six months after fertilisation commenced, analysing for the activity of enzymes related to carbon, nitrogen and phosphorus cycling, and based on absolute values as well as enzyme stoichiometric

ratios I infer the extent to which soil microbial activity is limited by the availability of different nutrients. With the results of this chapter I therefore discuss the impact of soil microorganisms affecting organic matter cycling in central Amazon forests and how this feedback could ultimately influence plant productivity.

Finally, **Chapter 6** presents the overarching conclusions and recommendations derived from this research.

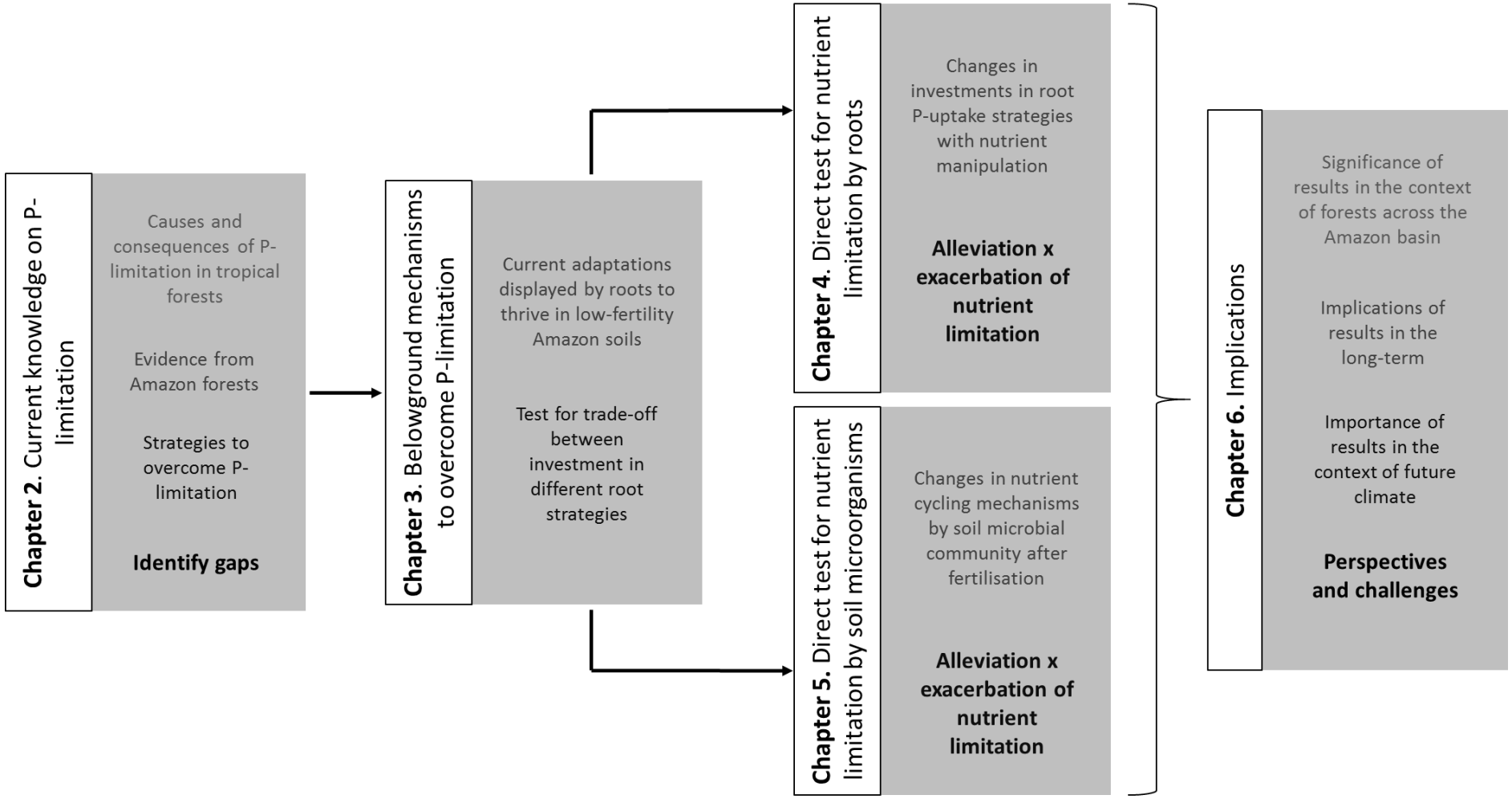

Figure 1.1. Conceptual diagram that summarises the specific aims of each chapter. Each box represents a chapter as indicated in the figure.

Chapter 2. Phosphorus cycling and limitation in Amazon forests: current knowledge and challenges for experiments and models

2. Phosphorus cycling and limitation in Amazon forests: current knowledge and challenges for experiments and models

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2.1. Abstract

Nitrogen (N) and phosphorus (P) are usually considered the most limiting elements to plant growth, with N limitation being more common in temperate forests, whilst P is usually found to limit tropical forests productivity. The predicted stimulation of tropical plant growth under increased atmospheric CO_2 could be ultimately controlled by the amount of available P in soil and how plants could efficiently acquire and use P. Moreover, understanding P cycling and limitation in tropical soils would greatly contribute to better predict biomass and forest production in dynamic vegetation models. Therefore, the aim of this review was to describe the mechanisms related to P limitation in tropical forests, with emphasis on Amazon forests growing on low-fertility soils. To achieve this goal, here I summarise what is currently known about tropical P limitation from empirical data, nutrient fertilisation experiments, CO_2 enrichment experiments and models, finally suggesting new measurements and approaches needed to improve understanding of Amazon forest functioning and nutrient cycling. Up to now, our knowledge about P-limitation in Amazon forests comes from indirect correlations between P concentrations in soils and aboveground plant traits and wood productivity. In addition, the few nutrient manipulation experiments installed in lowland tropical forests so far did not capture definite evidence for P limiting plant growth, although P addition was found to affect belowground plant compartments. The effects of increasing atmospheric CO_2 concentrations on forest functioning are, up to now, based on experiments that took place in temperate or less complex ecosystems, usually N- rather than P-limited. The installation of nutrient and CO_2 fertilisation experiments in Amazon forests are

critical to enhance our mechanistic understanding about how Amazon forests thrive in such highly weathered soils. Greater attention should be given to the plasticity of plants and soil microorganisms in scenarios of changing climate and soil nutrient availability, with studies focusing on enzyme and carboxylate exudation, root morphological adaptation as well as association with mycorrhizas. Combining our current knowledge with new information derived from a range of initiatives applied to different levels (species-specific to ecosystems) and different time scales (short- and long-term experiments) will directly help us elucidate the role of P limitation controlling tropical forests functioning and how it will consequently affect C storage by these forests under future climate.

2.2. Introduction

Tropical rain forests are the most species rich and productive terrestrial ecosystem on Earth, being the largest terrestrial carbon sink (2.83 Pg C per year) with the Amazon forest alone contributing about 40-50% of this total C uptake (Pan et al. 2011). Climate change is expected to have large impacts in tropical forests (Huntingford et al. 2013) and knowledge of their responses to ambient and elevated CO_2 is key to understanding their current and future functioning (Baker et al. 2004; Cox et al. 2013). Stimulation of tropical plant growth and increased net primary productivity (NPP) under atmospheric CO_2 enrichment (Kimball and Idso 1983; Ainsworth and Long 2005; Norby et al. 2005; Lloyd and Farquhar 2008), will be ultimately controlled by the amount of available nutrients in soil (Hungate et al. 2006) as well as how efficiently plants can acquire and use those nutrients.

Nitrogen (N) and phosphorus (P), for instance, are used in a wide-range of key physiological and metabolic plant processes and are usually considered the most limiting elements to plant growth (Walker and Syers 1976; Vitousek and Howarth 1991). Most of our understanding on how nutrient availability limits ecosystem productivity, however, comes from temperate and high-latitude ecosystems, which tend to be N-limited. The main natural source of N into terrestrial ecosystems is via biological fixation and atmospheric deposition (Vitousek and Howarth 1991; Galloway et al. 2004), as this element is found in very low concentrations in igneous and metamorphic rocks (Morford et al. 2011).

Rates of N fixation and mineralisation of organic N within soils decline with decreasing temperatures explaining why cooler ecosystems are often limited by N availability (Walker and Syers 1976; Vitousek and Sanford 1986). In the lowland tropics, high temperatures result in large rates of fixation and mineralisation and therefore, it is not N availability that is generally considered to be limiting plant growth in these areas (Vitousek 2004; Lambers et al. 2008).

Whilst the dominant input of N into terrestrial ecosystems is via atmospheric deposition and bacterial N fixation, rock-derived elements such as calcium (Ca), magnesium (Mg), potassium (K) and P enter the system almost exclusively through the weathering of parent material (Jordan 1982). Thus, along soil development, N tends to accumulate in undisturbed forests, while P and cations availability decreases (Figure 2.1) by leaching or occlusion in minerals and organic matter, potentially constraining tropical forest productivity. Particularly, P plays a key role in an array of plant processes, including intracellular energy transport (ATP), respiration, photosynthesis, N-fixation, nucleic acids synthesis and activation/inactivation of many enzymes (Vance et al. 2003; Raghothama and Karthikeyan 2005; Lambers et al. 2006). There are many processes that can further promote P limitation in most tropical forests, such as formation of soil layers that prevent P access by roots, low P concentrations in the parent material, immobilisation of available P by other minerals and enhanced supply of other resources that can in turn exacerbate P limitation (Vitousek et al. 2010). Furthermore, it has been suggested that P limitation in tropical forests could become more intense under elevated CO_2 concentrations (Gentile et al. 2012; Sardans et al. 2012; Ellsworth et al. 2017) with possible major impacts on future forest productivity. Although the concept of P limitation in tropical forests is widely accepted, there is a paucity of data to test its validity but also to explain the mechanisms by which these forests have adapted and thrive under low soil fertility.

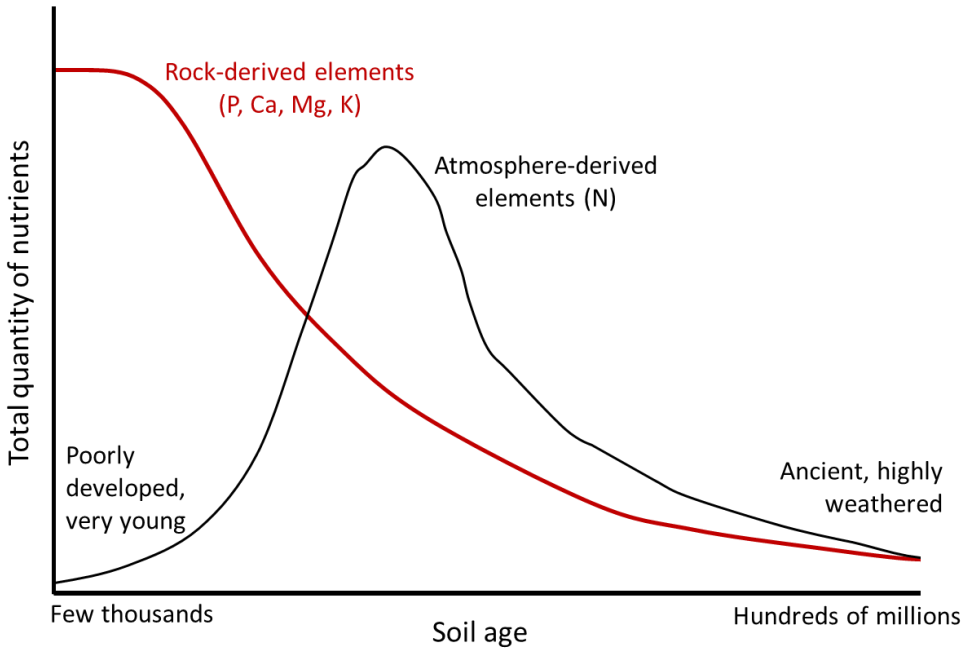


Figure 2.1. Changes in total concentrations of nutrients with soil age. Red line indicates soil elements derived from the weathering of rocks (P: phosphorus; Ca: calcium; Mg: magnesium; K: potassium). Black line indicates soil elements which the main source is the atmosphere, rather than rock weathering (N: nitrogen). Adapted from Lambers et al. (2008).

Large scale nutrient manipulation experiments allow quantification of nutrient limitation in different ecosystems (Vitousek and Farrington 1997) under present and future climate conditions. However, up to now they have been mostly conducted in temperate ecosystems with only six large-scale nutrient manipulation experiments performed in undisturbed tropical lowland forests including experiments in Borneo (Mirmanto et al. 1999), Cameroon (Newbery et al. 2002), Panama (Yavitt et al. 2011) and Costa Rica (Alvarez-Clare et al. 2013). However, it is only very recently that the two Amazon experiments have been installed, near Manaus in Brazil and in French Guiana, whilst there are also ongoing efforts to install the first Free Air CO_2 Enrichment (FACE) experiment in the Amazon (Hofhansl et al. 2016). Therefore, at present our understanding of the effects of soil nutrient availability on Amazon forest dynamics comes mainly from the study of natural gradients and from extrapolation of the results from experiments in other tropical forests.

Although the importance of P to ecosystem functioning is becoming increasingly recognised (Reed et al. 2015), P cycling is currently not represented in most dynamic global vegetation models (DGVMs). Therefore, adding the P cycle to existing vegetation dynamic models is expected to make projections more realistic in regards to biomass and forest production, particularly in the tropics and other areas where productivity may be limited by P (Quesada et al. 2012). However, the lack of information on the nature and magnitude of processes involved in P cycling is likely to increase uncertainty in DGVMs predictions. In addition, the high interdependence of N and P cycling in terrestrial ecosystems is likely to have an impact on the responses of forest productivity and C assimilation to climate change (Houlton et al. 2008). Therefore, the aim of this review is to highlight the importance of different mechanisms that determine P availability and the role they may play controlling Amazon forests functioning. More emphasis is given to belowground nutrient uptake mechanisms rather than mechanisms related to P-use efficiency aboveground. I first present a review of literature on terrestrial P cycling with a focus on evidence for P limitation in Amazon forests. I then summarise current empirical and experimental data explaining key processes of the P cycle in tropical forests, finishing with suggestions of measurements and approaches needed to improve understanding of Amazon forest functioning and nutrient cycling, crucial for a realistic representation of this important ecosystem in DGVMs under present and future climate.

2.3. Soil development and phosphorus cycling in Amazon forests

2.3.1. Phosphorus dynamics in soils

Phosphorus is considered a key element in pedogenic transformations due to its high importance to ecosystem functioning. In contrast to N, P is supplied to soil almost exclusively by the weathering of the parent material, with contributions from atmospheric deposition being very small and varying greatly in space and time (Walker and Syers 1976). Saharan dust input has been suggested to be a significant or even the main source of P in Amazonia (Newman 1995; Okin et al. 2004). Koren et al. (2006) and Ben-Ami et al. (2010) showed that dust from the Bódélé region could reach the Amazon but its quantification is difficult with its

actual role as a source of P being hard to detect in central Amazon forests. Moreover, Yu et al. (2015) report that the imported dust would provide an input equivalent to 23 (7-39) g P ha^{-1} $year^{-1}$ to the Amazon, comparable to the out-of-basin losses that range between 8 and 40 g P ha^{-1} $year^{-1}$ (Jordan 1982; Vitousek and Sanford 1986). These findings suggest that the role of atmospheric P input in the Amazon is only enough to prevent soil P depletion (Yu et al. 2015), with limited impact to enhance the actual nutritional status of the forest. In the timescale of soil pedogenesis, however, these seemingly small inputs could affect P stocks in low-fertility soils, especially in the eastern portion of the basin.

Phosphorus in soils can be found in many different organic and inorganic forms and its distribution is ultimately controlled by factors such as soil pH, vegetation type, climate and microbial activity (Bolan 1991). However, inorganic labile P in the soil solution, coming from both organic and mineral sources, is the only form of P assimilated by plants and microorganisms (Hinsinger 2001). The many chemical mechanisms influencing soil P availability and fluxes between the P pools are shown in Figure 2.2. The importance of soil parent material as a source of nutrients declines considerably with soil development and P concentrations tend to be high in moderately weathered soils, considerably declining with soil age (Walker and Syers 1976). In the timescale of soil development (millions of years), precipitation-dissolution plays a key role in releasing P strongly bound inside primary minerals to P adsorbed on mineral surfaces. Because of its very strong chemical bond, precipitation-dissolution reactions requires a lot of energy and occur very slowly during soil weathering. Moreover, the contribution of the primary minerals in most tropical soils can be considered virtually zero because of their intense weathering (represented by the dashed arrows in Figure 2.2) and high P-fixation capacity of tropical soils.

Adsorption-desorption refers to the transfer of P ions in solution to and from mineral-P sorbing surfaces. However, adsorption, rather than desorption is highly promoted in tropical soils rich in iron (Fe) and aluminium (Al) oxides due to the strong affinity between P ions and metal oxides and clay minerals. This further decreases concentrations of P available for plants and microorganisms (Hinsinger 2001). Because of the relatively high concentrations of organic P, rather than inorganic P, immobilisation-mineralisation can be considered the main process replenishing P in soil solution in tropical soils. Immobilisation is the

conversion of inorganic P into the organic fraction within the soil-plant and microbial systems, and mineralisation is the release of phosphate ions from organic P form via hydrolysis of organic P substrates resulting in inorganic phosphate release to the soil solution (Condron and Tiessen 2005). As a consequence of the intense weathering of Amazon soils, the majority of Amazon forests become essentially more dependent on cycling of organic P, as mineral P becomes increasingly depleted and the remaining mineral P fractions become occluded (Condron and Tiessen 2005).

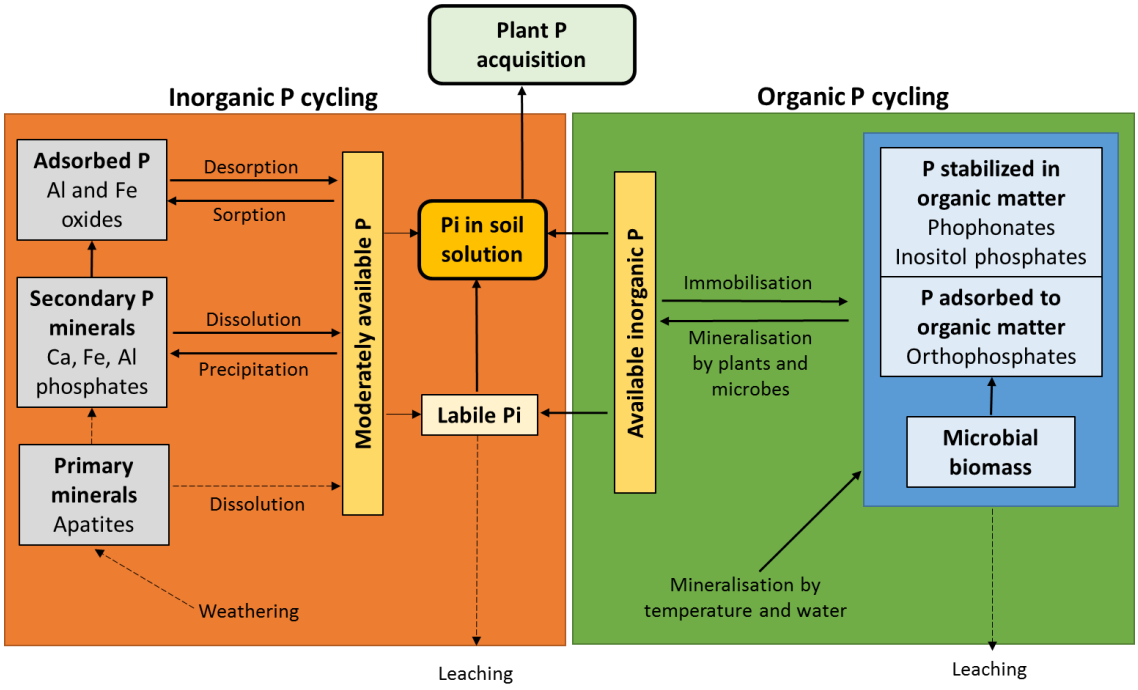


Figure 2.2. Schematic representation of the main phosphorus cycling pools and fluxes in soil, adapted from Shen et al. (2011). Soil P availability is affected by the balance between the processes of: 1) precipitation-dissolution (from strongly bound P inside minerals to P adsorbed in mineral surfaces); 2) adsorption-desorption (from mineral-P sorbing surfaces to P ions in soil solution); and 3) immobilisation-mineralisation (from organic matter to inorganic P). Available inorganic P can occur as phosphates in soil solution (HPO_4, H_2PO_4). The left orange portion represents inorganic P dynamics and green portion represents organic P dynamics. Dashed arrows represent processes and pools unlikely to occur in very weathered soils. Pi: inorganic P.

2.4. Variation in phosphorus availability across the Amazon basin

Overall, total phosphorus concentrations in Amazon soils are in the lower range of soil P reported in lowland tropical forests, but large differences can be found across the basin. The lowest total P concentrations can be found in the oldest Amazon soils and highest P concentrations in young to intermediate age soils (Quesada and Lloyd 2016), following the model of soil development proposed by Walker and Syers (1976). Hence, the current P content in Amazon soils is a result of the interaction between soil age and the quality of the parent material (Augusto et al. 2017), which in turn have been affected by a range of geological events spanning millions to billions of years of soil formation (Quesada et al. 2011; Quesada and Lloyd 2016).

The western region of the Amazon basin is formed by very young sediments, that still experienced uplift in the Pliocene, with ages varying between 1 and 2 million years, and even more recent flooded areas (in the past 5,000 years), which explains the higher fertility of this area. The north and southern edges of the basin cover very old soils on the Guyana and Brazilian crystalline shield, formed in the Proterozoic era. Despite being old (2,000 - 2,500 million years), these soils are less developed due to its crystalline formation, showing intermediate fertility (Quesada et al. 2010). Central and east portions of the Amazon basin were formed more than 50 million years ago (Irion 1978; Sombroek 2000; Quesada et al. 2011) from re-worked sediments from the Guyana and Brazilian Shields, explaining the very low fertility of the majority of Amazon soils. These areas also show high topographic stability which favours high levels of soil development. Those factors, combined with the hot and humid climate of the Amazon, result in intense weathering and nutrient leaching from naturally nutrient-poor parent material (Jordan and Herrera 1981).

Total P concentrations across the Amazon basin vary from 1,630 mg kg^{-1} at the base of the Andes to 10 mg kg^{-1} in some white sand forests in Guyana (Quesada et al. 2010; Quesada et al. 2012; Quesada and Lloyd 2016). As a result of the high variability in total soil P concentrations, P pools and cycling mechanisms also vary in importance along the extremes of the soil fertility spectrum found in the Amazon. For example, Gleysols, Cambisols and Plinthosols are predominant in the western portion of the Amazon (covering about

23% of the basin area) and because these soils are poorly developed, the weathering of primary and secondary minerals is still the main source of P to the forests growing in these soils (Quesada et al. 2010). At the other extreme of soil fertility, highly weathered Podzols and Arenosols, cover < 5% of the basin area and are considered to be the most infertile soils in Amazonia (Quesada et al. 2011). These soils are usually associated with white sand forests that rely almost exclusively on the recycling of organic P from decomposing litter to meet their nutritional demands (Stark and Jordan 1978; Sombroek 2000; Quesada et al. 2011). However, 60% of Amazon soils are in between these two extremes (Quesada et al. 2011), covering the central and eastern portion of the basin and therefore, due to their large spatial extent this review particularly focuses on P dynamics in highly-weathered Acrisols and Ferralsols.

Although direct evidence of P limitation can only be demonstrated via manipulation experiments, a negative relationship between soil fertility and rates of aboveground biomass accumulation was found across the Amazon basin (Quesada et al. 2012). Amazon forest dynamics have been found to be strongly correlated with total soil P concentrations, resulting in a gradient of tree growth rates across the basin: sites with high soil P concentrations have fast tree turnover rates and wood production but shorter stem residence time with low wood density and therefore low aboveground biomass stocks; contrary, P-poor sites have slow tree turnover rates and coarse wood production but longer stem residence time with high wood density and aboveground biomass stocks (Malhi et al. 2004; Quesada et al. 2010; Quesada et al. 2012; Johnson et al. 2016). Soil P concentrations and tree growth gradients along the Amazon basin result in a suite of different nutrient cycling strategies spanning two extremes scenarios, varying from an “open” system in high fertility soils to a “closed” system in low fertility soils (Jordan and Herrera 1981; Quesada et al. 2012). An “open” system refers to forests where there are losses of nutrients to the atmosphere, surface water and groundwater and the nutrients lost are replenished mainly by soil mineral weathering, which is usually poorly developed with high P and cations concentrations. “Closed” systems, on the other hand, are characterised by virtually no nutrient inputs from rock weathering since such soils are very old and developed with low concentrations of P and cations. In “closed” systems, nutrient cycling is very efficient, being almost leak-proof, and nutrient losses (if existing)

are compensated by atmospheric deposition (Jordan 1982; Quesada et al. 2012). The main mechanisms and adaptations used by plants to cycle nutrients efficiently in such “closed” systems are discussed in depth in the next section.

2.5. Strategies to overcome P limitation

As a consequence of low P availability in the majority of Amazon soils, plants are expected to display an array of mechanisms to adapt to low-P conditions. Plants are able to alter their morphological, physiological, biochemical and molecular properties to optimise nutrient acquisition (Aerts 1999; Raghothama 1999). Aboveground, plants can use strategies to maximise P-use efficiency in low-fertility soils, including C investment to produce secondary metabolites that therefore reduce levels of herbivory, low nutrient concentrations in leaves as well as longer leaf life spans (Reich et al. 1992; Paoli and Curran 2007). Belowground strategies for P acquisition include root morphological modifications such as changes in root architecture, increased root hair production, root hair elongation, cluster root formation and association with mycorrhizal fungi (Raghothama 1999; Richardson et al. 2011); physiological, biochemical and molecular responses including increase secretion of organic acids and production of phosphatase enzymes by roots, responsible for releasing mineral-bound P and organic P, respectively (Quiquampoix and Mousain 2005). The next section includes detailed key belowground and aboveground strategies adopted by plants to maximise P use and more importantly, P uptake under limiting nutrient availability in tropical forests (Figure 2.3).

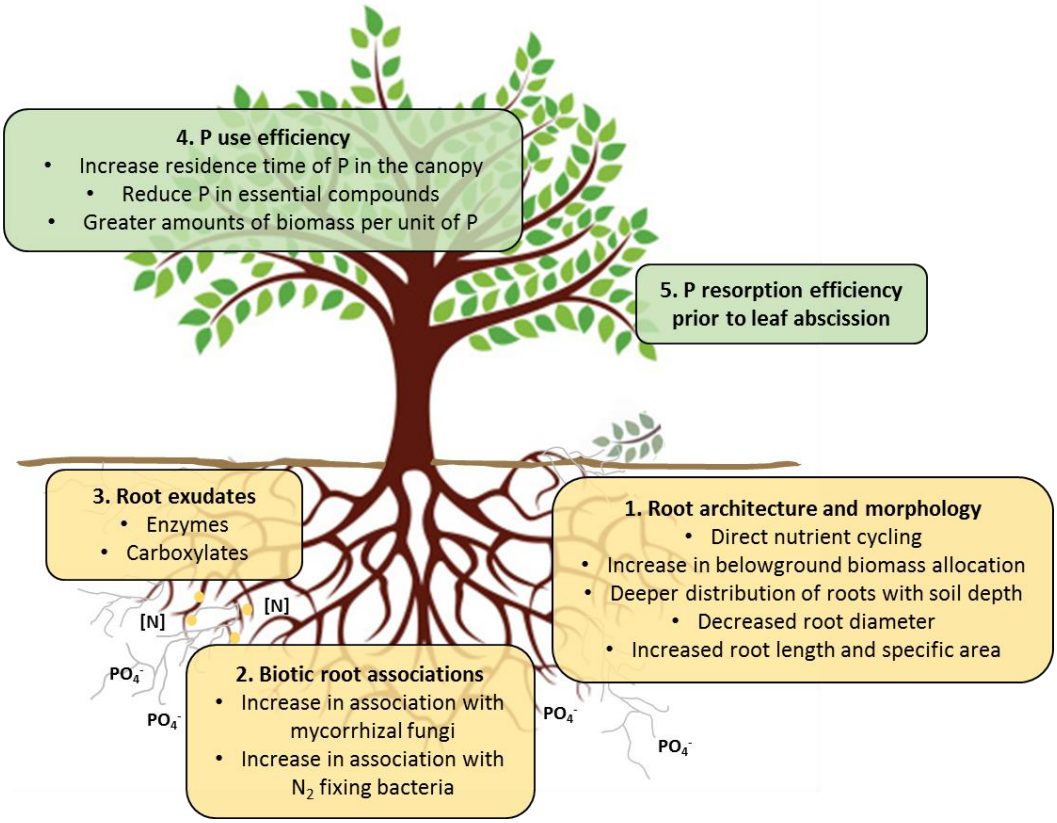


Figure 2.3. Main aboveground and belowground strategies used by plants to overcome soil P limitation. Green boxes represent aboveground and yellow boxes represent belowground strategies. Numbers are linked to sections 2.5.1 and 2.5.2. Grey lines represent mycorrhizal fungi hyphae and yellow dots represent N_2 fixing bacteria associated with roots in the soil.

2.5.1. Belowground strategies: Plant P-uptake

1) Root architectural and morphological modifications: The main mechanism of P movement in the soil is diffusion (Bolan 1991; Hinsinger 1998), however, due to its very low mobility in soils, roots preferentially move towards P (Aerts 1999). On average, about 33% of global annual plant NPP is allocated to fine root production (Jackson et al. 1997) and trees in nutrient-poor soils are often expected to allocate more biomass belowground, aiming to increase nutrient uptake (Hendricks et al. 1993; Aerts 1999). In a Colombian Amazon forest, Jimenez et al. (2009) and Jiménez et al. (2014) found evidence to support this differential allocation: under similar climatic conditions, forests on low-nutrient soils allocated three times more biomass to fine roots production than forests

found in more fertile soils. Root distribution along the soil profile can also change in the face of nutrient limitation, with trees mining deeper soil layers to take up nutrients that are still being weathered from minerals (Iversen 2010; Iversen et al. 2015). However, exploring deeper soil layers is more effective when there is still nutrient release from parent material weathering, which is unlikely to happen in central and eastern Amazon forests. In these low-fertility soils instead, roots grow into the litter layer, above the mineral soil, where organic P is being mineralised through organic matter decomposition (Went and Stark 1968; Stark and Jordan 1978; Jordan and Herrera 1981). This has been termed “direct nutrient cycling” (Went and Stark 1968) and describes nutrient mineralisation from decomposing leaves and its immediate transfer to roots and mycorrhizal hyphae, and ultimately back to the trees. The dense root mat and humus layer on top of these soils also acts as an important conservation mechanism, preventing nutrients leaching out of the system (Stark and Jordan 1978; Jordan and Herrera 1981; Tobón et al. 2004).

Root morphological adaptations include the investment in root hairs, especially in plants lacking mycorrhizal associations, providing competitive advantage in exploring soil volumes that would not be explored by hairless roots (Bates and Lynch 2001). Changes in root diameter and specific root length (SRL - amount of root length invested per unit root mass) towards thinner and longer roots facilitate the exploration of further patches of soil with potentially higher moisture and nutrient availability (Hodge 2004; Metcalfe et al. 2008). Thin-roots tend to have high absorptive capacity because of their high investment in root length and surface area, but they also come at a high cost to the plant as these roots have high respiration rates, tend to be short-lived and are susceptible to pathogens and herbivores attack (Maherali 2014; Laliberte et al. 2015; McCormack et al. 2015). On the other hand, thicker roots, that show more conservative traits, tend to have longer life span and may be involved more in nutrient transport inside the plant rather than nutrient uptake, and thus species with thicker roots may be highly dependent on biotic associations if they are to explore the soil volume and acquire nutrients (Hodge 2004; Liu et al. 2015; McCormack et al. 2015).

2) Root biotic associations: Trees with thick roots are expected to be highly dependent on forming associations with mycorrhizal fungi to meet nutrient

demands (Hodge 2004; Comas et al. 2014; Eissenstat et al. 2015; Liu et al. 2015; Kong et al. 2016). The C-investment in mycorrhizal fungi per unit of hyphal surface is lower when compared to the C-costs of building roots (Li et al. 1991; Jansa et al. 2003; Jansa et al. 2011). In this symbiotic association, plants benefit from nutrients acquired efficiently by mycorrhizas and in exchange, mycorrhizas benefit from C supplied from the host-plant. Arbuscular mycorrhizal (AM) fungi are the most common form of fungi associated with higher plants (Brundrett 2004; Soudzilovskaia et al. 2015; van der Heijden et al. 2015), being very abundant in lowland *terra firme* forests in the Amazon, commonly associated with intermediate and low-fertility soils (Stürmer and Siqueira 2006). Ectomycorrhizal associations in Amazonia, on the other hand, are most predominant in white sand forests, where soil fertility is in the lowest range for the Amazon basin (Quesada et al. 2011). Ectomycorrhizas can show greater ability to hydrolyse organic P and scavenge for inorganic P compared with AMs (Singer 1988; Becerra and Zak 2011; Roy et al. 2016). Generally, plant-mycorrhizal associations contribute to expand the soil volume explored by roots (Smith and Read 2010), increasing the amount of P uptake by mycorrhizal roots when compared to non-mycorrhizal roots (Bolan 1991).

Plant association with N_2-fixing microorganisms is also a widespread mechanism initially recognised for its importance in forests limited by N (Galloway et al. 2004). However, in old tropical soils, the association between N_2-fixing bacteria and trees is very common (Houlton et al. 2008) and it has been suggested that N_2-fixers can allow for greater investment in mycorrhizal association and/or exudation of P-related enzymes via allocation of the extra N fixed, giving these trees a competitive advantage in mobilising P from soil solution (Nasto et al. 2017). However, more recent studies failed to find support for the idea that root phosphatase activity is higher in N_2-fixers at the individual and species level in tropical forests (Batterman et al. 2013a; Batterman et al. 2018; Nasto et al. 2014), suggesting that the interactions between soil N and P availability could be revisited. Moreover, despite the fact that Fabaceae is one of the most common families in Amazonian forests, not all legumes are able to fix nitrogen, with some species even being facultative N_2-fixers (Batterman et al. 2013b).

3) Root exudates: Since P in tropical soils is largely bound in organic compounds or adsorbed to mineral surfaces (Cross and Schlesinger 1995), roots invest in compounds to mineralise organic P or liberate adsorbed P prior to assimilation (Jones and Oburger 2011). Roots can exude water, sugars, carboxylates, enzymes, hormones, mucilage and phenolic compounds and the exudation of such compounds can represent up to half of C allocated belowground by plants (Lynch and Ho 2005). Carboxylates are compounds released by roots that can be an important plant reaction under P deficiency (Jones and Oburger 2011; Turner et al. 2012; Lambers et al. 2015). Carboxylates release P strongly bound to Al and Fe minerals in acid soils, or Ca in alkaline soils, by directly replacing P in those minerals or by altering soil pH (Lambers et al. 2008). However, due to the complexities related to measuring carboxylate exudation in field conditions, only few studies attempted to understand this process (Lambers et al. 2012; Wang et al. 2013) and therefore, the importance of carboxylates exudation influencing P availability in Amazon soils is still unknown.

The main mechanism controlling P bioavailability in tropical soils is the hydrolysis of organic P through phosphatase enzyme activity, a constitutively N-rich enzyme (about 15%) (Olander and Vitousek 2000; Treseder and Vitousek 2001) that can be released by plants, some mycorrhizal fungi and microorganisms in soils (Vance et al. 2003). Despite its potential importance for the recycling of organic P, studies reporting root phosphatase activity in the tropics are rather limited (Kitayama 2013; Nasto et al. 2014; Ushio et al. 2015; Nasto et al. 2017; Batterman et al. 2018) and non-existent for Amazon forests.

2.5.2. Aboveground strategies: P-use efficiency

4) Phosphorus use efficiency (PUE): PUE is usually determined as plant growth per unit of nutrient taken up (Aerts 1996; Vitousek 2004). Plants under nutrient limitation can increase the residence time of P within the canopy (Vitousek 2004), reduce or replace P in essential compounds (White and Hammond 2008) and produce greater amounts of biomass per unit of P (Kitayama et al. 2000; Kitayama and Aiba 2002; Vitousek 2004; Kitayama 2013). Amazon forests under low-fertility soils have high PUE, resulting in a tight

coupling between forest functioning and soil fertility (Quesada and Lloyd 2016). In agreement with this, leaf and litter P levels have been found to be positively correlated with soil P concentrations in the Amazon (Fyllas et al. 2009), with leaf P concentrations being more strongly correlated with soil P available pools rather than total soil P concentrations (Quesada 2008). Such aboveground mechanisms of efficient P-use and conservation might greatly influence forest productivity and biomass accumulation in Amazon forests, consequently affecting the way these forests will respond to climate change.

5) Phosphorus resorption efficiency (PRE): This metric is defined as the amount of nutrients resorbed by plants prior to leaf abscission, and determined by comparing nutrient concentration in fresh litter and in mature fresh leaves (Killingbeck 1996; van Heerwaarden et al. 2003; Kitayama et al. 2004; Richardson et al. 2008; Reed et al. 2012). In general, PRE decreases with increasing phosphorus availability in tropical forest (Kobe et al. 2005; Vergutz et al. 2012; de Campos et al. 2013). In Amazon forests, a greater proportion of P was generally resorbed when compared to N (64-69% against 42-58% on average) (Reich et al. 1995). Quesada (2008), studying different soils across the Amazon basin, found strong positive correlations between P concentrations in soils and litter and P concentrations in leaves and litter. As a consequence of P resorption, litter C:P ratios in tropical regions are high when compared to temperate forests (Jackson et al. 1997; McGroddy et al. 2004; Marklein et al. 2016). This situation might induce a positive feedback where P limited plants could potentially become even more limited: high C:P ratios would induce slow rates of litter decomposition which could therefore lead to intense competition for soil nutrients between roots and microorganisms (Han et al. 2013).

2.6. Evidence of nutrient limitation in tropical forest

2.6.1. Nutrient manipulation experiments

Correlations between soil fertility, plant properties, microbial community and forest processes can add valuable knowledge about nutrient limitation, but real causation can only be achieved by nutrient addition experiments (Cleveland et al. 2011; Sullivan et al. 2014). Nutrient limitation in forests is usually determined by the increase in plant growth (Elser et al. 2007; Lebauer and Treseder 2008)

and changes in foliar nutrient concentration after nutrient manipulation (Townsend et al. 2007; Ostertag 2010). However, Sullivan et al. (2014) also suggested that belowground processes and compartments should be accounted for when studying nutrient limitation. Long-term monitoring as well as long-term nutrient manipulation experiments are useful tools to elucidate the causes and consequences of P limitation in the tropics, contributing enormously to our understanding of forests functioning under present and future climate.

In a global analysis of nutrient limitation in different ecosystems, Elser et al. (2007) pointed out that 173 fertilisation experiments have been installed in terrestrial ecosystems. From the total, 43 were located in forest ecosystems and up to now, only six large-scale nutrient-addition experiments have been placed in undisturbed tropical lowland forests (Mirmanto et al. 1999; Newbery et al. 2002; Wright et al. 2011; Alvarez-Clare and Mack 2015). From these six experiments, results obtained by the four published nutrient manipulation experiments, none of which are in Amazonia, suggest that multiple nutrients can potentially limit plants in different life stages in tropical forests (Alvarez-Clare et al. 2013) and that responses also differ with short and long-term nutrient addition. Moreover, these experiments indicate that above and belowground compartments show different magnitude of responses, with root and leaf traits and the function of soil microbial communities being amongst the first to show signs of alleviation of nutrient limitation.

The longest running nutrient fertilisation experiment started in Panama in 1998 (Yavitt et al. 2011) and results suggest that P is not the only nutrient limiting this forest (Wright et al. 2011). After 11 years of nutrient manipulation, K addition reduced fine root biomass and K-only and K+N addition increased small-diameter tree growth, whilst P addition increased root biomass and litterfall production mostly from large trees and lianas (Wright et al. 2011; Yavitt et al. 2011). However, after 14 years of nutrient addition, Wurzburger and Wright (2015) found decrease in fine root biomass after N+P+K addition, inducing a shift in root traits from a conservative strategy towards nutrient-rich and short-lived roots capable of rapid resource acquisition. Phosphorus addition also affected root-mycorrhizal interactions in Panama, increasing root association with mycorrhizas even after 14 years of nutrient addition (Wurzburger and Wright 2015). Furthermore, soil microbial community responses in Panama also indicate that P strongly limits this

compartment, since soil microbial nutrients increased and enzyme activities decreased after 10 years of P addition (Turner and Wright 2014). More recently, evidence for P-limitation on species-specific tree growth in Panama was found, but since some species grow more rapidly than others, even under low-fertility soils, P-limitation was not captured at the community-level (Turner et al. 2018). In Borneo, after four years of P addition, litterfall production increased (Mirmanto et al. 1999), whilst in Cameroon, no effects on NPP or tree growth were detected after two years of P addition (Newbery et al. 2002). In Costa Rica, the addition of P increased growth from small diameter trees in forests, whilst no effects were observed for large trees (Alvarez-Clare et al. 2013; Alvarez-Clare and Mack 2015).

The different responses obtained after P addition could be a result of varying P sorption and occlusion in soil as suggested by the results found in Panama by Mirabello et al. (2013). Other possible causes for the lack of conclusive evidence for P limitation in the tropics might be due to i) high diversity of tree species that could therefore use different strategies to deal with P limitation, ii) increase in herbivory after nutrient addition making it hard to detect any increase in leaf and litterfall production, iii) shifts in plant community to species adapted to new soil fertility conditions and iv) limitation by other important environmental properties, such as light or other soil nutrients (Harpole et al. 2011). These experiments took place on soils with total P concentrations of 80 mg kg^{-1} in Borneo (Mirmanto et al. 1999), 277 mg kg^{-1} in Cameroon (Newbery et al. 2002), 600 mg kg^{-1} in Panama (Wright et al. 2011) and 1,600 mg kg^{-1} in Costa Rica (Alvarez-Clare et al. 2013), while total P concentrations in central Amazon soils are only 100 mg kg^{-1} on average (Quesada et al. 2010; Quesada and Lloyd 2016).

In addition to differences in soil fertility, mean absolute tree growth rate (≥ 10 cm diameter at breast high) in tropical forests where nutrient manipulation experiments have been installed are comparatively higher than tree growth in central Amazonia: 7.1-7.4 mm yr^{-1} in Borneo (Mirmanto et al. 1999), 2.16 mm yr^{-1} in Cameroon (Newbery et al. 2002), 1.4-9.2 mm yr^{-1} in Panama (Condit et al. 1995) and 5-18 mm yr^{-1} in Costa Rica (Clark and Clark 1999), contrasting with 1.64 mm yr^{-1} in a central Amazon forest (Da Silva et al. 2002). I therefore hypothesise that results from those sites do not necessarily represent the potential role of P availability in controlling plant and microbial processes in

Amazonian forests. Because of their natural differences in environmental properties and the array of responses obtained by these manipulation experiments, conducting similar long-term experiments in Amazon forests under very-low fertility soils becomes crucial, before any generalisation about P limitation in tropical forests could be made. Recently, two nutrient manipulation experiments were set up in the Amazon forest, one in Brazil (AFEX) and one in French Guiana (IMBALANCE-P) and results derived from these experiments will soon contribute to an improved understanding of nutrient limitation in Amazon forests.

2.6.2. CO_2 fertilisation experiments

Under future atmospheric CO_2 concentrations, tropical plant growth and NPP are expected to increase (Kimball and Idso 1983; Ainsworth and Long 2005; Norby et al. 2005; Lloyd and Farquhar 2008). However, to sustain increased plant growth under elevated CO_2, phosphate ions would have to be mineralised from complex organic and inorganic molecules and consequently released to soil solution, in order to keep a relatively constant concentration of P in the soil solution (Lloyd et al. 2001). Up to now, Free air CO_2 enrichment (FACE) experiments were installed predominantly in temperate ecosystems, providing an insight on the responses of forests under N, rather than P limitation (Norby et al. 2015). FACE experiments in boreal and temperate ecosystems have indicated the presence of CO_2 and nutrient feedbacks towards both 1) sustaining the growth of forests through increased N mineralisation from organic matter but also of 2) decline in tree growth due to decrease in N availability (Norby et al. 2010; Van Groenigen et al. 2014; Norby et al. 2015; Terrer et al. 2017).

More recently, results from the EucFACE experiment installed in a P-poor *Eucalyptus* forest in Australia demonstrated that after 18 months of elevated CO_2, available P concentrations in soils increased 54% (Hasegawa et al. 2016). However, microbial enzyme activities related to C, N and P cycling did not change with increasing CO_2, suggesting that higher P concentrations in soils could be driven by plant investments belowground, especially carboxylates and H^+ exudation under elevated atmospheric CO_2 (Drake et al. 2016; Hasegawa et al. 2016). Moreover, after three years of CO_2 fumigation, Ellsworth et al. (2017)

captured increased photosynthetic rates in the EucFACE experiment, but the higher levels of soil P availability and stimulation in photosynthesis did not translate into increased tree growth. Together, these results suggest that P availability might be still constraining the predicted positive effect of CO_2 on aboveground growth, but more importantly, CO_2 fertilisation could be shifting NPP belowground towards alleviating P limitation (Ellsworth et al. 2017).

It is proposed that under elevated CO_2, more humic acids and organic matter could be produced due to higher plant productivity (Lloyd et al. 2001). In tropical soils, these compounds compete with phosphate ions for sorption sites in soil minerals and thus, higher CO_2 could lead to higher C in soil, which in turn displaces and releases more P to soil solution. In this scenario, soil C stocks increase and forest growth is enhanced because of increased P availability in the soil solution (Lloyd et al. 2001). This mechanism, however, would be limited by the concentration of sorbed P in soils and further increases in atmospheric CO_2 concentrations could make nutrients in soil less abundant relative to C, suggesting that nutrient limitation could ultimately increase with global climate change (Hungate et al. 2003; Luo et al. 2006). However, we currently have very limited understanding of the likely dynamics of sorbed P under future climate change in tropical soils and the exact direction of these responses remains to be tested.

The exudation of other compounds by plants, such as organic acids and anions is hypothesised to play a role on plant nutrition, but up to now, the importance of these exudates releasing significant amounts of P is unclear. The significance of these processes, however, is also expected to increase under elevated CO_2, since belowground C allocation towards root exudates could be even greater (Phillips et al. 2011), having the potential to offset or delay nutrient limitation effects for a period of time. However, under future climate, the role of the microbial biomass could shift from a source to sink of nutrients, with plant biomass increase depending on the balance between microbial nutrient immobilisation and mineralisation via organic matter decomposition. Evidence from nutrient manipulation experiments support the hypothesis that soil microbial community might already be limited by P in tropical forests in Panama (Turner and Wright 2014), which as a consequence could intensify P limitation by plants via competition under elevated CO_2.

Furthermore, CO_2 enrichment is also predicted to change root distribution along the soil profile, with roots accessing deeper layers to mine nutrients (Iversen 2010; Nie et al. 2013). Such belowground adaptation was described so far only in N-limited temperate ecosystems (Iversen et al. 2008; Iversen 2010) and the actual importance of investment in deeper roots in tropical forests remains uncertain. Since the weathering of parent material in the majority of Amazon soils is not expected to currently contribute to plant nutrition, investment in deeper roots might not be as beneficial for tropical plants as it is in temperate ecosystems. However, when considering the role of increasing temperature and droughts, trees could indeed explore deeper soil layers in order to acquire water, and this could interact with nutrient acquisition.

Also of great to importance to nutrient cycling is the association of plants with mycorrhizal fungi and how this relationship could be affected by increasing atmospheric CO_2 concentrations (Treseder 2004; Terrer et al. 2016; Ellsworth et al. 2017). Elevated CO_2 was found to substantially increase mycorrhizal colonisation in a range of different temperate and boreal ecosystems, but such increase in mycorrhizal investment was ultimately affected by nutrient availability, decreasing with N and P addition (Treseder 2004). More specifically, responses from plants associating with ectomycorrhizas and arbuscular mycorrhizas were found to vary greatly and interact with N availability, with CO_2 enrichment stimulating biomass accumulation in plants associated with ectomycorrhizas independently of soil N concentrations, whereas plants associated with arbuscular mycorrhizas did not respond to the increase in atmospheric CO_2 concentrations due to N limitation (Terrer et al. 2016). It is still unknown if the interaction between elevated CO_2 and P availability in tropical forests would follow similar patterns as found in N-limited temperate and boreal ecosystems. Additionally, because the total amount of P in terrestrial ecosystems is fairly constant on the time scale of years and centuries, forest responses to elevated CO_2 are likely to be controlled by changes in the availability of mineral and organic P, and more importantly, by the amount of organic matter inputs to soil that will, eventually, reach equilibrium (Yang et al. 2014). Since tropical forests and savannahs represent about 60% of global gross primary productivity (GPP) (Beer et al. 2010), the need to understand the consequences of P limitation under global climate change in these areas will only grow in importance.

2.6.3. Current representation of P-cycling in DGVM

Phosphorus limitation, when represented in dynamic vegetation models, is usually included via a “supply-demand approach”, where the total amount of nutrient demand by vegetation and microorganisms is compared to the supply: if the supply does not meet the potential GPP, the demand is therefore reduced (Goll et al. 2012; Goll et al. 2017). Since P limitation to plant growth in the tropics has been predicted to become more intense or frequent under elevated CO_2 concentrations (Gentile et al. 2012; Sardans et al. 2012; Ellsworth et al. 2017), some models, such as CASA-CNP, JSBACH-CNP and CLM-CNP now incorporate P dynamics and C-N-P interactions (Wang et al. 2010; Goll et al. 2012; Yang et al. 2014; Goll et al. 2017).

Aboveground, leaf P concentrations directly affect biochemical photosynthetic capacity and electron transport (Farquhar et al. 1980; Domingues et al. 2010; Mercado et al. 2011), ultimately affecting plant C assimilation. The representation of aboveground pools of different stoichiometry and lability is of great importance for nutrient cycling, once these pools enter the litter fraction and become re-cycled in the system. Phosphorus pools above and belowground are represented in models in many different forms (leaves, litter, fine and coarse roots, woody tissues), with the main distinction being between live and dead plant tissues. Representation of different plant P pools are based on plant functional types (PFTs) and soil groups (with some parameters varying also within a given range for each biome) (Wang et al. 2010; Goll et al. 2012; Yang et al. 2014). Phosphorus retranslocation before leaf senescence, a crucial mechanism by which plants recycle P more efficiently in tropical forests, was also recently included in JSBACH-CNP and CLM-CNP models (Goll et al. 2012; Yang et al. 2014).

Belowground, CLM-CNP model incorporates five soil inorganic P pools (solution P, labile P, secondary mineral P, parent material P and occluded P) and P in organic matter (Yang et al. 2014). An important advantage of this model is the representation of P fractions based on the Hedley fractionation method, and since this method is becoming extensively used, it aids model parameterisation and evaluation. Mineralisation of organic P and desorption of inorganic occluded

P pools is crucial to keep the balance between P supply from soil and P demand from plants and microorganisms. Accordingly, biological and biochemical P mineralisation processes are becoming increasingly included in models: if P is not limiting, biochemical mineralisation occurs at a constant rate, but if limitation increases, mineralisation rates also increase until a maximum rate (Wang et al. 2010; Goll et al. 2012; Yang et al. 2014). Once available, labile P can be immobilised by plants and microbes, simulating the competition for P. Since P mineralisation is mediated by N-rich phosphatase enzymes, some biogeochemical models now include an interdependence between mineralisation rates and N cycle, where the stimulation of P mineralisation decreases when N is also limiting (Wang et al. 2010; Yang et al. 2014).

The addition of P-cycling in these DVGMs greatly affected ecosystem responses under future climate (Wang et al. 2010; Goll et al. 2012; Yang et al. 2014; Wang et al. 2015; Yang et al. 2016). For example Goll et al. (2012), using JSBACH-CNP model which couples N and P cycles and considers two P fractions in soils (available and sorbed), found a 25% decrease in global land C uptake when comparing to simulations without nutrient limitation. The CASA-CNP model, including for example P pools by availability to plants and microbes and P biological and biochemical mineralisation found a decrease in C uptake in terrestrial ecosystems varying from 13% to 25%, depending on the CO_2 emission scenario considered for the simulations (Zhang et al. 2014). Additionally, Yang et al. (2016) using the CLM-CNP model to investigate the role of P limitation on C cycling in Amazon forests, found that despite the positive effects of elevated CO_2 on C accumulation, this was reduced by 26% when compared to simulations using only C and N cycling. These authors point out that despite strong P limitation, Amazonian forests are still able to work as a sink of atmospheric CO_2 because of important ecological processes, such as the release of phosphatase enzymes and biochemical P mineralisation.

Since P cycling in tropical forests is considered somewhat closed, with virtually no gains or losses, the stimulated C assimilation from elevated CO_2 conditions would result in progressive P limitation via increased P immobilisation due to changes in C:nutrient ratios in plant tissues and soils. As described in the previous sections, this hypothesised effect of exacerbation of P limitation could be ultimately offset by including in DGVMs the representation of increased

biomass allocation towards roots, investments in enzymes and carboxylate exudation as well as increasing plant-mycorrhizal associations. However, because different tree species display various levels of soil P-affinity, ranging from trees adapted to low-P conditions (low-P specialists), tree species that occur in different levels of P availability (P-generalists) and species adapted to high levels of soil P (high-P specialists), the thresholds by which P availability becomes limiting to tree growth and the possible mechanisms that plants would use to adapt to different soil conditions are likely to be species-specific (Turner et al. 2018). The inconsistent evidence for P-limitation in tropical forests at the species and the community-level (Turner et al. 2018) therefore suggests that the inclusion of P-limitation in vegetation models might not be as simple.

2.7. Opportunities for moving forward

Despite the general assumption that P availability strongly limits tropical forests, there is still a lot to be learned about the mechanisms involved in P cycling in Amazon forests. Processes related to P turnover and acquisition are very dynamic and challenging to measure and even our current best approaches are limited. The same applies to models, where current models that incorporate P cycling still offer a poor representation of a very complex system, lacking several key processes of ecological importance, above all in a changing climate. Based on the empirical, experimental and modelling data gathered in the previous sections, below I describe what I believe are the main gaps related to the understanding of P cycling in Amazon ecosystems, together with suggestions of new approaches towards expanding our knowledge on belowground P cycling (Table 2.1).

Table 2.1. Summary of recommendations regarding P acquisition by plants and soil microorganisms, listing experimental and modelling approaches needed to improve understanding and representation of P cycling.

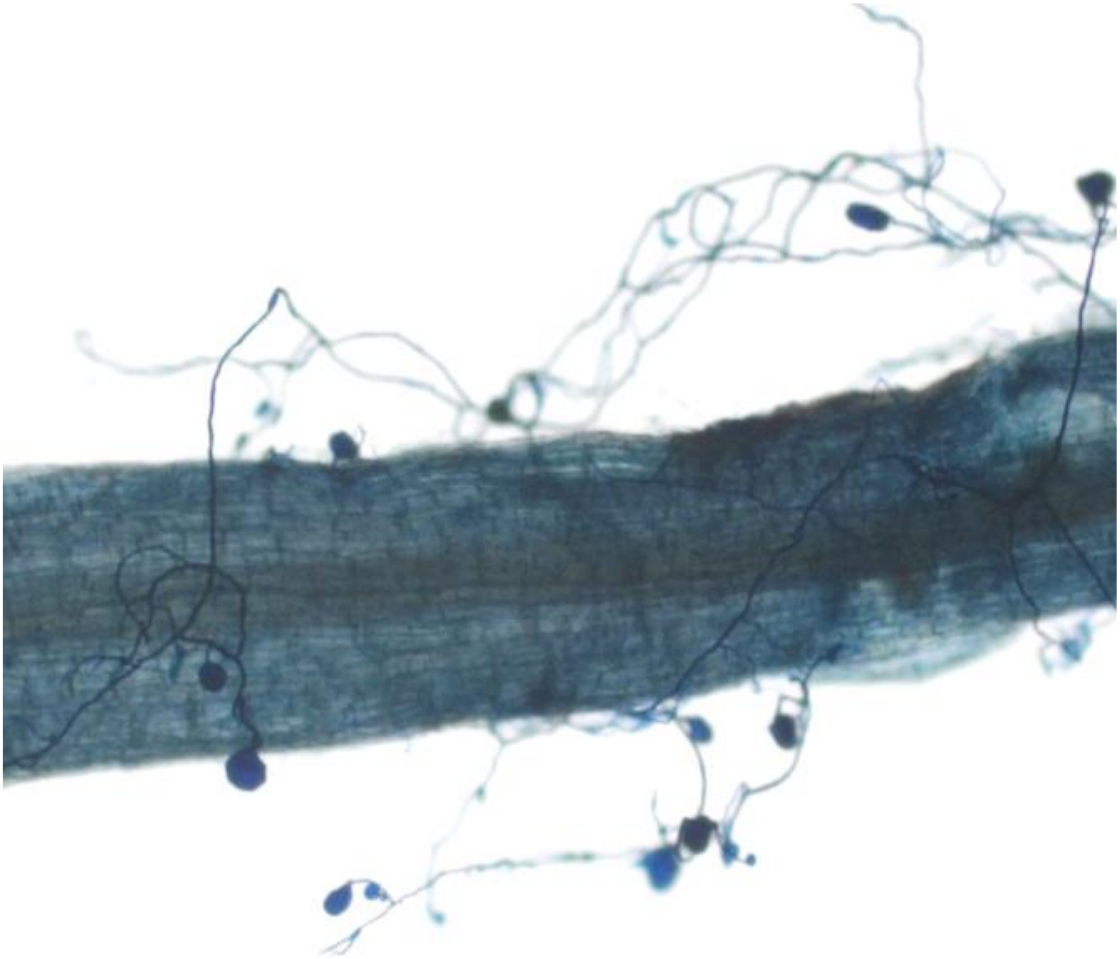
	Empirical data needed for process understanding	**Data and processes needed to improve P cycling in models**

P fractions	• Biological significance of P fractions, seasonality and influence of other soil properties	• Inclusion of P pools related to plants and microbial demand • P pools dynamic in time and dependence on soil properties
P transformation processes	• Factors affecting P mineralisation and immobilisation	• Soil P transformations (mineralisation, immobilisation, occlusion) based on plant and soil microbes demand and supply
Microorganisms	• Role of microorganisms as source and sink of nutrients • Extent of plant-microbe competition • Links between microbes and organic matter decomposition	• Incorporate microbial biomass in the balance of plant supply-demand for P • Potential for microbes increasing competition with plants or accelerating organic matter decomposition
Mycorrhizal colonisation	• Costs and benefits of plant-fungi interaction in acquiring P from soil • Degree of efficiency in P uptake	• C-costs of root-mycorrhizal association • P-uptake efficiency by mycorrhizas
Root morphology and architecture	• Plasticity of root morphology and architectural properties • Trade-off between root life span, turnover, morphology and nutrient uptake • Role of roots in direct P-uptake from litter and organic matter • C allocation to roots	• Differential biomass allocation • Incorporation of root biomass directly affecting nutrient uptake • Root distribution along soil depth
Exudates	• Energy- and N-costs involved • Importance to plant nutrition	• Allocate part of NPP to exudation • Differentiate between exudates released by roots and soil microbes

Recent model studies suggest that nutrient limitation may reduce the actual capacity of forests to accumulate carbon in the future (Yang et al. 2016) and this would mean that allowable future human CO_2 emissions would need to decrease further to avoid the most dangerous consequences of climate change. However, since not much is known about the nature of the processes involved, there is still high uncertainty in model predictions and specifically the limited understanding of P cycling means that the actual role of tropical forests as a future sink or source of carbon remains unclear. However, to go beyond the current state of the art in C-N-P modelling, more empirical data are crucial to strengthen the linkages between climate, soil biogeochemical processes and forest functioning. More measurements, new approaches, experiments and modelling efforts are key tools needed to fill the gap of knowledge related to nutrient limitation and Amazon forest functioning.

In an attempt to fill the gap of knowledge regarding Amazon forest functioning and the complexity of the mechanisms behind P cycling, two major experiments are currently being initiated in lowland Amazon forests in Brazil: the first large-scale factorial nutrient addition experiment (AFEX - Amazon Fertilisation Experiment) and the first free air CO_2 enrichment in mature tropical forests (AmazonFACE). In addition, another nutrient manipulation experiment is currently being installed in French Guiana (IMBALANCE-P). These initiatives will enhance our mechanistic understanding about how Amazon forests thrive in such highly weathered soils and help us elucidate if P limitation will affect atmospheric CO_2 assimilation by these forests. Based on the results summarised here, the following chapters aim to fill some of the scientific gaps regarding the role of belowground mechanisms in nutrient cycling in Amazon forests, both in naturally low-fertility soils and in response to soil nutrient manipulation.

Chapter 3 - Multiple phosphorus acquisition strategies adopted by fine roots in low-fertility soils in central Amazonia



3. Multiple phosphorus acquisition strategies adopted by fine roots in low-fertility soils in central Amazonia

An edited version of this chapter has been accepted in **Plant and Soil** journal as: Lugli, L. F., Andersen, K. M., Aragão, L. E. O. C., Cordeiro, A. L., Cunha, H. F. V., Fuchslueger, L., Meir, P., Mercado, L. M., Oblitas, E., Quesada, C. A., Rosa, J. S., Schaap, K. J., Valverde-Barrantes, O. and Hartley, I. P. 2019. Multiple phosphorus acquisition strategies adopted by fine roots in low-fertility soils in central Amazonia. Plant and Soil special issue S74 – Amazon.

3.1. Abstract

Ancient Amazon soils are characterised by low concentrations of soil phosphorus (P). Therefore, it is hypothesised that plants may invest a substantial proportion of their resources belowground to adjust their P-uptake strategies, including root morphological, physiological (phosphatase enzyme activities) and biotic (arbuscular mycorrhizal (AM) associations) adaptations. Since these strategies are energy demanding, I hypothesise that trade-offs between morphological traits and root phosphatase exudation and symbiotic associations would occur. Specifically, I expected that trees which invest in finer roots, and therefore have greater ability to explore large soil volumes, would have a high investment in physiological adaptations such as enhanced phosphatase production. In contrast, I expected that trees with predominantly thicker roots would invest more in symbiotic associations, in which carbon is traded for P acquired from AM fungal communities. I collected absorptive roots (<2mm diameter) from a lowland central Amazon forest near Manaus, Brazil, measuring fine root diameter, specific root length (SRL), specific root area (SRA), root tissue density (RTD), root phosphatase activity (PHOS) and arbuscular mycorrhizal (AM) fungi colonisation. Root morphological traits were related to PHOS activity, with higher PHOS activity in roots with higher SRL and SRA but lower RTD. However, the degree of AM colonisation was not related to any measured root morphological trait. Fine absorptive roots likely benefit from having low RTD, high SRL, SRA and PHOS exudation to acquire P efficiently. However, because AM colonisation was not related to root morphology, I suggest that investment in

multiple P-uptake strategies is required for maintaining productivity in central Amazon forests.

3.2. Introduction

Amazon forests are highly productive ecosystems, crucial for global carbon (C), nutrient and water cycling (Malhi et al. 2004) and C storage (Pan et al. 2011). Yet, about 60% of the soils in the Amazon basin are characterised by concentrations of phosphorus (P) and cations that are considered limiting to plant growth (Quesada et al. 2011). Phosphorus is used in an array of plant processes, including photosynthesis and respiration (Vance et al. 2003; Raghothama and Karthikeyan 2005), and, as a result, P availability in soils is usually considered to play an important role controlling growth rates in Amazon forests (Quesada et al. 2010; Quesada et al. 2012). In young ecosystems, phosphorus is supplied to soil almost exclusively by the weathering of the parent material (Walker and Syers 1976), with small contributions from atmospheric deposition, varying greatly in space and time (Yu et al. 2015). Generally, during soil development, the importance of parent material as a source of nutrients declines considerably over time; total soil P concentration decreases due to weathering and leaching, and the remaining P pools become gradually more recalcitrant, adsorbed or occluded within mineral matrices and organic matter (Walker and Syers 1976; Tiessen et al. 1984; Quesada et al. 2010). Hence, over geological time scales, tropical forests become essentially dependent on the recycling of organic P from litter decomposition.

The concept of P limitation in tropical forests is widely accepted, but still, the mechanisms that allow Amazon forests to thrive in such P-poor environments are not clear. Plants can respond in two ways to overcome P limitation in soils: 1) increasing P-use efficiency aboveground, and/or 2) by improving P uptake belowground (Vitousek et al. 2010; Kitayama 2013). Plants can enhance aboveground P-use efficiency by increasing the residence time of P within the canopy (Kitayama et al. 2000; Kitayama and Aiba 2002; Vitousek 2004; Kitayama 2013), maximising P resorption in leaves before senescence (Aerts 1996; Raghothama and Karthikeyan 2005; Hayes et al. 2014) or reducing or replacing P in essential compounds with other elements (White and Hammond 2008;

Lambers et al. 2012). In addition to aboveground phosphorus efficient use, plants under low-fertility soils may be able to alter their root morphology and physiology, and/or vary their reliance on mycorrhizal fungi to optimise nutrient acquisition (Raghothama 1999; Richardson and Simpson 2011). Although belowground plant nutrient uptake mechanisms could be important contributors to tropical forest productivity (Bardgett et al. 2014), there is limited understanding of the role of different root traits in P uptake in tropical forests, especially in the Amazon.

Phosphorus is very immobile in soils, and for this reason roots preferentially move towards P (Aerts 1999). Root morphological traits, such as total root length, surface area and diameter directly control the efficiency by which P is assimilated by plants (Eissenstat 1992; Eissenstat and Yanai 1997), with small diameter roots usually being efficient at exploring large soil volumes (Bates and Lynch 2001; Hodge 2004; Liu et al. 2015). However, the only form of P assimilated by plants and microorganisms is inorganic phosphate, which is largely bound in organic compounds within tropical soils. Consequently, these compounds cannot be directly taken up by plants and microorganisms and need to be degraded before being assimilated by roots (Lambers et al. 2006). The main process through which organic P is hydrolysed in soils is via the activity of phosphatase enzymes released by microbes and plant roots (Olander and Vitousek 2000; Hinsinger 2001; Treseder and Vitousek 2001; Vance et al. 2003). Therefore, the coordination between small root diameter and large SRL and SRA together with phosphatase exudation may give fine roots a competitive advantage over thick roots by enabling them to explore the soil volume and mobilise organic P more efficiently (Liu et al. 2015; Kong et al. 2016).

Species with thick absorptive root systems, on the other hand, are not very efficient at nutrient foraging and are expected to preferentially invest in mycorrhizal associations to meet nutrient demands (Comas et al. 2014; Eissenstat et al. 2015; Kong et al. 2016). The very fine hyphal network typical of arbuscular mycorrhizas allow the fungi to acquire P away from P-depleted zones around roots, resulting in high inorganic P uptake in exchange for photosynthetically fixed C from the host plant (Hodge 2004; Smith and Read 2010; Eissenstat et al. 2015). In addition to their role in P uptake, AM fungi could benefit plants by altering the microbial community in the rhizosphere, changing soil aggregation and decreasing plant susceptibility to pathogens (Koide 1991;

Herre et al. 2007; Laliberte et al. 2015). Because of the multiple benefits that AM fungi confer to plants, this symbiotic association is extremely common and it is estimated that about 74% of all plant species are colonised by these fungi (Brundrett 2009; Smith and Read 2010).

Root morphological adaptations, as well as investment in phosphatase enzyme production and association with mycorrhizal fungi are, however, resource-costly (Jakobsen and Rosendahl 1990; Treseder and Vitousek 2001; McCormack et al. 2015). For instance, the construction of fine acquisitive roots might require less C per unit root length but these roots also display higher rates of respiration and faster turnover when compared to coarser roots (Laliberte et al. 2015; McCormack et al. 2015). Mycorrhizal fungi can receive up to 25% of C assimilated by plants (Jakobsen and Rosendahl 1990) and root exudates can represent up to half of belowground C allocation (Lynch and Ho 2005). As a result, it has been suggested that many trees do not invest energy in multiple nutrient acquisition mechanisms, but rather show a trade-off between one strategy at the expense of another (Ryan et al. 2012; Ushio et al. 2015; Nasto et al. 2017). One such trade-off in nutrient foraging strategies between thin and thick absorptive roots has been shown for a few tree species (Ushio et al. 2015; Chen et al. 2016) but the generality of such a mechanism at the ecosystem-level has yet to be tested.

Since the role of fine roots in forest functioning in Amazonia is poorly understood, this study aims to describe root strategies used by trees to overcome P-limitation in central Amazonia, testing for possible trade-offs between alternative strategies. To address this I investigated root morphological traits represented by root diameter (mm), specific root length (cm mg^{-1}), specific root area (cm^2 mg^{-1}) and root tissue density (mg cm^{-3}), as well as root phosphatase activity and association with arbuscular mycorrhizal fungi. My hypothesis was that under similar environmental conditions, a trade-off between resource investment in root morphological traits and P-uptake strategies would occur, with different predominant P-uptake strategies being associated with roots of different morphologies. I would therefore expect that root phosphatase activity (PHOS) should be negatively correlated with root diameter and RTD, but positively correlated with SRL and SRA and that AM colonisation should correlate positively with root diameter and RTD and negatively with SRL and SRA. Under an

alternative scenario, I hypothesised the lack of such a trade-off because of the very low P availability in soils and/or the many potential roles that AM fungi could play, resulting in roots investing in multiple P-uptake mechanisms independently of their morphology.

3.3. Material and methods

3.3.1. Study site description

This study was carried out in the Cuieiras Reserve at ZF-2, ca. 60 Km north of Manaus, Amazonas, Brazil, maintained by the National Institute of Amazonian Research (INPA). The vegetation is an old growth, *terra firme* lowland forest with mean air temperature of 26ºC and mean annual precipitation around 2,400 mm (Araújo 2002). The plots at the study site are characterised by high species diversity and the soils are classified as Ferralsols (World Reference Base for Soil Resources, 2006), with soil pH (in H_2O) ranging from 4 to 4.7. The soils in my study area show particularly low P concentrations in relation to other soils across the Amazon basin (Quesada et al. 2010; Quesada et al. 2011), being on the low range of fertility for Amazon soils. For instance, total P in the top 10 cm of soil along the basin varies from 29 - 968 mg kg^{-1} (Quesada et al. 2010), with total P in my study site varying from 118 - 217 mg kg^{-1} in the same soil horizon (Supplementary Table 3.1.; methodology as described in the Appendix 1).

I sampled 32 plots, measuring 40 m x 40 m, established in areas with similar soil, vegetation and terrain (all plateaus), with plots at least 50 m away from each other (Figure 3.1). The similar environmental conditions and consistently low-P concentrations in soils across the plots allowed us to investigate the extent to which, under similar soil conditions, different P-uptake strategies are used by trees at the ecosystem level. Due to the small variation in soil fertility among plots in my study site, relationships between soil P concentrations and root traits are only briefly discussed and are shown in Supplementary Figure 3.1.

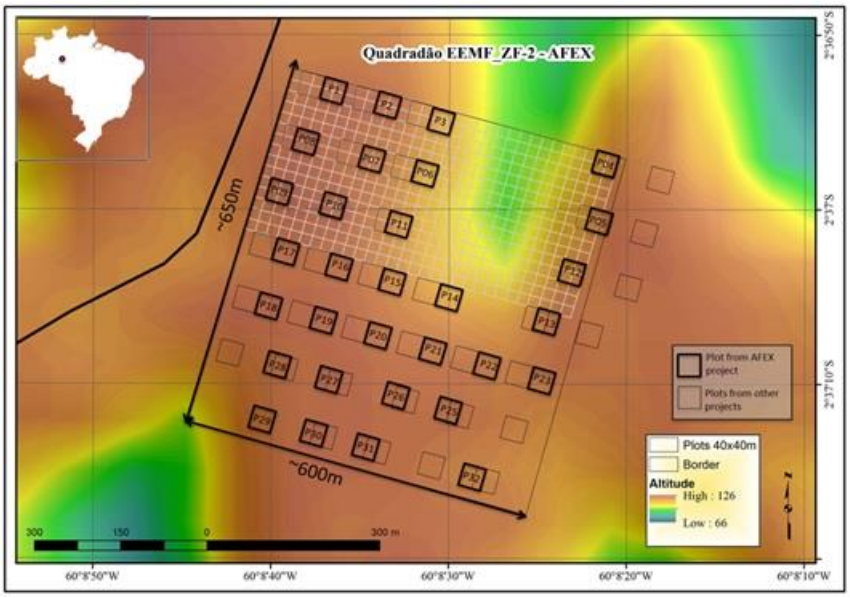


Figure 3.1. Distribution of 32 plots in a central Amazon forest at the ZF-2 reserve, 60 Km from Manaus, Brazil. All plots were installed in plateau areas with similar environmental properties.

3.3.2. Root sampling

Freshly produced fine-roots were sampled in May, 2016 using the ingrowth core technique (Metcalfe et al. 2007). In each plot (*n*=32), five 12 cm-diameter, 30 cm-deep, root-free ingrowth cores (2 cm plastic mesh bag) were installed in January-February 2016 in the central 20 m x 20 m plot area. Ingrowth cores were collected after 3-4 months of installation and all fine roots were manually extracted and root-free soil reinserted into the existing holes (Metcalfe et al. 2007). Root samples were divided by depth (0-5, 5-10, 10-20 and 20-30 cm) and cleaned by removing soil particles. Fine roots are usually defined as ≤ 2 mm in diameter, but because I sampled relatively young roots (< 4 months old), my samples represented only first to third order roots, as classified by Pregitzer et al. (2002). I therefore defined these roots by their function as absorptive fine roots, directly involved in nutrient uptake (McCormack et al. 2015). I restricted my analyses for subsamples from 0-10 cm depth interval, since the majority of roots were found in the top 10 cm soil layer. Five replicate cores were combined per plot (*n*=32) and subsamples were analysed for mycorrhizal colonisation and fine root acid phosphatase activity (PHOS). Another subsample was scanned for

morphology analysis and then dried at 60ºC to determine dry root mass. Root productivity was calculated as dry mass of roots (mg) produced per day for the top 10 cm soil layer.

3.3.3. Root morphology

Root samples from the 0-10 cm layer were scanned twice to determine root morphological traits. First an image was obtained with all roots from the sample, subsequently, some root fragments were picked to analyse for mycorrhizal colonisation and the sample was scanned again to generate a second image on which root fragments used for AM analysis were absent. The morphological traits for the fragments used for AM analysis (methodology as described in the Appendix 1) were calculated using the difference between the two scanned images. In addition, another small subsample of root tips were used for the enzyme assay and scanned separately. All images were analysed using WinRHIZO (WinRHIZO Regular, Regent Instruments, Canada) to determine specific root length (SRL), specific root area (SRA), root tissue density (RTD) and mean root diameter (Metcalfe et al. 2008). SRL (cm mg^{-1}) was calculated as the length per unit root dry mass; SRA (cm^2 mg^{-1}) was calculated as root superficial area per unit dry mass; RTD (mg cm^{-3}) was calculated as root dry mass per unit root volume. With these three sets of root morphological measurements I was able to discuss general morphological traits at plot and ecosystem level but also look at correlations between the root morphological traits, AM colonisation and PHOS activity.

3.3.4. Root phosphatase activity

Potential root acid phosphomonoesterase activity (PHOS) was measured for roots from 0-10 cm soil depth (*n*=32) using a fluorimetric microplate assay adapted following protocols by German et al. (2011) and Turner and Romero (2010). All clean root samples were kept refrigerated (4ºC) to avoid tissue degradation and enzyme activities were measured within two weeks of root sampling using triplicate subsamples per plot. About 10 mg of each root sample (washed, fresh weight basis) were placed into a sterile 2 mL Eppendorf snap-cap

vial with 1 ml of buffer and 0.25 ml of Methylumbelliferyl-phosphate (MUF-phosphate; 2mM). A further identical root sample was prepared as control by adding 1.25 ml of buffer. In addition, buffer blanks (*n*=3) and substrate blanks (*n*=3) were prepared as 1.25 ml of buffer (no roots, no substrate) and 1 ml of buffer + 0.25 ml of MUF-phosphate (no roots), respectively. Samples were incubated for 30 min at ~25ºC while gently shaking, then 50 µL of 1 M NaOH were added to all samples and standard vials to terminate the reactions. Aliquots of the sample solution were pipetted into a black 96-well microplate and 20 min post termination, fluorescence was read on a fluorometer (Tecan Infinite® 200 PRO, Grödig, Austria), at 365 nm excitation and 450 nm emission. Roots were then removed from vials, rinsed with Milli-Q water and dried at 60ºC for 72 hours. Root PHOS activity per plot is represented in µmol MUF g^{-1} dry root mass h^{-1}. Based on the images from the scanned root subsamples, root PHOS activity was also calculated per cm root length and cm^2 root area (Supplementary Table 3.2. in Appendix 1).

3.3.5. Mycorrhizal colonisation

To determine AM colonisation, root samples from the 0-10 cm soil depth were cleaned and segments of fresh absorptive roots from the first three orders were cut and stored in 50% ethanol. Samples were then transferred to small cassettes and if needed, subsamples were separated by colour/appearance: dark roots, red/brown and clear roots. The clearing and staining processes, designed to highlight only the mycorrhizal structures, were adapted to tropical roots based on Brundrett et al. (1984) and Wurzburger and Wright (2015). The cassettes were placed in a 2.5% KOH solution and autoclaved at ~120° C for ± 10 minutes, with processing time depending on the darkness of the roots. If roots were still darkly pigmented after the clearing process, they were placed in alkaline H_2O_2 solution for further bleaching for ± 30 minutes, checking them constantly so no material was lost due to over clearing or bleaching. Before staining, roots were acidified by placing cassettes in 2% HCl solution for 30 minutes and then were added to a beaker with Trypan Blue 0.05% for 30 minutes. When consistently blue, roots were rinsed well and stored in distilled water in the fridge (4°C) for slide preparation. The time between root staining and AM colonisation analysis never

exceeded two weeks. Roots were placed in rows across the length of the slide and fixed using polyvinyl alcohol (PVA). To quantify mycorrhizal colonisation, 50-100 intersections per sample were read using the microscope micrometer (vertical cross-hair) to analyse the sections (McGonigle et al. 1990). At each intersection the colonisation was scored according to the following categories: no mycorrhizal structures, hyphae only, hyphae + arbuscules, hyphae + vesicles, hyphae + vesicles + arbuscules. Mycorrhizal colonisation was assessed as the percentage of the total root intersections along the root length that had mycorrhizal fungi. Only total AM colonisation (sum of colonisation by hyphae, arbuscules and vesicles structures) is discussed in detail in the results, and colonisation by each AM fungal structure are shown in Supplementary Table 3.2. in the Appendix 1.

3.4. Statistical analyses

Linear models were used to test for the influence of soil P fractions on root traits. Bivariate relationships between root morphological properties, root phosphatase activity and AM colonisation were described using Standard Major Axis (SMA) line fits using the package 'smatr' (Warton et al. 2006; Warton et al. 2012). Standard Major Axis analysis is generally used in plant allometry studies when there is no clear causation among the variables tested. By using the SMA test, I aimed to estimate the line of best fit between traits, taking into account inherent error associated with both axes. All statistical analyses were conducted in R version 3.3.3. (R Core Team, 2017).

3.5. Results

3.5.1. Root traits variation

Despite the common soil conditions in my study site (Table 3.1 and Supplementary Table 3.1.), I found marked variation in root morphology (Table 3.2 and Supplementary Table 3.2.), however, no significant relationships between soil P fractions and root traits were observed, with the exception of a positive relationship between bicarbonate Pi (one of the most readily available forms of P) and RTD (Supplementary Figure 3.1.). Correlations between root

morphological traits are not discussed here but are shown in Supplementary Figures 3.2-3.4 in the Appendix 1 for each set of morphological data. Fine root diameter varied from 0.39 to 1.1 mm, with RTD displaying a three-fold variation, ranging from 141.78 – 419.22 mg cm^{-3} (Table 3.2 and Supplementary Table 3.2.). Specific root area and root PHOS activity showed a four-fold variation, varying from 0.14 – 0.56 cm^2 mg^{-1} and 15 - 66 µmol g^{-1} dry root h^{-1} respectively. Among all traits measured, SRL was the most variable, ranging from 0.59 to 4.15 cm mg^{-1}. All sampled roots showed some degree of AM colonisation, ranging between 10 to 80% of root length colonised by AM, with an average of 44.31% (Table 3.2 and Supplementary Table 3.2.).

Table 3.1. Soil total P and soil P fractions following Hedley et al. (1982) methodology (details are in Appendix 1). Values represent mean (± SE) and ranges for each P fraction (mg kg^{-1}) extracted from all plots (*n*=32). Pi= inorganic P; Po= organic P. P fractions are shown in decreasing order of availability, with resin P being the most available fraction and residual P being the least available. Total P is the sum of all fractions; readily available P is calculated as the sum of resin P and bicarbonate Pi and Po (fractions that are very to moderately available for plants); total extractable P is calculated as the sum of all fractions except residual P. SE= standard error.

	Mean	**SE**	**Minimum**	**Maximum**
Resin P	4.46	0.17	2.59	6.44
Bicarbonate Pi	1.33	0.10	0.39	2.51
Bicarbonate Po	6.07	0.26	3.73	10.26
Hydroxide Pi	15.76	0.40	11.27	20.73
Hydroxide Po	15.27	0.49	9.27	19.09
HCl P	1.20	0.06	0.75	2.26
Residual P	104.4	3.11	68.9	166.7
Total P	148.4	3.41	118.5	217.4
Readily available P	11.86	0.35	7.84	16.22
Total extractable P	44.09	0.85	32.73	53.87

Table 3.2. Root productivity, morphological, physiological and biotic properties from 32 plots in a central Amazon forest. Values represent means and ranges for each root trait. SRL = specific root length (cm mg^{-1}); SRA = specific root area (cm^2 mg^{-1}); RTD = root tissue density (mg cm^{-3}), PHOS = acid phosphatase activity (µmol g^{-1} h^{-1}) and AM colonisation = arbuscular mycorrhizal fungi colonisation (%). SE = standard error. Mean root morphological data (diameter, SRL, SRA and RTD) and root productivity are from the whole ingrowth core sample. PHOS activity and AM colonisation were determined in roots subsampled from the total ingrowth core sample. All measurements, with the exception of AM colonisation and diameter, are shown in root dry weight basis.

	Mean	**SE**	**Minimum**	**Maximum**
Root productivity (mg day^{-1})	7.85	0.91	2.77	16.01
SRL (cm mg^{-1})	2.09	0.14	0.58	4.15
SRA (cm^2 mg^{-1})	0.33	0.01	0.13	0.56
RTD (mg cm^{-3})	239.5	10.5	141.8	419.2
Diameter (mm)	0.69	0.04	0.39	1.11
PHOS (µmol g^{-1} h^{-1})	36.05	2.58	15.46	65.98
AM colonisation (%)	44.31	2.93	10.17	80.95

3.5.2. Relationship between root morphological properties and P-uptake strategies

In partial corroboration with my hypothesis, I found that root morphological traits were related to the levels of root PHOS exudation (Figure 3.2). Root PHOS activity decreased with increasing RTD (r= 0.14, *P*= 0.035), and increased with increasing SRL (r= 0.16, *P*= 0.02) and SRA (r= 0.25, *P*= 0.003), with no significant relationship between root PHOS and root diameter (Figure 3.2a). In contrast, no relationship between total AM colonisation levels and root morphological traits was found (correlations between root morphological traits and AM colonisation by different fungi structure are not discussed here but are shown in Supplementary Figure 3.5. in the Appendix 1). The lack of a trade-off between root morphological traits measured here and the degree of root AM colonisation (Figure 3.3) therefore supports my alternative hypothesis that AM colonization is independent of morphological strategies in this study site.

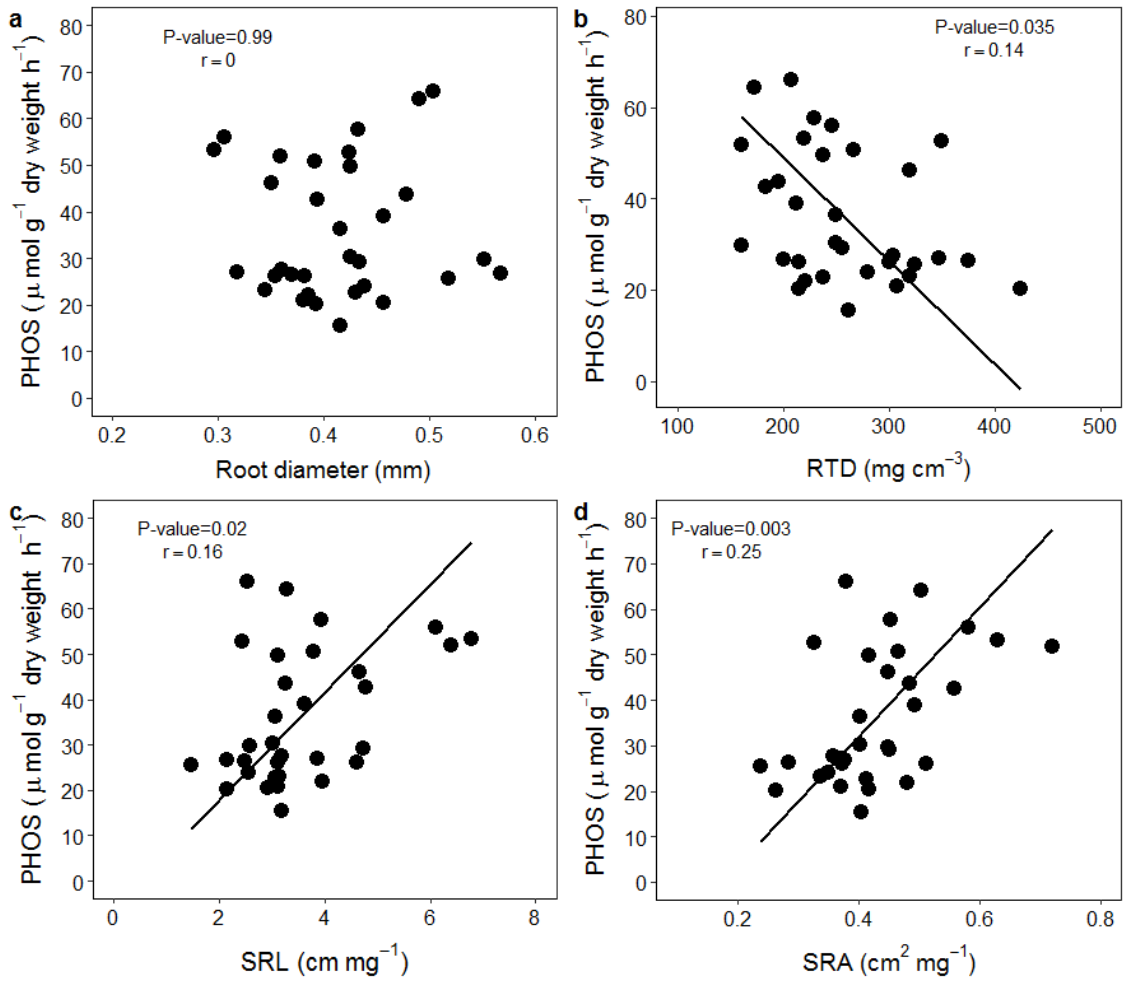


Figure 3.2. Standardised major axis (SMA) relationships between root phosphatase activity and root morphological properties. Each dot represent a mean of root traits per plot (0-10 cm soil depth) extracted from ingrowth cores (ecosystem-level measurement). PHOS = acid phosphatase activity (µmol g^{-1} dry root h^{-1}); SRL = specific root length (cm mg^{-1}); SRA = specific root area (cm^2 mg^{-1}); RTD = root tissue density (mg cm^{-3}). Solid lines indicate significant relationships (p<0.05).

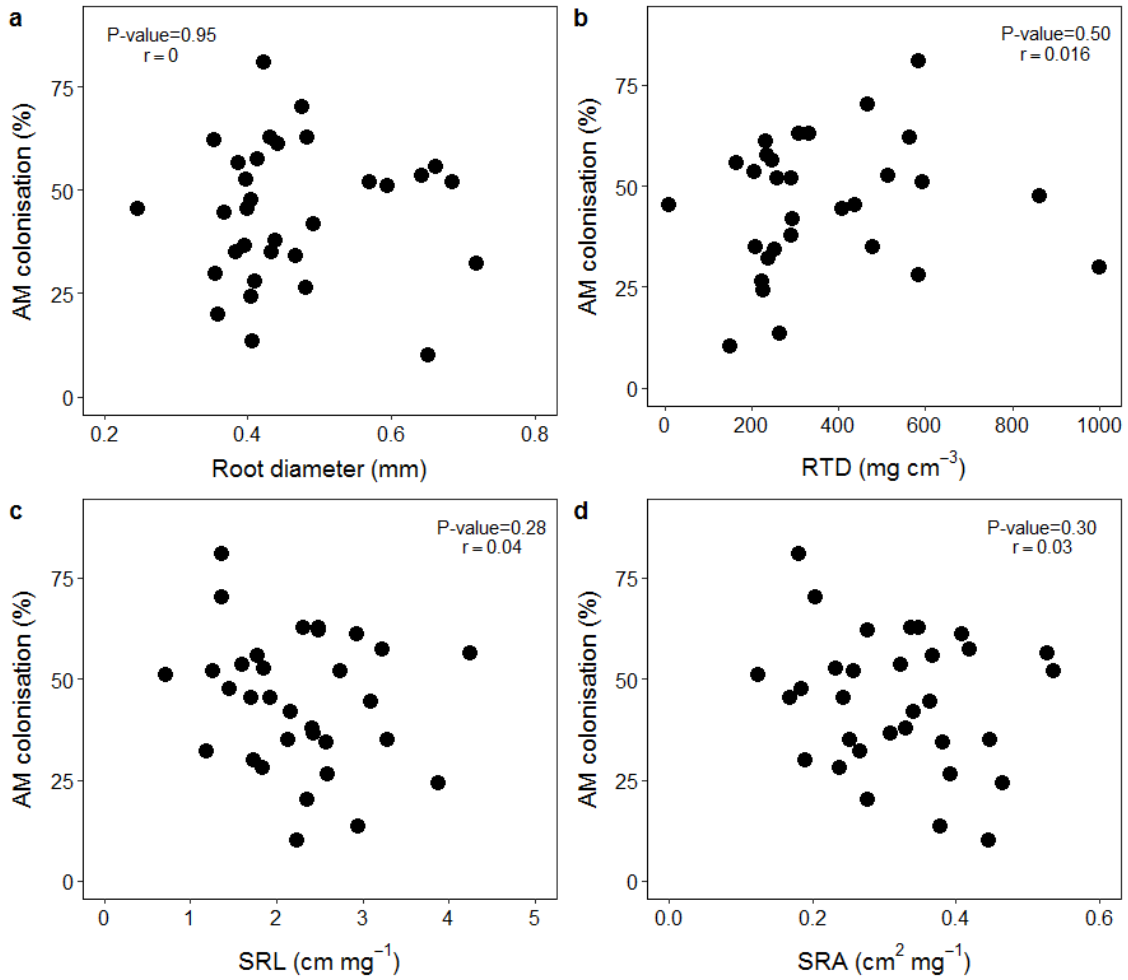


Figure 3.3. Standardised major axis (SMA) relationships between total root AM colonisation (% of root length) and root morphology properties. Each dot represent a mean of root traits per plot (0-10 cm soil depth) extracted from ingrowth cores (ecosystem-level measurement). SRL = specific root length (cm mg^{-1}); SRA = specific root area (cm^2 mg^{-1}); RTD = root tissue density (mg cm^{-3}).

3.5.3. Trade-offs between root P-uptake strategies

Since root morphological traits were found to influence root PHOS activity but not AM colonisation, the existence of a trade-off between both root P-uptake strategies was not supported by my results. This is further demonstrated by the lack of a clear relationship between PHOS activity and AM colonisation (Supplementary Figure 3.6. in the Appendix 1) although it should be noted that it was not possible to carry out the enzyme and mycorrhizal assays on the same root subsamples. Therefore, the idea that trees with different root morphologies

would invest in one dominant P-uptake mechanism at the expense of another was not supported.

3.6. Discussion

Fine root traits are often reported to reflect plant efficiency in acquiring the most limiting soil nutrients (Lambers et al. 2006; Raghothama 1999). Based on a presumed trade-off in energy investments between root trait adaptations to improve P uptake, I aimed to test if in central Amazon forests trees would also preferentially display one dominant root P-uptake strategy. Despite the similar topography and the relatively narrow variation in P availability in my study site, root traits did not converge towards a single P-uptake strategy but rather displayed multiple complementary strategies to overcome apparent P limitation. I therefore suggest that the variations in root traits found in this central Amazon forest reflect the dominant features of a much larger diversity of adaptations displayed by different plant species in low fertility soils.

3.6.1. Variation in root morphology

Although I found SRL and SRA varying across plots, mean values were similar to the ones found by Metcalfe et al. (2008) in a clay-rich Ferralsol in Para, Brazil. Also using the ingrowth core technique, these authors found SRL varying from 1 to 3.5 cm mg^{-1} and SRA varying from 0.25 to 1.5 cm^2 mg^{-1} during a year-round sampling campaign, while mean values found here for one sampling interval (3-4 months) were 2.09 cm mg^{-1} and 0.33 cm^2 mg^{-1} for SRL and SRA, respectively. Wurzburger and Wright (2015) reported mean values of 1.5 cm mg^{-1} for SRL and 150 mg cm^3 for RTD at a site in Panama, but this was based on measurements made on standing stocks rather than in ingrowth cores. Additionally, Kong et al. (2014) also using standing stock cores, found mean SRL and RTD of 5.6 cm mg^{-1} and 358.5 mg cm^{-3} respectively for trees at a subtropical site in China, contrasting with 2.09 cm mg^{-1} and 239.47 mg cm^{-3} found in this study. The data reported here and from other studies in tropical forests are, however, not directly comparable because of the difference in methods used (ingrowth core *versus* standing stock biomass). I aimed to investigate young,

absorptive roots, making ingrowth cores the most appropriate method for my study, but further research on established root systems in future studies would also be valuable.

3.6.2. Relationship between root morphology and phosphatase activity

I hypothesised that root phosphatase activity would be related to root morphological traits, with higher PHOS activity in roots with smaller diameter and lower tissue density and therefore higher SRL and SRA. Although my samples refer to plot means at the ecosystem level, I found partial support for the first hypothesis, since three of the four root morphological traits analysed here were correlated as expected with PHOS activity, with the exception of root diameter. My results confirm the findings of Ushio et al. (2015) who found lower PHOS activity with increasing RTD, and higher PHOS activity with increasing SRA at the species level in tropical montane forests in Borneo. On the other hand, Ushio et al. (2015), studying fine roots up to 2 mm diameter, reported that PHOS activity was negatively correlated with root diameter, whilst no such relationship was found in this study. The lack of a significant relationship between root diameter and PHOS found here could be due the small variation in root diameter from my root subsamples (0.29 – 0.57 mm), since only first order acquisitive roots were analysed.

A decline in RTD with increasing PHOS could be seen as a strategy to maximise belowground biomass investment per root volume (Eissenstat 1992) thereby reserving energy for phosphatase exudation and consequently P uptake. Despite the low C-costs of construction, fine roots have higher costs of maintenance when compared to coarse roots, displaying high rates of respiration and turnover (Eissenstat and Yanai 1997; McCormack et al. 2015). For instance, because of their lower mechanical strength, fine roots are more susceptible than coarse roots to attack by soil-borne pathogens and herbivores (Laliberte et al. 2015; Ushio et al. 2015). The high investment in producing roots with high SRA and SRL associated with high risks of carbon loss could be compensated by high-P acquisition resulting from higher PHOS activity (Ushio et al. 2015). Because samples in this study represent many different plant species, correlation coefficient values obtained for PHOS activity and morphological traits were low,

but despite the species-specific noise, my analysis still captures the dominant signal on the relationships tested at the ecosystem level. Nevertheless, my results suggest that investing in fine long roots and PHOS exudation seem to be an efficient P-uptake mechanism, possibly overcoming the potential costs of root maintenance.

The efficiency of plants in acquiring P increases under P limitation and therefore, higher root PHOS activity is usually found in soils with low P concentrations (Raghothama and Karthikeyan 2005; Kitayama 2013). Root PHOS activity at the site studied here varied from 15 to 65 µmol g^{-1} dry root h^{-1}, which was lower than the values found in a montane forest in Borneo (100 - 200 µmol pNPP g^{-1} h^{-1}) (Kitayama 2013; Ushio et al. 2015) but higher than root PHOS activity found in plants associated with AM in Panama (10 µmol pNPP g^{-1} h^{-1}) (Steidinger et al. 2015) and also higher than in forests in Costa Rica (5-12 µmol 4-MUF g^{-1} h^{-1}) (Nasto et al. 2014; Nasto et al. 2017). Total soil P concentrations in forests in Borneo were very low, ranging from 10-60 mg kg^{-1}, which could explain the higher investment of plants in root PHOS activity reported by Kitayama (2013) and Ushio et al. (2015). In addition, litter decay rates and consequently organic P mineralisation is slower in montane forests because of the lower temperatures (Grubb 1977; Vitousek and Sanford 1986), which could also translate into higher phosphatase exudation in Borneo. On the other hand, total P concentrations in soils in Costa Rica (665-1600 mg kg^{-1}) (Nasto et al. 2014), were much higher than those found in this study (118-227 mg kg^{-1}). Thus, the low root PHOS activity in Costa Rica and Panama could therefore suggest that (1) central American forests are not as P-limited as the central Amazon forest studied here or (2) the greater P availability allows trees in these central American forests to invest in P-use/acquisition strategies other than PHOS activity.

3.6.3. Relationship between root morphology and total mycorrhizal colonisation

I also hypothesised that thick, dense and short absorptive roots would have higher AM colonisation than small diameter roots. Despite substantial variation in root morphological traits, no significant relationships were observed between AM colonisation and the other root traits, irrespective of whether relationships with

total mycorrhizal colonisation rates or rates of colonisation by different AM structures (arbuscules, vesicles, hyphae; see Appendix 1) were investigated. Total AM root colonisation in my study site ranged from 10-80%, with a mean of 44%, whilst earlier studies have shown that AM colonisation in Brazilian native woody species could range from absent to about 80% in different levels of soil-P (Siqueira and Saggin-Júnior 2001). Apart from soil conditions, root development (length, diameter, root hair density and nutrient uptake capacity), environmental characteristics (soil nutrient availability, plant competition, soil microbial biota, soil depth, seasonality) and fungal hyphae properties (root colonisation rate, growth rate and nutrient uptake capacity) have all been reported to influence AM colonization (Hoeksema et al. 2010; Johnson 2010; Smith et al. 2011; Li et al. 2017). Evidence for the positive correlation between root diameter and AM colonisation exists for some species in central Amazon (St John 1980), but it is possible that relationships at the ecosystem level may be different or harder to detect.

Various studies have suggested that fine and thick absorptive roots display different strategies for nutrient acquisition, with thin roots depending on directly acquiring nutrients and thicker roots depending more on mycorrhizal fungi (Eissenstat et al. 2015; Liu et al. 2015; Chen et al. 2016; Kong et al. 2016). The absence of a significant relationship between AM colonisation and root morphological traits found in this study was also reported for Brazilian native woody species studied under greenhouse conditions (Siqueira and Saggin-Júnior 2001) and in a meta-analyses of studies with plants from different biomes (Maherali 2014). Siqueira and Saggin-Júnior (2001) and Maherali (2014) reported certain coarse-root tree species being completely AM-independent, whereas other species with small root diameter showed high AM colonisation. Arbuscular mycorrhizal fungi form associations within root cortical cells only, and for this reason, root diameter does not necessarily represent an appropriate proxy for mycorrhizal colonisation (Brundrett 2002; Guo et al. 2008; Comas et al. 2014). For roots with similar diameters, those with a greater cortex to stele ratio, are usually expected to support higher AM colonisation (Valverde-Barrantes et al. 2016; Kong et al. 2017). Therefore, differences in key root properties between species may obscure relationships between diameter and colonisation rates. Other relevant properties of roots, such as root architecture (e.g. branching

frequency, root hair density and length), could also change in response to AM colonisation (Maherali 2014), but were beyond the scope of this study. As an alternative, there is evidence suggesting that trees lack efficient mechanisms to control AM colonization (Johnson 2010; Valverde-Barrantes et al. 2016), meaning that all root cortical tissues created could be potentially colonized independently of P availability. Furthermore, the relative importance of internal AM structures *versus* extraradical hyphae for nutrient foraging is still not completely understood (Smith and Read 2010), and since I did not sample soil hyphae, I assumed that the percentage of internal colonisation was a proxy for P uptake.

3.6.4. Trade-off between root P-uptake strategies and the consequences for ecosystem functioning under P-limitation

Investment in nutrient acquisition by plants demands the allocation of other resources, such as C and nitrogen (N) (Nasto et al. 2017). For instance, it has been suggested that mycorrhizal fungi can receive up to 25% of C assimilated by plants (Jakobsen and Rosendahl 1990) and root exudates can represent up to half of belowground C allocation (Lynch and Ho 2005). In addition, the N costs of P uptake via PHOS activity could range between 1 to 16 g N per g of P (Treseder and Vitousek 2001). Based on the possible resource costs of investing in multiple P-uptake strategies simultaneously, I hypothesised the existence of a trade-off between PHOS activity and AM colonisation (Nasto et al. 2017). However, because only PHOS activity was related to root morphological traits with no influence on AM colonisation, I can infer that these two strategies were not negatively correlated as I hypothesised, suggesting that at the ecosystem level there is not a trade-off between these P-uptake mechanisms at my study site.

With soil development, the contribution of primary minerals as a source of P diminishes to very low levels (Walker and Syers 1976) and plants may rely more on recycling organic P from vegetation inputs to meet their P demand. Inorganic phosphate (mainly PO_4^{-3}) is the only form of P assimilated by plants and, therefore, organic P needs to be hydrolysed by phosphatase enzymes to release inorganic P (Lambers et al. 2006). For this reason, I would expect root PHOS activity to play a major role in P cycling in this central Amazon forest if compared

to the role of AM fungi. The average level of AM root colonisation in my study site was 44%, lower than compared to the 70% AM colonisation observed in nutrient-rich soils in Panama (Wurzburger and Wright 2015). However, independent of root morphology, all plots in my study site displayed moderate levels of AM colonisation, suggesting that besides improving inorganic P uptake, AM association could play other roles. Arbuscular mycorrhizal fungi could benefit plants by increasing the uptake of micronutrients (e.g. zinc, copper), change the microbial community in the rhizosphere, increase plant chemical defence against pathogens and herbivores (Koide 1991; Herre et al. 2007; Laliberte et al. 2015) and also possibly by influencing phosphatase production by plants (Joner et al. 2000; Nasto et al. 2014). Fine roots are more susceptible to soil pathogen and herbivore attack (Laliberte et al. 2015) and the potential role of AM increasing plant defence could therefore explain why small diameter roots were found to be colonised by AM in my study site. The multiple functions of AM fungi associating with roots could also obscure the relationship between AM colonisation rates and P uptake.

Additionally, the lack of correlation (neither negative nor positive) between AM colonisation and root PHOS could suggest the possible complementary role of both strategies (Turner 2008; Steidinger et al. 2015). In this scenario, roots would benefit by investing in both strategies at the same time, since AM fungi and PHOS are known to explore different forms of P in soils: roots release phosphatase enzymes to mineralise organic P whilst AM fungi could benefit plants by exploiting inorganic P pools that do not need to be hydrolysed by enzymes or, more importantly, that have already been released by phosphatase enzymes (Nasto et al. 2014). This mechanism may be of special importance for fine absorptive roots in my study site, which displayed intermediate levels of AM colonisation and phosphatase exudation. This could also suggest that fine roots with high SRL and SRA may still not ensure adequate nutrient supply for some species in low fertility soils in the tropics, making them also dependent on AM (Siqueira and Saggin-Júnior 2001).

Investing in both enzyme production and AM association at the same time could also suggest that plants have access to enough resources, such as C and N, to support both strategies. In addition, mechanisms such as the exudation of organic acid/anions by roots could be an important plant reaction under P

deficiency (Lambers et al. 2006; Aoki et al. 2012), but were not analysed in this study. Since my measurements were made at the ecosystem level, I was not able to analyse the individual relationships between root morphology, phosphatase activity and AM colonisation at the species level and therefore these interpretations could be different if I could control for host species. For instance, not all tree species have the same P requirements and P-use-efficiencies and therefore the investment in strategies to scavenge P could differ. Furthermore, plant benefits due to association with AM fungi would vary according to the tree and fungus identity, with similar levels of AM colonisation theoretically resulting in different P-uptake rates for different species (Siqueira and Saggin-Júnior 2001; Herre et al. 2007; Maherali 2014). Given the multiple and increasing recalcitrant P forms in highly weathered soils, enzyme production, association with AM fungi and a high diversity of root morphological traits, combined could maximize P uptake (Kong et al. 2014; Zemunik et al. 2015; Li et al. 2017), indicating that in soils with very low P availability, such those in central Amazon, more than one P-uptake strategy is needed. Ultimately, understanding how root traits vary at the ecosystem level and how they relate to each other is critical to expand our knowledge about P cycling mechanisms, especially in the context of P-limitation in tropical forests with very high tree diversity.

3.7. Conclusions

In a P-poor tropical forest in central Amazonia, I show that morphological traits of absorptive fine roots are related to root phosphatase activity. However, these roots are also associated with AM fungi, independent of their morphology. I suggest that: 1) the substantial investment in multiple root P-uptake strategies further emphasises the importance of P-limitation to the function of these forests; 2) mycorrhizal association could have other benefits for plants other than only P uptake; 3) the relative abundance of other resources such as water, light, carbon and nitrogen may explain how plants are able to invest resources in multiple P-uptake mechanisms; and 4) the high diversity of tree species and the resulting diversity and combinations of P-uptake strategies makes detecting possible trade-offs between individuals or species challenging, which highlights the importance of ecosystem-level estimates (i.e. inability of add up all possible

strategies in a meaningful way). Overall, I conclude that because of the many forms that P is found in Amazon soils and the multiple steps needed to make P available for plants, roots also display a range of adaptations to enhance P-uptake under limitation. The hydrolysis of organic P by root phosphatase exudation and inorganic P uptake by AM fungi could be seen as complementary mechanisms contributing to the functioning of this central Amazon forest, at even very low P availability. Finally, after describing root adaptations to low-P availability in naturally low-fertility soils in this chapter, the following chapter (Chapter 4) aims to detect evidence for alleviation or exacerbation of P-limitation in a central Amazon forest, by capturing any changes in root traits related to P-acquisition following nutrient addition.

Chapter 4 - Fine root responses to phosphorus, nitrogen and cations addition in a lowland forest in central Amazon

4. Fine root responses to phosphorus, nitrogen and cations addition in a lowland forest in central Amazon

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4.1. Abstract

Phosphorus (P) is hypothesised to be the main nutrient limiting tropical forest productivity, but more recent evidence suggests that multiple nutrients could regulate forest functioning. Root functional traits usually represent a balance between maximising the acquisition of limiting resources and minimising root tissue construction and maintenance. On low-fertility sites, plant economic theory predicts that if the supply of the limiting nutrient in soils is increased, plant investment in root biomass and nutrient uptake strategies should decrease. To test this, I investigated the response of fine root properties to the addition of nutrients in slow-growing primary rainforest established on low fertility soils in central Amazon. Using the ingrowth core method, I sampled young fine roots (<2 mm diameter) and measured root morphological traits (root diameter, specific root length, specific root area and root tissue density), root biomass production, root phosphatase enzyme activity and root mycorrhizal colonisation in 32 plots after seven months of phosphorus, nitrogen (N) and cations addition in a fully factorial design. I hypothesised that the addition of P would reduce root productivity, phosphatase activity and mycorrhizal colonisation, with root morphology shifting as a sign of alleviation of P limitation by increasing root diameter and tissue density and decreasing specific length and area. I also hypothesised that the addition of N and/or cations would exacerbate P limitation and therefore root productivity and phosphatase exudation would increase, with morphological traits changing towards finer and longer roots. Contrary to expectations, root productivity in the first seven months post-fertilisation was

>50% higher in plots where cations were added, with no effects of P or N addition observed. The addition of cations and P increased root diameter, mainly for the 0-10 cm soil layer, with no significant effects on other root morphological traits. As predicted, root phosphatase activity strongly decreased with P addition, indicating alleviation of P limitation. Mycorrhizal colonisation, on the other hand, increased with P addition, suggesting a potential shift in P-uptake strategies, from mining (e.g. release of phosphatases) to foraging root traits (e.g. association with mycorrhizas). These results support the hypothesis that P limits some aspects of plant functioning in this central Amazon forest, but also suggest that cations could play an important role in controlling fine root production and the expression of root traits. In conclusion, multiple nutrients may limit belowground processes in central Amazon forests and even slow-growing tropical rainforest can respond rapidly to changes in soil nutrient availability.

4.2. Introduction

Tropical rain forests are the most diverse and productive terrestrial ecosystem on Earth (Malhi and Grace 2000), representing the largest terrestrial carbon sink (2.83 Pg C per year), with the Amazon forest alone contributing about 40-50% of this total C uptake (Pan et al. 2011). It is hypothesised that tropical plant growth and net primary production (NPP) may be further stimulated under atmospheric CO_2 enrichment (Kimball and Idso 1983; Ainsworth and Long 2005; Norby et al. 2005). However, future uptake may ultimately be controlled by the amount of available nutrients in soil (Hungate et al. 2006) as well as how efficiently plants can acquire and use nutrients. The vast majority of Amazon forests occupy soils characterised by very low concentrations of phosphorus (P) and cations that could limit plant growth (Quesada et al. 2011), ultimately influencing forest responses to elevated CO_2. While in temperate forests nitrogen (N) is usually considered to limit plant growth, P is considered the primary limiting nutrient in tropical lowland forests (Walker and Syers 1976; Vitousek and Sanford 1986). Phosphorus is supplied to soil almost exclusively by weathering of the parent material (Walker and Syers 1976) and the intense soil weathering, combined with high topographic stability and the hot and humid climate of the Amazon results in

decreasing P availability with soil development (Irion 1978; Sombroek 2000; Quesada et al. 2011).

As a consequence of low P availability in the majority of Amazon soils, plants are expected to display an array of mechanisms to adapt to low-P conditions. In low-fertility soils plants are able to alter their morphological, physiological, biochemical and molecular properties to optimise nutrient acquisition (Aerts 1999; Raghothama 1999). Belowground, root functional traits represent a balance between maximising the acquisition of limiting resources and minimising the costs of root tissue construction and maintenance (Aerts and Chapin 2000; Wurzburger and Wright 2015). Because of the low mobility of P in soils, roots usually move towards P (Aerts 1999) and therefore changes in root diameter and specific root length (SRL - amount of root length produced per unit root mass) towards thinner and longer roots facilitate the exploration of greater soil volume and potentially small patches with higher moisture and nutrient availability (Hodge 2004; Metcalfe et al. 2008). Moreover, roots can also typically benefit from association with arbuscular mycorrhizas to meet nutrient demands (Hodge 2004; Comas et al. 2014; Eissenstat et al. 2015; Liu et al. 2015; Kong et al. 2016). The very fine hyphal network typical of arbuscular mycorrhizas (AM) allow the fungi to acquire P away from P-depleted zones around roots, resulting in high inorganic P uptake in exchange for photosynthetically fixed C from the host plant (Hodge 2004; Smith and Read 2010; Eissenstat et al. 2015).

Phosphorus can be found in soils in different organic and inorganic pools with different availabilities, but only inorganic P can be directly assimilated by plants and soil microorganisms (Lambers et al. 2006). In P-poor tropical soils, large fractions of P are bound in organic compounds which cannot be directly taken up by plants and microorganisms and consequently these compounds need to be degraded before being assimilated by roots (Lambers et al. 2006). The hydrolysis of organic P happens through the activity of phosphatase enzymes released by microbes and plant roots (Olander and Vitousek 2000; Hinsinger 2001; Treseder and Vitousek 2001; Vance et al. 2003). Therefore, some plant species invest in long thin roots, which can explore small soil pores and the organic soil layer, releasing phosphatases to mobilise organic P (Kong et al. 2016; Liu et al. 2015). Root production, morphological adaptations, as well as investment in phosphatase enzyme production and association with mycorrhizal fungi are,

however resource-costly (Jakobsen and Rosendahl 1990; Treseder and Vitousek 2001; McCormack et al. 2015). Therefore, there are likely to be trade-offs between uptake strategies, and on low-fertility soils, when the supply of the limiting nutrient is increased, plant investment in root biomass and nutrient uptake strategies is predicted to decrease (Bloom et al. 1985).

Nutrient manipulation experiments are one of the most robust methods to directly test for nutrient limitation in terrestrial ecosystems (Cleveland et al. 2011; Sullivan et al. 2014; Wright et al. 2018). A diverse range of fine root responses to nutrient manipulation in tropical forests has been detected from the few established experiments in lowland tropical forests. For instance, potassium (K) was found to significantly decrease root biomass production and increase root tissue turnover in a large-scale nutrient experiment in tropical forests in Panama (Wright et al. 2011; Yavitt et al. 2011; Sayer et al. 2012). The addition of P, on the other hand, was found to increase fine root biomass production (Yavitt et al. 2011), and affect root morphology at the same site in Panama (Wurzburger and Wright 2015). In another fertilisation experiment in a tropical forest in Costa Rica, the addition of N and P increased nutrient concentration in roots, but no effect on root biomass production was detected after two years (Alvarez-Clare and Mack 2015). Although the hypothesis of P-limitation in tropical forests is widely accepted, clear evidence from large-scale experiments is variable and limited, mainly due to differences in soil properties, forest structure and plant diversity, with very little information about the contribution of belowground plant mechanisms to nutrient uptake in Amazon forests. The responses obtained by those studies reinforce the importance of conducting a similar experiment in central Amazonian forests under very-low fertility soils, before any generalisation about P limitation in tropical forests is made.

The aim of this study was to determine if there were any changes in the nutrient cycling mechanisms adopted by trees after nutrient addition in a central Amazon forest. To investigate how root properties relate to nutrient acquisition, I measured morphological traits (root diameter, specific root length, specific root area and root tissue density), root biomass and productivity, root phosphatase enzyme activity and arbuscular mycorrhizal colonisation in the first large-scale nutrient manipulation experiment in central Amazon forests (AFEX). Measurements were conducted seven months after nutrient addition and

therefore represent short-term ecosystem responses to fertilisation. My first hypothesis was that P limitation should be manifested in plots fertilised with P by a decrease in investment in root phosphatase activity and AM colonisation. My second hypothesis was that following nutrient addition, root productivity and morphology would change with roots shifting from acquisitive to more conservative traits in plots where P was added. As a consequence of alleviation of P limitation, I expected a decrease in root productivity and an increase in mean root diameter and root tissue density (RTD), but a decrease in specific root length (SRL) and specific root area (SRA). Finally, I predicted that the addition of other nutrients, including N and cations would exacerbate P limitation, resulting in increased root phosphatase activity, AM colonisation and root production with roots retaining more acquisitive morphological traits (e.g. high specific root length).

4.3. Material and methods

4.3.1. Site description and experimental design

This research was part of the first large-scale nutrient addition experiment in central Amazon, AFEX (Amazon Fertilisation Experiment) installed ca. 70-90 km north of Manaus/Amazonas, Brazil at the BDFFP Reserve (Biological Dynamics of Forest Fragments Project) at ZF-3, a collaborative project between the National Institute for Amazon Research (INPA) and the Smithsonian Institute (STRI). The vegetation is an old growth, *terra firme* forest, associated with dystrophic soils with very low total P content (~ 85 mg kg^{-1}; see Supplementary Table 4.6. in the Appendix 2 for more information about baseline soil properties before the start of the fertilisation) and mean soil pH in water of 4.2. Mean air temperature is 26ºC and mean annual precipitation is 2,400 mm (Araújo, 2002). AFEX is composed of thirty-two 50 m x 50 m plots distributed among four blocks. Each of the four blocks has eight plots representing the eight treatments applied in a factorial design: control, N, P, cations (Ca, Mg, K), N+P, N+cations, P+cations, and N+P+cations (Figure 4.1). All plots (four plots per treatment) were established in areas with similar soil, vegetation and terrain. Key monitoring measurements were limited to the central 30 m x 30 m (900 m^2 area). Nutrients were added at the following rates, separated into three applications over the course of each wet

season: (1) N: 125 kg ha^{-1} yr^{-1} as Urea; (2) P: 50 kg ha^{-1} yr^{-1} as triple superphosphate, and (3) Cations: 160 kg ha^{-1} yr^{-1} as dolomitic limestone for Ca and Mg, plus 50 kg ha^{-1} yr^{-1} as potassium chloride for K. The level of nutrient addition has been considered carefully and was calculated from previous gradient work (Aragão et al. 2009, McGroddy et al. 2004). Nutrients were added in May and June 2017 with further additions in December 2017-January 2018.

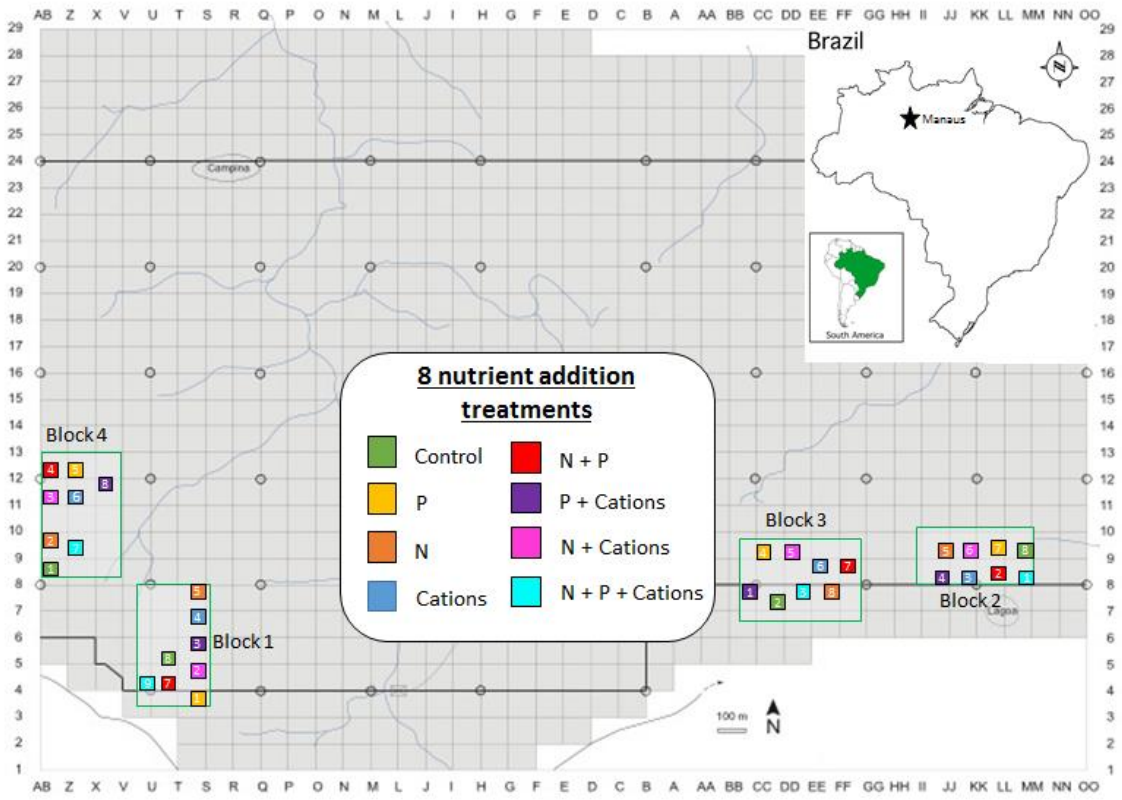

Figure 4.1. Details of the study area at BDFFP, showing the distribution of blocks, treatments and plots in a central Amazon forest near Manaus, Brazil as part of the AFEX project.

4.3.2. Root sampling

In each plot (*n*=32), five 12 cm-diameter, 30 cm-deep, root-free ingrowth cores (2 cm plastic mesh bag; Figure 4.2) were installed in August 2017 in the central 30 m x 30 m plot area (Metcalfe et al. 2007). Ingrowth cores were collected every 3 months after installation and the five core replicates were homogenised in the field by plot and by soil layer (0-10 and 10-30 cm; *N*=64). Only fine roots

produced in the three month interval between November 2017 and February 2018 (middle of the wet season) were used in this study. All fine roots were manually extracted during a period of 60 minutes in intervals of 15 minutes and root-free soil reinserted into the existing holes (Metcalfe et al. 2007). The cumulative root biomass sampled in each time interval was used to estimate the amount of roots not sampled during 60 minutes (Metcalfe et al. 2007). This estimate was made using the Michaelis-Menten asymptotic curve (Equation 1), extrapolating the amount of roots sampled for 180 minutes. After sampling, roots were washed in water and cleaned by gently brushing to remove soil particles.

$$y = \frac{\alpha * x}{\beta + x} \qquad \text{Equation 1.}$$

where *y* is total fine root biomass estimated in each sample after 180 minutes of sampling; *x* is accumulated time (15 to 180 minutes), α and β are parameters from the adjusted equation for each plot and depth.

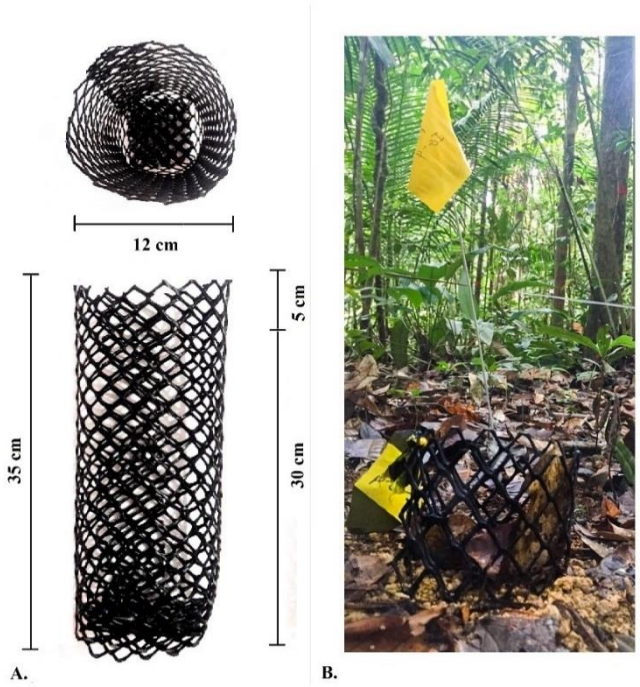

Figure 4.2. Detail of the ingrowth core and its dimensions on the left panel; ingrowth core installed in the field in the right panel. Photo credit: J. S. Rosa.

4.3.3. Root subsampling

Fine roots in both soil layers were separated into different diameter classes <1 mm and 1 mm to 2 mm, rather than by root order because of the difficulty to assign order to species-rich samples (Wurzburger and Wright 2015). Root samples were subsampled for a) enzyme assays, b) mycorrhizal colonisation, c) morphology + dry weight and d) dry weight only. Enzyme assays and mycorrhizal colonisation were determined in root fragments <1 mm diameter which usually represent absorptive fine roots responsible for nutrient acquisition (McCormack et al. 2015). Root subsamples in all diameter classes (<2 mm) were used to determine root morphological traits and subsequently dried at 60ºC to determine dry root mass. The remaining roots from each sample (<2 mm) were also oven dried at 60ºC to determine root productivity in different soil depths.

4.3.4. Root morphology

After cleaned, root samples (<1 and 1-2 mm diameter separately) were spread homogeneously in a plastic tray from where about ¼ of root biomass was separated for subsequent scanning. Images were analysed using WinRHIZO (WinRHIZO Regular 2015, Regent Instruments, Canada) to determine specific root length (SRL), specific root area (SRA), root tissue density (RTD) and mean root diameter (Metcalfe et al. 2008). SRL (cm g^{-1}) was calculated as root length per unit root dry mass, SRA (cm^2 g^{-1}) was calculated as root superficial area per unit dry mass and RTD (g cm^{-3}) was calculated as root dry mass per unit root volume.

4.3.5. Fine root productivity

Fine root productivity (<2 mm) was calculated as dry mass of roots (mg) produced per day for the whole ingrowth core sample and also by depth (0-10 and 10-30 cm). Root productivity was also expressed in cm per day and cm^2 per day by extrapolating root length and area produced per plot, based on the dry weight, length and area of root subsamples scanned for determining root morphology traits. Root net primary productivity was calculated for the 3 month

interval between ingrowth core samplings (November 2017-February 2018) and was expressed in Kg ha^{-1} $month^{-1}$ but also in m m^{-2} $month^{-1}$ and m^{-2} m^{-2} $month^{-1}$.

4.3.6. Root phosphatase activity

Potential root acid phosphomonoesterase activity (PHOS) was measured using a fluorimetric microplate assay following protocols by German et al. (2011) and Turner and Romero (2010) as described in Chapter 3 (Lugli et al. accepted). After cleaning, root enzyme activities were measured within 3 days of root sampling using triplicate subsamples per plot. About 10 mg of the root sample (washed, fresh weight basis) were placed into a sterile 2 mL Eppendorf snap-cap vial with 1 ml of buffer and 0.25 ml of Methylumbelliferyl-phosphate (MUF-phosphate; 2mM) which was used as an analogue substrate for phosphomonoesterase. A further identical root sample was prepared as control by adding 1.25 ml of buffer. In addition, buffer blanks (*n*=3) and substrate blanks (*n*=3) were prepared as 1.25 ml of buffer (no roots, no substrate) and 1 ml of buffer + 0.25 ml of MUF-phosphate (no roots), respectively. Samples were incubated for 30 min at ~25ºC while gently shaking, then 50 µL of 1 M NaOH were added to all samples and standard vials to terminate the reactions. Aliquots of the sample solution were pipetted into a black 96-well microplate and 20 min post termination, fluorescence was read on a fluorometer (Tecan Infinite® 200 PRO, Grödig, Austria), at 365 nm excitation and 450 nm emission. Roots were removed from vials, rinsed with Milli-Q water, scanned and subsequently dried at 60ºC for 72 hours. Root phosphatase activity per plot is expressed in µmol MUF g^{-1} dry mass h^{-1}. Based on the images from the scanned root subsamples, root phosphatase activity was also expressed in cm root length and cm^2 root area.

4.3.7. Mycorrhizal colonisation

To determine AM colonisation, root subsamples from 0-10 cm and 10-30 cm soil depths were cleaned and scanned, and segments were stored in 50% ethanol. Roots were transferred to small cassettes separated by colour/appearance, since processing time depended on the clarity of the roots (i.e. dark roots took more time to clear than brown and clear roots). The clearing

and staining processes, designed to highlight only the mycorrhizal structures, were adapted for tropical roots based on Brundrett et al. (1984) and Wurzburger and Wright (2015). The cassettes were placed in a 2.5% KOH solution and autoclaved at ~120 °C for ± 10 minutes. If roots were still dark pigmented after the clearing process, they were placed in alkaline H_2O_2 solution for further bleaching for ± 30 minutes, checking them constantly to make sure that no material was lost due to over clearing or bleaching. Clearing and bleaching time varied depending on root thickness and pigmentation. Before staining, roots were acidified by placing cassettes in 2% HCl solution for 30 minutes and were then added to a beaker with Trypan Blue 0.05% for 30 minutes. When consistently blue, roots were rinsed well in distilled water and stored at 4°C until slide preparation. The time between root staining and slide preparation never exceeded two weeks. Stained roots were then cut in 1 cm length fragments and 10 fragments were placed vertically in a microscope slide and fixed using polyvinyl alcohol (PVA). Each slide contained 10 fragments of 1 cm (total 10 cm roots per slide) and each sample was prepared in duplicates (2 slides per sample). Each root fragment was checked lengthwise at 1 mm intersections (40x optical) (McGonigle et al. 1990). At each intersection, AM colonisation was scored according to the following categories: no mycorrhizal structures, hyphae only, hyphae + arbuscules, hyphae + vesicles, hyphae + vesicles + arbuscules. Mycorrhizal colonisation was assessed as the percentage of the total root intersections along the root length that had mycorrhizal fungi. Due to time limitations, it was only possible to test for mycorrhizal colonisation for the 0-10 cm soil layer in the control and +P plots (n=4 per treatment).

4.4. Statistical analysis

The effect of each treatment was tested on root morphology traits, biomass, productivity, phosphatase production and AM colonisation using linear mixed-effect models using ‘lme4’ and ‘lmerTest’ packages (Bates et al. 2013; Bates et al. 2015; Kuznetsova et al. 2015). Treatments (n = 8) were used as a fixed factor, and block (n = 4) as random factor as follows: lmer (root trait~treatment + (1|block). Data were checked for normality and the selection for the best model was made based on functions from ‘LMERConvenienceFunctions’ package

(Tremblay and Ransijn 2015). When the model was significant, the Dunnett post-hoc test was conducted to detect for differences between treatments and control plots (*n*= 4 plots per treatment; mean of both soil depths). Paired t-tests were used to detect differences in the variables tested between the two soil depths.

Linear mixed-effect models were also used to test the effect of the interaction between nutrients added using the factorial design N*P*cations as follows: lmer (root trait~N*P*cations +(1|block)When the addition of a single nutrient or the interaction between them were significant, a backward elimination of all effects of the linear mixed model was performed using the *step* function from the 'lmerTest' package and only the significant effects of nutrient additions are reported. When the addition of an element and/or their interaction resulted in significant effects, a post-hoc Tukey pairwise comparison of means between all the contrasts was performed using the package 'emmeans'. All analyses were conducted in R version 3.4.4 (R Core Team 2018).

4.5. Results

4.5.1. Root productivity

Mean root productivity (roots <2 mm diameter) among all treatments was 293.03 ± 23.88 Kg ha^{-1} $month^{-1}$ for the 0-30 cm soil depth, varying from 181.64 Kg ha^{-1} $month^{-1}$ in the +N treatment to 419.72 Kg ha^{-1} $month^{-1}$ in the P+cations treatment (Figure 4.3). However, no significant differences were detected between treatments for the full 0-30 cm profile ($F_{7,21}$=1.39, p=0.26), nor when analysing the 0-10 cm ($F_{7,21}$=2.15, p=0.08) and 10-30 cm soil layers separately ($F_{7,21}$=0.45, p=0.86). Higher root productivity was detected for the 0-10 cm than for the 10-30 cm soil layer for all treatments, with significantly more root biomass produced in the superficial layer in control, N+P and N+P+cations plots (Supplementary Table 4.1. in the Appendix 2). Root productivity in terms of total root length and root area for 0-10 cm and 10-30 cm soil layers are also shown in Supplementary Table 4.1. in the Appendix 2 and responses follow similar patterns to the ones found for root mass productivity.

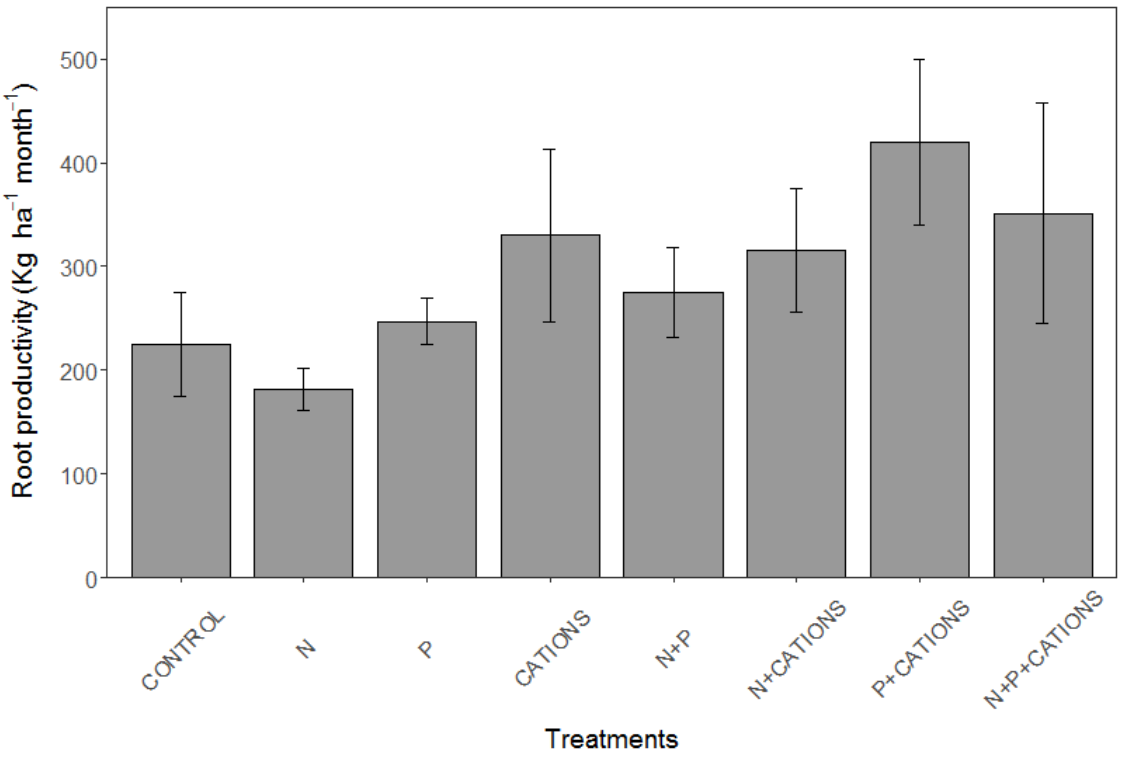

Figure 4.3. Fine root productivity in Kg ha^{-1} $month^{-1}$ for the 0-30 cm soil layer in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. *n*=4 per treatment. Error bars represent standard error.

When testing for the effect of the factorial N*P*cations addition, no significant effects were found for root productivity in dry mass for 0-30 cm depth with the addition of N and P ($F_{1,21}$=0.28, *p*=0.59 and $F_{1,21}$=1.72, *p*=0.20 respectively, Figure 4.4). However, root productivity significantly increased with the addition of cations only ($F_{1,21}$=7.11, *p*=0.01). Total root productivity for the 30 cm soil depth was 52% higher in plots with cations addition when compared to plots where cations were not added (354.06 ± 38.92 versus 231.99 ± 18.54 Kg ha^{-1} $month^{-1}$), with no significant interactions between nutrients (Figure 4.4). A similar trend of increased root production with cations addition was found when analysing root productivity in length and area ($F_{1,21}$=3.64, *p*=0.07 and $F_{1,21}$=5.06, *p*=0.03 respectively; Supplementary Table 4.2. in the Appendix 2).

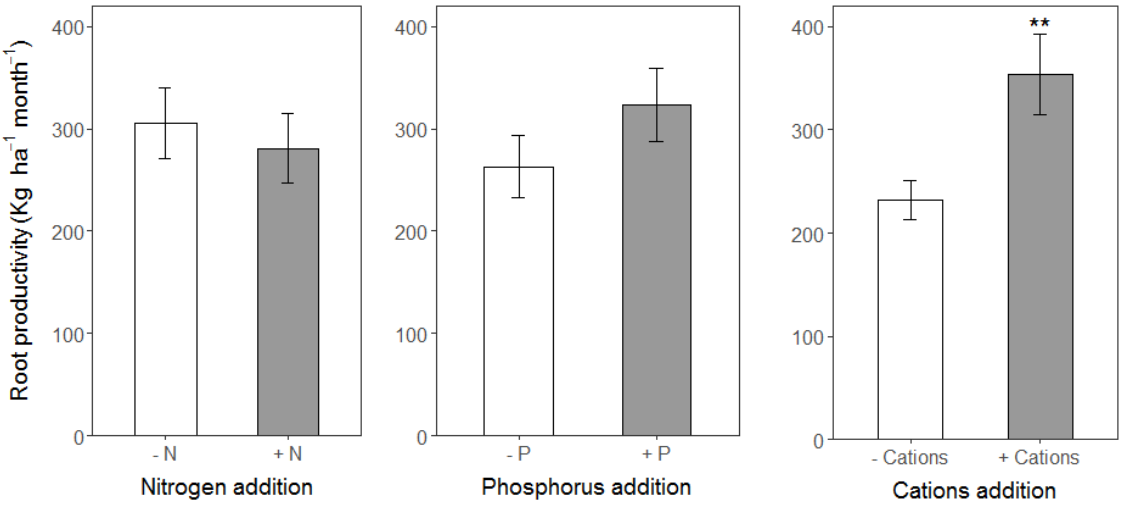

Figure 4.4. Fine root productivity in Kg ha^{-1} month^{-1} for the 0-30 cm soil depth in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

The significant increase in mean root productivity with cations addition for the whole core was mainly driven by changes in the 0-10 cm soil layer, with no significant effect in the 10-30 cm layer with the addition of any nutrient (Figure 4.5). The addition of cations-only significantly increased root productivity for the 0-10 cm soil layer ($F_{1,21}$=10.89, p=0.003) from 132.41 ± 11.54 to 220.17 ± 23.005 Kg ha^{-1} month^{-1}. Similarly, the addition of cations also increased root productivity in the 0-10 cm soil layer when expressed in terms of length ($F_{1,21}$=6.67, p=0.01) and area ($F_{1,21}$=8.71, p=0.007). Phosphorus addition caused marginally significant increases in total root length and total root area ($F_{1,21}$=3.00, p=0.09 and $F_{1,21}$=3.89, p=0.06 respectively; Supplementary Figures 4.1. and 4.2. in the Appendix 2). When comparing soil depths in each nutrient addition treatment, significant differences were found using paired t-tests for all cases, with the exception of -P plots, where root productivity in the 0-10 and 10-30 cm layers did not differ significantly (Supplementary Table 4.2.).

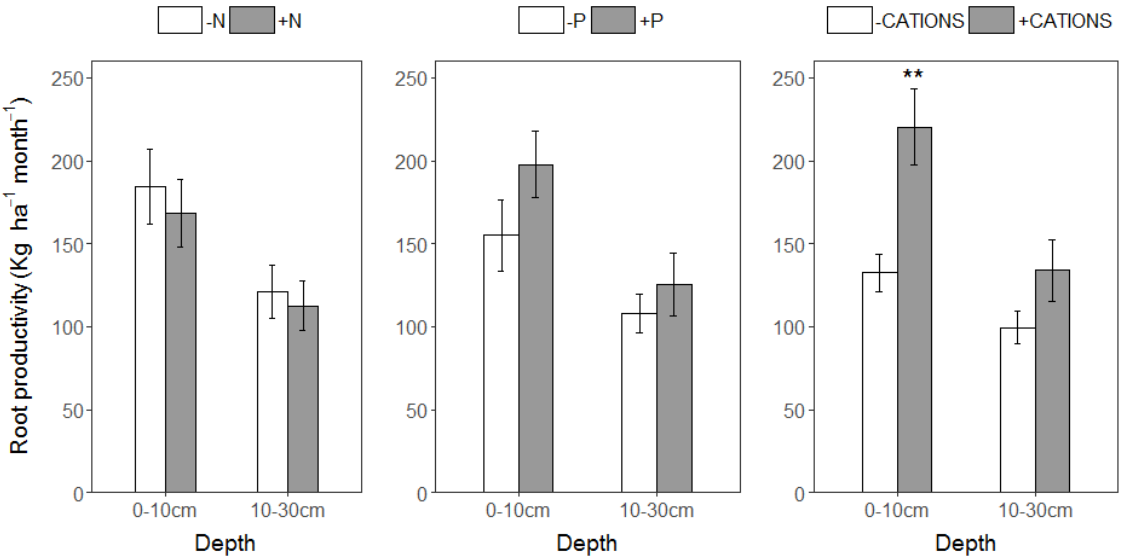

Figure 4.5. Fine root productivity in Kg ha^{-1} $month^{-1}$ for the 0-10 and 10-30 cm soil depths in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of nutrients in each depth. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

4.5.2. Root morphological traits

Among the root morphological traits analysed here (diameter, SRL, SRA and RTD), no differences were found between treatments for the mean 0-30 cm soil depth nor for each depth separately (0-10 and 10-30 cm layer; Table 4.1). Paired t-tests were conducted to detect any differences in root morphological traits between soil depths in each of the treatments, but no significant differences were found.

Table 4.1. Mean root diameter (mm), SRL (cm g^{-1}), SRA (cm^2 g^{-1}) and RTD (g cm^{-3}) ± standard errors in eight treatments and two soil depths (0-10 cm and 10-30 cm). *n*= 4 per treatment per depth. No differences between treatments and between soil layers were detected.

Treatment	Depth	Diameter	SRL	SRA	RTD

CONTROL	0-10 cm	1.01 ± 0.04	1,289.94 ± 88.31	312.01 ± 18.17	0.153 ± 0.009
	10-30 cm	0.99 ± 0.06	1,326.65 ± 137.79	309.27 ± 25.38	0.157 ± 0.013
N	0-10 cm	0.95 ± 0.10	1,356.03 ± 348.41	324.51 ± 57.61	0.152 ± 0.010
	10-30 cm	1.07 ± 0.08	1,206.01 ± 105.02	308.92 ± 15.94	0.143 ± 0.005
P	0-10 cm	1.05 ± 0.03	1,193.50 ± 46.77	298.23 ± 20.30	0.158 ± 0.013
	10-30 cm	1.07 ± 0.07	1,280.56 ± 172.18	315.21 ± 27.12	0.149 ± 0.010
CATIONS	0-10 cm	1.00 ± 0.11	1,065.91 ± 108.42	264.48 ± 16.25	0.179 ± 0.003
	10-30 cm	1.08 ± 0.06	1,112.40 ± 73.71	290.91 ± 8.57	0.149 ± 0.010
N+P	0-10 cm	1.11 ± 0.04	1,184.58 ± 24.26	297.33 ± 7.24	0.147 ± 0.006
	10-30 cm	1.04 ± 0.03	1,507.06 ± 127.45	356.27 ± 36.53	0.138 ± 0.012
N+ CATIONS	0-10 cm	1.13 ± 0.04	1,299.94 ± 142.32	314.06 ± 20.52	0.144 ± 0.008
	10-30 cm	1.16 ± 0.04	1,242.82 ± 176.94	310.76 ± 34.79	0.143 ± 0.013
P+ CATIONS	0-10 cm	1.13 ± 0.03	1,048.28 ± 169.67	285.09 ± 32.69	0.148 ± 0.013
	10-30 cm	1.09 ± 0.08	1,306.88 ± 176.93	317.50 ± 20.09	0.143 ± 0.001
N+P+ CATIONS	0-10 cm	1.17 ± 0.03	1,396.27 ± 244.42	343.00 ± 53.09	0.140 ± 0.012
	10-30 cm	1.17 ± 0.04	939.96 ± 145.89	252.93 ± 25.54	0.173 ± 0.017

When testing the effect of the factorial N*P*cations addition for both soil depths together (0-30 cm), mean root diameter increased significantly only with cations addition ($F_{1,21}$=7.86, P=0.01; Figure 4.6), with a marginal effect of P addition ($F_{1,21}$=3.32, P=0.08; Figure 4.7), and no influence of N addition, or any significant interaction between elements. When analysing each soil layer separately, the positive effect of cations addition on root diameter was marginally significant for both 0-10 cm and 10-30 cm depth ($F_{1,21}$=3.59, P=0.07 and $F_{1,21}$= 3.70, P=0.07, respectively; Figure 4.6). The addition of P only significantly increased root diameter for the 0-10 cm soil layer ($F_{1,21}$=4.59, P=0.04; Figure 4.7), with no significant effect for the 10-30 cm depth ($F_{1,21}$=0.14, P=0.70; Figure 4.7). No changes were found for RTD, SRL and SRA when analysing soil depths together or separately with the addition of any nutrients (Supplementary Table 4.3. in the Appendix 2). Paired t-tests were conducted to detect any differences

in root morphological traits between soil depths with the addition of each nutrient, but no significant differences were found (Supplementary Table 4.3. in the Appendix 2).

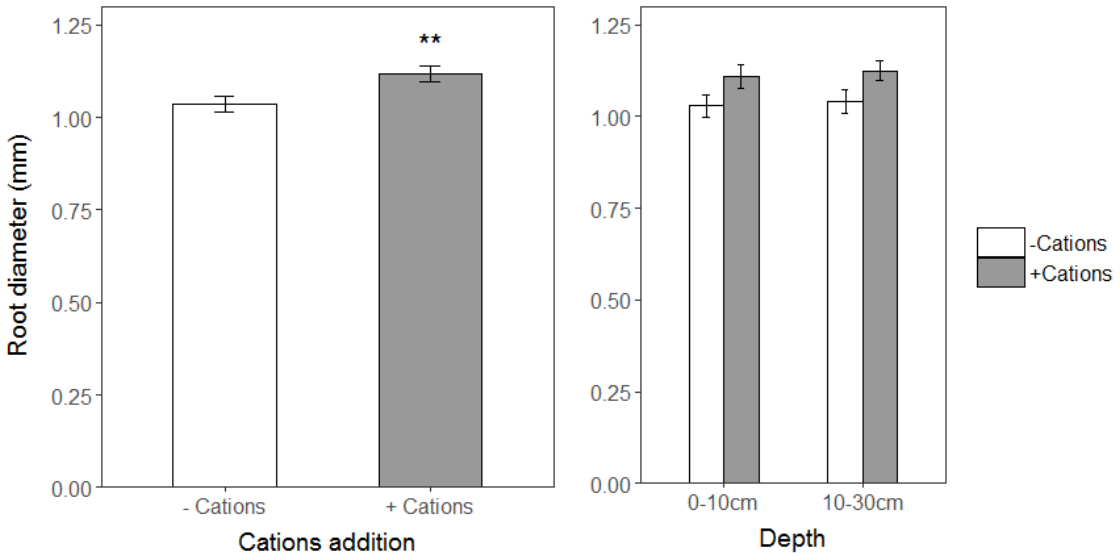

Figure 4.6. Mean fine root diameter (mm) in response to cations addition in a lowland tropical forest in central Amazon, Brazil. Left panel shows the mean of root diameter in the 0-30 cm soil layer; right panel shows mean of root diameter for the 0-10 and 10-30 cm soil layers separately. Each panel contrasts 16 plots with and without the addition of cations. Error bars represent standard errors. Significant effects between treatments are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

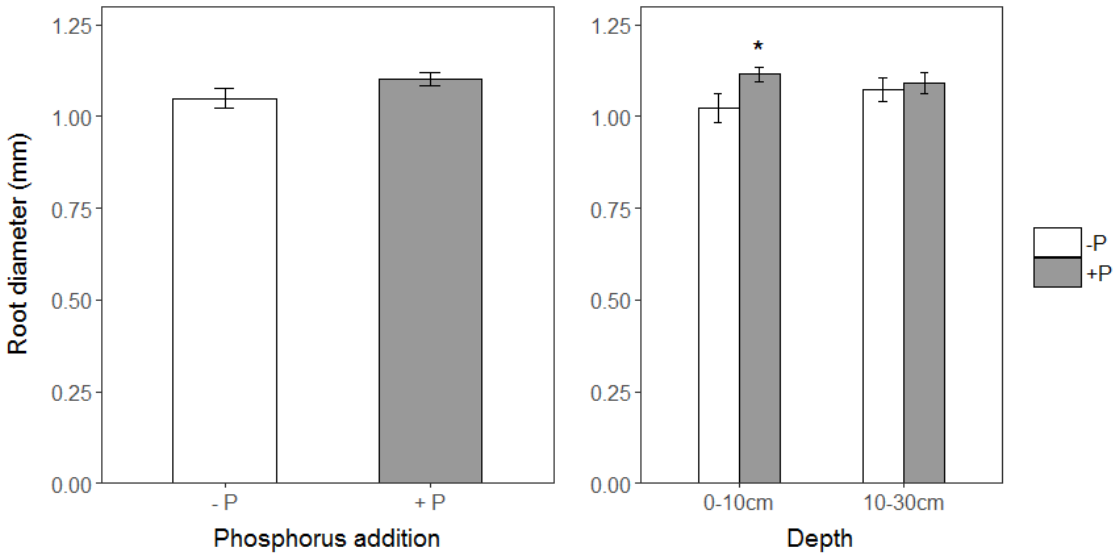

Figure 4.7. Mean fine root diameter (mm) in response to phosphorus addition in a lowland tropical forest in central Amazon, Brazil. Left panel shows the mean of root diameter in the 0-30 cm soil layer; right panel shows mean of root diameter for the 0-10 and 10-30 cm soil layers separately. Each panel contrasts 16 plots with and without the addition of phosphorus. Error bars represent standard errors. Significant effects between treatments are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

4.5.3. Root phosphatase activity

Root phosphatase activity (dry weight basis) did not differ between treatments for the mean 0-30 cm soil depth ($F_{7,21}$=0.94, p=0.49; Figure 4.8), nor when comparing treatments in the 0-10 cm and 10-30 cm soil layer separately ($F_{7,21}$=1.45, p=0.24 and $F_{7,21}$=0.48, p=0.83 respectively; Supplementary Table 4.4. in the Appendix 2). Mean phosphatase activity across all treatments was 36.05 ± 1.57 µmol g^{-1} root dry weight $hour^{-1}$. No differences between soil layers in each treatment were detected using paired t-tests. Similar responses were found for root phosphatase activity expressed on a root length and root area basis (Supplementary Table 4.4.).

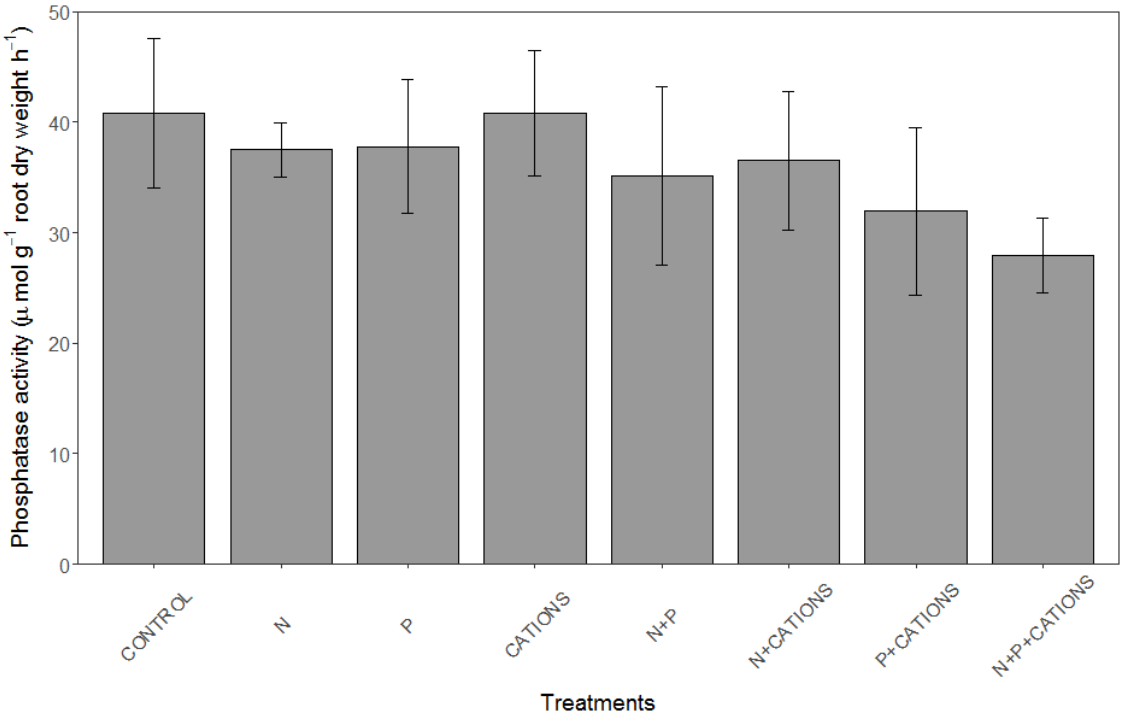

Figure 4.8. Mean root phosphatase activity in µmol g^{-1} root dry weight $hour^{-1}$ for the 0-30 cm soil layer in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. *n*=4 per treatment. Error bars represent standard error.

The factorial addition of N, P and cations did not significantly affect root phosphatase activity for the mean 0-30 cm soil layer (Figure 4.9), although only a marginal decrease was detected with P addition ($F_{1,21}$=3.21, *p*=0.09).

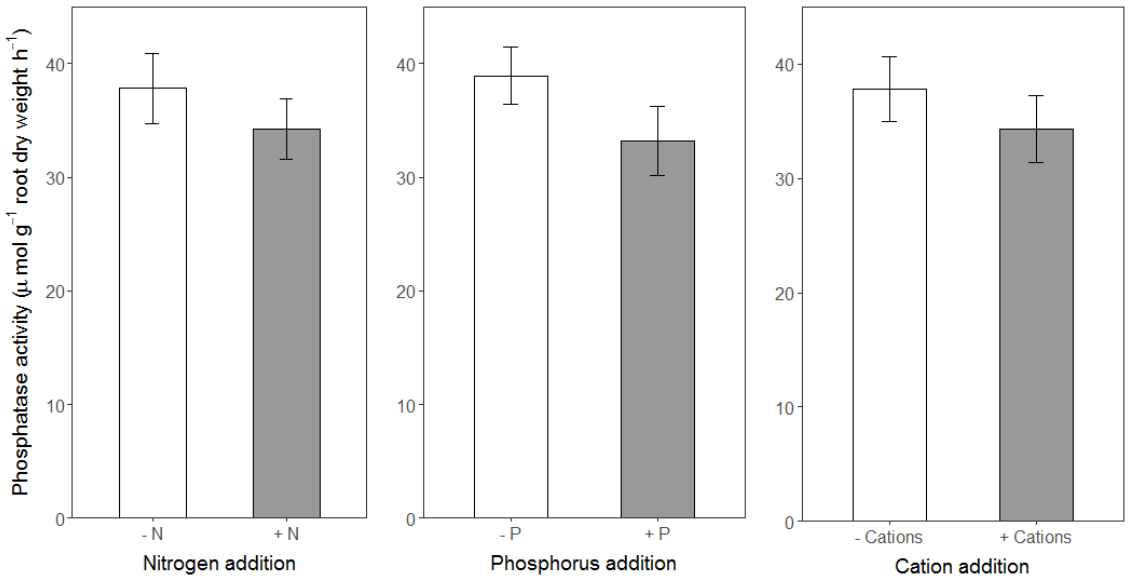

Figure 4.9. Mean root phosphatase activity (0-30 cm soil depth) in µmol g^{-1} root dry weight $hour^{-1}$ with and without the addition of N, P and cations. Each panel contrasts 16 plots with and without the addition of each nutrient. Error bars represent standard errors.

When analysing soil depths separately, the addition of P significantly decreased phosphatase activity in the 0-10 cm soil layer, from 41.84 ± 2.70 in -P plots to 31.97 ± 2.95 µmol g^{-1} root dry weight $hour^{-1}$ in +P plots ($F_{1,21}$=6.78, p=0.01; Figure 4.10; Supplementary Table 4.5.). Phosphorus addition also decreased root phosphatase activity per root length and area in the 0-10 cm soil layer ($F_{1,21}$=4.16, p=0.05 and $F_{1,21}$=7.37, p=0.01 respectively; Supplementary Figures 4.3. and 4.4. in the Appendix 2). No effect of the addition of N or cations was found for the 0-10 cm soil layer. Root phosphatase activity was not significantly affected by the addition of any nutrient in the 10-30 cm soil layer (Figure 4.10). No differences between soil layers were detected using paired t-tests for root phosphatase activity with and without the addition of each nutrient (Supplementary Table 4.5.).

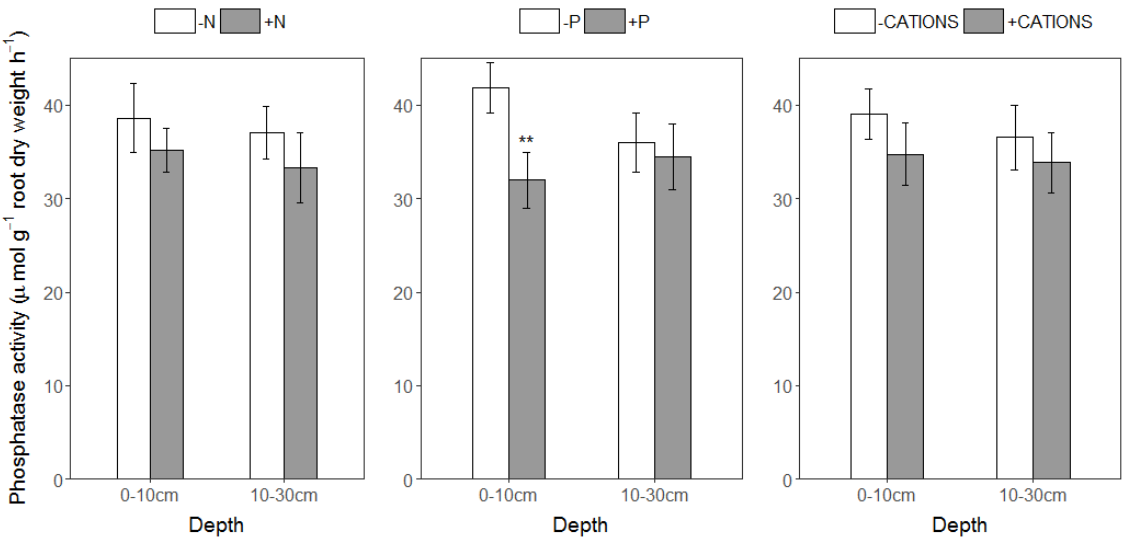

Figure 4.10. Mean root phosphatase activity in µmol g^{-1} root dry weight $hour^{-1}$ in two soil layers (0-10 and 10-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots per depth with and without the addition of nutrient. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

4.5.4. Mycorrhizal colonisation

No significant effects were detected when comparing total AM colonisation between control and +P plots for roots at the top 10 cm soil layer ($F_{1,3}$ = 3.76, P = 0.14; Figure 4.11.). Mean AM colonisation in +P plots was 53.2 ± 7.9% and 34.6 ± 5.3% in control plots. When analysing AM structures separately, a marginally significant difference was found for the percentage of roots colonised by hyphae-only ($F_{1,3}$ = 6.31, P = 0.08; Figure 4.11), with 46.6 ± 5.9% of colonisation in +P plots *versus* 26.3 ± 5.4% in control plots.

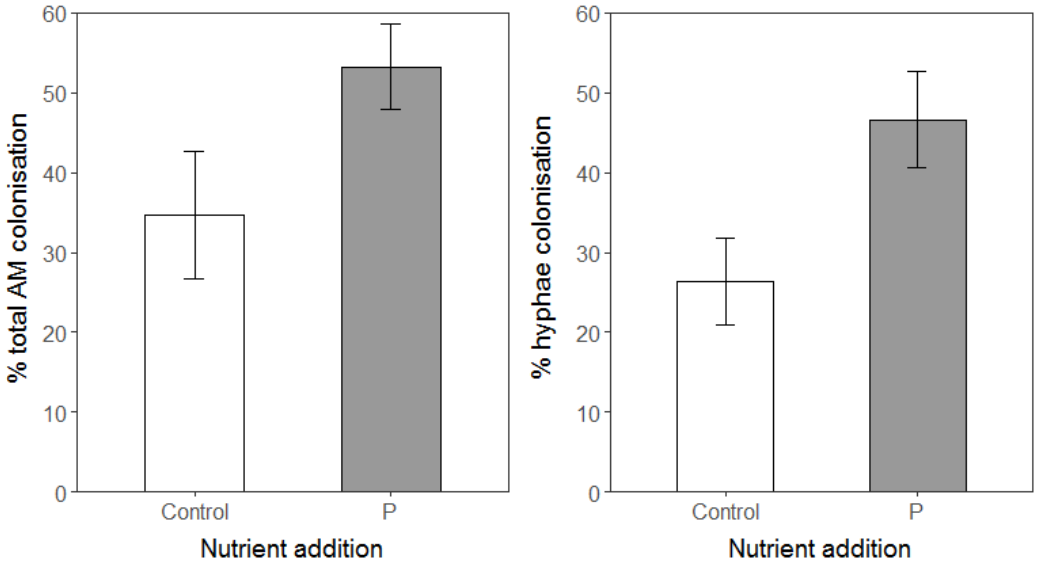

Figure 4.11. Total root mycorrhizal colonisation (left panel) and hyphae colonisation (right panel) in % root length for the 0-10 cm soil layer in control plots and +P plots in a lowland tropical forest in central Amazon. Each panel contrasts 4 plots with and without the addition of phosphorus. Error bars represent standard errors.

4.6. Discussion

4.6.1. Changes in root productivity with nutrient addition

Based on the P-paradigm which assumes that P is the most limiting nutrient in tropical forests, I predicted that investment in root biomass would be mainly affected by P addition. According to the plant economic theory proposed by Bloom et al. (1985), a decline in the investment in root biomass and nutrient uptake strategies would be expected when the supply of the limiting nutrient in soils is increased. Contrary to my predictions, however, root productivity did not decline with P addition, but in fact increased substantially after seven months of cations addition. This short-term positive response of root productivity could be analogous to the increased growth usually seen following the natural pulse of nutrient release from the decomposing leaf litter biomass that occurs during the dry-wet season transition in central Amazon (Luizão and Schubart 1987). This results in substantial amounts of the elements released from litter decomposition

being removed by the action of fine roots, mycorrhizas, microorganisms and arthropods (Luizão and Schubart 1987).

The increase in root biomass production with cations addition found in this study could also be due to the fact that measurements were made only seven months after fertilisation started. In the long-term, however, it is likely that chronic nutrient addition would favour alleviation of nutrient limitation, changing the direction of the responses found here. In this scenario, plants would potentially invest less in root biomass and allocate the extra carbon aboveground (Ostertag 2001; Jimenez et al. 2009; Wurzburger and Wright 2015). In concert to that rationale, the addition of K alone and in combination with N and P decreased fine root biomass (<2 mm diameter) for the 0-10 cm soil layer after four (Yavitt et al. 2011) and ten years of nutrient addition in lowland forests in Panama (Wright et al. 2011). At the same site, after 14 years of fertilisation the responses were even stronger: the addition of K-only and N+P decreased fine root biomass, with a reduction of 50% with the addition of the three elements combined (Wurzburger and Wright 2015). Such results point to different nutrients playing important roles controlling belowground plant biomass allocation in response to changes in soil nutrient availability. In a factorial NP fertilisation experiment in lowland forests in Costa Rica, however, no significant changes in fine root biomass were detected after two years of nutrient addition (Alvarez-Clare and Mack 2015).

The main processes by which roots acquire nutrients from soils might differ depending on the chemical properties of ions of each element, as well as other edaphic properties such as water content and soil pH (Hinsinger 1998). In general, Ca and Mg are less retained by soil particles and are usually supplied to plants by root interception and mass flow (Bolan 1991; Barber 1995; Hinsinger 1998). Root interception refers to the direct contact between root surface and a nutrient, while mass flow refers to the movement of a nutrient to the root surface via water flow during water uptake by plants (Bolan 1991). On the other hand, P and K are supplied to the plants mainly by diffusion, which is the movement of nutrients to the root surface caused by the difference in concentration gradients between the soil solution and the root surface (Bolan 1991). Since the cation treatment is mainly constituted of calcium and magnesium and the concentration of such nutrients in soils in my study area were very low (mean of 0.03 $cmol_c\ kg^{-1}$ and 0.06 $cmol_c\ kg^{-1}$ for available Ca and Mg, respectively, across all plots before

fertilisation; Supplementary Table 4.6. in the Appendix 2), I hypothesise that the increase in root biomass production after cation addition could be a response to short-term alleviation of cation limitation with the extra roots produced acting towards increasing nutrient supply to plants (Bloom et al. 1985). Although less likely, the increased root production after cation addition could also be interpreted as a sign of exacerbation of P limitation.

4.6.2. Responses of root morphological traits to nutrient addition

My hypothesis was that if nutrients were limiting this forest, root traits would shift from acquisitive to conservative morphological traits with nutrient addition. Although no differences were found for SRL, SRA and RTD, root diameter increased with cation and phosphorus addition, suggesting that multiple nutrients could affect the expression of root morphological traits. The addition of cations, however, affected mean root diameter for the whole 0-30 cm soil layer, whilst the addition of P only affected root diameter in the 0-10 cm soil layer. Such responses could be attributed to differences in nutrient mobility in soils, suggesting that i) contrary to P, cations are more mobile in soils and could percolate the soil profile, ultimately influencing roots in deeper layers (Chadwick et al. 1999) and ii) that P is mainly absorbed by roots in the superficial soil layers (Stark and Jordan 1978). Moreover, such changes in root morphology expression could indicate that in this central Amazon forest, plants could be limited by P but also by cations availability.

The increase in root diameter and biomass production (significantly for cation addition and marginally significantly for the P treatment when expressed as productivity in root length and area) suggests that after nutrient addition plants are investing in more roots with larger diameter. Although small diameter roots are more efficient in exploring larger soil volumes (Bates and Lynch 2001; Hodge 2004; Liu et al. 2015), the increase in root diameter detected here could confer trees other benefits, such as i) increased mechanical protection against pathogens and herbivores (Laliberte et al. 2015; Ushio et al. 2015) and ii) increased number or size of root cortical cells which could consequently increase levels of mycorrhizal colonisation (Brundrett 2002; Guo et al. 2008; Comas et al. 2014). Relationships between root diameter and mycorrhizal colonisation in the

context of my results are discussed in more detail under the “Mycorrhizal responses” section in this chapter. In contrast to my findings, after 14 years of nutrient addition in a lowland forest in Panama, RTD decreased by 25% and SRL increased by 50% in response to added N+P+K in <2 mm diameter roots from bulk soils, with no significant effects on root diameter (Wurzburger and Wright 2015). The results from the experiment in Panama suggest that although less roots were produced with chronic N+P+K addition, indicating an alleviation of limitation by multiple nutrients, these roots are becoming less dense and with higher nutrient foraging precision due to increased SRL. Therefore, nutrient addition in Panama caused a change in the expression of root morphological traits towards more acquisitive strategies, contradicting the evidence found in my study that with short-term alleviation of nutrient limitation root traits in this central Amazon forest become more conservative (e.g. increase in root diameter).

4.6.3. Responses of root phosphatase activity with nutrient addition

Among the root traits analysed in this study, the significant decrease in root phosphatase activity with short-term phosphorus addition is the strongest evidence for alleviation of P limitation in this central Amazon forest. This is the first time that direct experimental evidence for P addition rapidly reducing root phosphatase activity has been reported in a tropical forest. The results found here support the idea that the exudation of phosphatase by plants plays an important role towards P acquisition in low fertility soils in central Amazon, and its rapid down-regulation suggests that this is indeed a resource-costly strategy.

Phosphatase activity in this study decreased only in the superficial 0-10 cm soil layer, with no effect for the 10-30 cm layer. This effect could again be due to the very low mobility of P in soils (Hinsinger 2001) and also the short-time between nutrient addition and my measurements. Although, it was not known to what extent or how rapidly tropical trees could down-regulate phosphatase production when P limitation was alleviated, the interdependence of root phosphatase activity and phosphorus availability in soils is well documented in soil fertility gradient studies (Kitayama 2013; Nasto et al. 2014; Ushio et al. 2015). Under P limitation, plants tend to be more efficient in acquiring P and higher root

phosphatase activity is usually found in soils with low P concentrations (Raghothama and Karthikeyan 2005; Kitayama 2013).

In the present study, total soil P concentrations varied from 52 to 137 mg kg^{-1} (before fertilisation started) whilst root phosphatase activity varied from 27 to 40 µmol g^{-1} h^{-1} among all treatments (Table 4.2). Similar phosphatase activity was found in another lowland forest in central Amazon forest studied here, despite the slightly higher soil P concentrations (see Chapter 3). In soils of intermediate fertility in Panama, root phosphatase activity varied from 10 to 44.7 µmol pNP g^{-1} h^{-1} (Steidinger et al. 2015; Batterman et al. 2018). In a montane forest in Borneo, however, total P concentrations were very low (<60 mg kg^{-1}), resulting in high levels of root phosphatase exudation, reaching up to 200 µmol g^{-1} h^{-1} (Kitayama 2013; Ushio et al. 2015). On the other extreme of soil fertility, total soil P concentrations in a lowland tropical forest in Costa Rica were at least six times higher than in the present study (Table 4.2), resulting in comparatively very low phosphatase activities, reaching a minimum of 5 µmol g^{-1} h^{-1} (Nasto et al. 2014; Nasto et al. 2017). The intermediate levels of enzyme activity in central Amazon forests and Panama, when compared to very high activity in Borneo and low phosphatase activity in Costa Rica, therefore suggest that i) montane forests in Borneo are extremely P-limited and ii) forests in Costa Rica are not as P-limited as forests in Panama and central Amazon.

Table 4.2. Root phosphatase activity (PHOS) in µmol g^{-1} root dry mass h^{-1} and total soil P (mg kg^{-1}) in different tropical forests. Data is shown from lower to higher phosphatase activity and compared to data found in this study in two different locations (ZF-2: Chapter 3; ZF-3: this chapter).

Study	Location	Forest type	Total P	PHOS
Nasto et al. 2017	Costa Rica	Lowland forest	665-1,600	5 - 12
Steidinger et al. 2015	Panama	Montane forest	n.a.*	10
Batterman et al. 2018	Panama	Reforestation experiment	n.a.	17 - 44
Ushio et al. 2015	Borneo	Montane forest	10-60	100 - 200
This study (ZF-2)	**Brazil**	**Lowland forest**	**118-217**	**15 - 66**
This study (ZF-3)	**Brazil**	**Lowland forest**	**52-137**	**27 - 40**

* total soil P concentrations were not available (n.a,) in the publications but available P is considered low (<0.1 mg kg^{-1} and 0.1-0.6 mg kg^{-1} in Steidinger et al. 2015 and Batterman et al. 2018, respectively)

I also hypothesised that the addition of N or cations could exacerbate P limitation. For example, the addition of N could reduce P availability by slowing down organic matter decay (Mori et al. 2018) and therefore higher phosphatase exudation was expected in +N plots as a sign of exacerbation of P-limitation. Phosphatase is a constitutively N-rich enzyme (about 15%) (Olander and Vitousek 2000; Treseder and Vitousek 2001) and alternatively, N inputs have been shown to increase phosphatase exudation by plants and soil microorganisms in a wide range of terrestrial ecosystems (Marklein and Houlton 2012). However, in my study, no significant increase in root phosphatase activity was observed after N or cations addition. The lack of a significant effect on root phosphatase activity with N addition found in this study therefore gives no support to the idea that additional N can be allocated to P acquisition. Such results could be attributed to i) the already comparatively high N status of soils sampled here, ii) the possibility that signals of exacerbation of P limitation would be captured only after chronic N addition or iii) P-uptake mechanisms in this central Amazon could be already operating in their maximum due to the very low soil P concentrations.

4.6.4. Mycorrhizal responses to phosphorus addition

Another prediction for this study was that AM colonisation would decrease with P addition as a sign of reduced investment in the symbiosis once P limitation was alleviated. Contrary to expectations, total AM colonisation and the levels of root colonised by hyphae-only increased after seven months of P addition, although this was only marginally significant when compared to control plots. The increase, rather than a decrease in AM colonisation with P addition could be attributed to: i) to the short-term nature of the nutrient addition and potentially, the opposite signal may be observed with chronic P-addition; ii) changes in AM community composition and morphology with P-addition (Li et al. 2019) or, iii) the increase in soil inorganic P concentrations relative to organic P after fertilisation.

Since AM fungi directly increase the plant-host capacity to forage for inorganic P, rather than to mine organic P (but see Joner and Johansen (2000) and references therein for evidence of AM fungi releasing enzymes to scavenge for organic P), the results suggest that plants in this central Amazon forests may be investing in fungal symbiosis at the expense of root morphological and physiological adaptations to increase P uptake. This argument is supported by the fact that an increase in mycorrhizal colonisation, was also found for mixed root cores after 14 years of nutrient addition in the forests in Panama (Wurzburger and Wright 2015). Mean mycorrhizal colonisation at my study site increased from 34% in control plots to 53% in +P only plots, while in Panama the lowest level of AM colonisation observed was ~60% (Wurzburger and Wright 2015). The low mycorrhizal colonisation rates in my study site and increases with P addition, may suggest that AM colonisation is more beneficial as soil P availability increases from very low levels.

Roots associated with AM fungi are known to be more efficient on nutrient uptake per unit length than non-mycorrhizal roots (Bucher 2007; Smith and Read 2010). In the specific case of P, because of its low mobility in soils, the fungal mycelium can increase the soil volume explored and also penetrate small soil pores not accessible by fine roots (Smith and Read 2010). The slightly increase in root diameter in the 0-10 cm soil layer detected in plots where P was added could also help explain the higher levels of AM colonisation found when compared to control plots. Root diameter is usually reported to be positively correlated with AM colonisation (Eissenstat et al. 2015; Liu et al. 2015; Chen et al. 2016; Kong et al. 2016) although such a correlation was not detected in a central Amazon forest growing on naturally low fertility soils (see Chapter 3). In addition, because only an increase in root diameter was captured, with no changes in root specific length or area, from a cost-benefit perspective, thicker absorptive roots would benefit more from associating with AM fungi towards increasing soil nutrient foraging (Eissenstat et al. 2015).

The decrease in root phosphatase activity and the evidence for higher AM colonisation levels with P addition found here could even indicate a trade-off between one strategy at the expense of another (Ryan et al. 2012; Ushio et al. 2015; Nasto et al. 2017). It is suggested that about 20% of plant C could be transferred to the fungi whilst root exudates can represent up to half of

belowground C allocation (Bago et al. 2003; Lynch and Ho 2005; Parniske 2008). In addition to the lower costs to the plants when compared to root exudates, mycorrhizal fungi can be a more efficient strategy by delivering up to 80% of plant P (Bucher 2007; Smith and Read 2010).The increase in the inorganic P pool after P addition appeared to favour plant association with AM fungi for foraging P, rather than investment in enzymes to scavenge for organic P. These differences in P-pools explored by each root strategy together with the cost-benefit for plants could explain the rapid potential shift from high investments in physiological P-uptake mechanisms to increased association with AM fungi after fertilisation.

Mycorrhizal symbiotic association with plants is extremely common and it is estimated that about 74% of all plants species are colonised by these fungi (Brundrett 2009; Smith and Read 2010). In addition to P uptake, AM mycorrhizal could benefit plants by increasing the uptake of micronutrients, altering the microbial community in the rhizosphere and increasing plant chemical defence or decreasing plant susceptibility to pathogens (Koide 1991; Herre et al. 2007; Laliberte et al. 2015). The multiple functions of AM fungi associating with roots could, however, also obscure the relationship between AM colonisation levels and P uptake. Because of the variation within treatments and the fact that only the responses of two out of 8 treatments were analysed, potential shifts in the role of AM fungi with N and cations addition and their interactions are still not completely clear for this central Amazon forest.

4.7. Conclusions

Mature tropical forests that grow in low fertility soils are characterised by their high species diversity and slow growth, making the detection of possible responses to nutrient addition hard to capture and interpret. In this study, however, root traits related to nutrient acquisition strongly responded to the short-term addition of nutrients. Recent evidence from large-scale nutrient manipulation experiments suggest that different biological processes may be limited by multiple nutrients in tropical forests. Accordingly, I found support for the hypothesis that P limits some aspects of plant functioning in this central Amazon forest, but cations also appear to play an important role in controlling root productivity and the expression of root traits, but no effects of N addition were

observed. My results suggest that plants rapidly respond to changes in soil nutrient availability by changing their investments in root enzyme exudation and symbiotic associations as well as the expression of root morphological traits. The decrease in root phosphatase exudation with P addition is clear evidence for alleviation of P limitation in this tropical forest. Additionally, the reduced investment in enzyme activity after P addition combined with increased root diameter and association with AM fungi points to a potential shift in the strategies adopted by roots to acquire P, from root traits associated more with mining of organic P to traits linked to foraging for mineral P. Finally, the strong effect of cation addition increasing root productivity and root diameter in this study suggests that the role of rock-derived elements other than P is usually underestimated, but critical if we are to deepen our understanding of plant-soil interactions in tropical forests. Long-term measurements will be crucial to understand the specific way by which, N, P and cations influence belowground plant processes and to capture possible changes in root nutrient uptake mechanisms in time. I therefore conclude that multiple nutrients may limit belowground processes in central Amazon forests and that even slow-growing tropical rainforest can respond very rapidly to changes in soil nutrient availability.

In addition to the responses of root traits to nutrient addition, the following chapter (Chapter 5) aims to detect evidence for alleviation or exacerbation of P-limitation in a central Amazon forest, by analysing the responses of soil microorganisms, more specifically, the activity of enzymes related to C, N and P cycling.

Chapter 5 - Short-term responses of soil microbial enzyme activity to phosphorus, nitrogen and cation additions in a lowland forest in central Amazon

5. Short-term responses of soil microbial enzyme activity to phosphorus, nitrogen and cation additions in a lowland forest in central Amazon

5.2. Abstract

Soil nutrient availability strongly influences above and belowground aspects of tropical forests functioning. The highly weathered status of central Amazon soils results in low concentrations of rock-derived nutrients such as P and cations which are likely to limit soil microbial activity. Microbial nutrient acquisition is regulated by the exudation of extracellular enzymes responsible for organic matter decomposition and nutrient mineralisation. To determine the extent to which the production of these enzymes is determined by soil nutrient status, I investigated the response of three soil hydrolytic enzymes related to carbon (β-glucosidase), nitrogen (N-acetyl β-glucosaminidase) and phosphorus (phosphomonoesterase) cycles to the addition of nutrients in slow-growing, primary rainforest established on low-fertility soils in central Amazon. This research took place within the Amazon Fertilisation Experiment (AFEX), in which 32 50 x 50 m forest plots were exposed to N, P and cations addition in a fully factorial design. Soils were sampled from two different depths (0-5 cm and 5-10 cm) 6 months after nutrient fertilisation commenced. I hypothesised that if the soil microbial community was indeed limited by P in this forest, the investment in P-enzymes would decrease with P addition, resulting in higher C:P and N:P enzyme ratios due to the disproportionate decrease in phosphatase. I also hypothesised that the added N would be allocated towards increased phosphatase production and the addition of cations would exacerbate P-limitation by changing soil resource stoichiometry. Phosphatase activity significantly decreased with the addition of P, but only when cations were not added in combination. β-glucosidase and N-acetyl β –glucosaminidase were not significantly affected by the addition of any nutrient, but also tended to decrease with P addition. Nitrogen addition did not affect enzyme production. Despite the reduction in phosphatase activity, C:P and N:P ratios remained constant after nutrient addition, indicating an overall reduction in enzyme production following P addition. The rapid responses of enzyme production to P addition in central Amazon forests suggests

that P availability does regulate their activity, but the results also suggest that cation availability strongly influences responses to P addition. Since soil microorganisms can act both as sink or source of nutrients, the alleviation of P limitation could ultimately affect the extent to which plants are also P-limited, impacting forest functioning and carbon assimilation.

5.3. Introduction

Soil nutrient availability is considered to play a key role influencing productivity in lowland tropical forests and recent studies suggest that nutrients may be important in regulating the response of forests to elevated atmospheric CO_2 concentrations (Gentile et al. 2012; Sardans et al. 2012; Ellsworth et al. 2017). For instance, nitrogen (N), phosphorus (P) and potassium (K) were found to affect different above and belowground components of plant productivity in the tropics (Wright et al. 2011; Santiago et al. 2012; Wurzburger and Wright 2015), whilst, to date, P has been found to be the sole nutrient strongly regulating soil microbial communities in lowland tropical forests in Costa Rica and Panama (Cleveland et al. 2002; Cleveland and Townsend 2006; Turner and Wright 2014). During soil development, total P concentration decreases and the remaining pools become gradually more recalcitrant (Walker and Syers 1976; Tiessen et al. 1984; Quesada et al. 2010). The intense soil weathering of the majority of the Amazon basin (central and east portions) results in about 60% of the soils having very low cation and total P contents (Irion 1978; Sombroek 2000; Quesada et al. 2011). Inorganic labile P is the only form of P that can be assimilated by plants and microorganisms (Lambers et al. 2006), but with soil age, the recycling of organic P becomes the main or even the only source of P for plants and microorganisms in low-fertility Amazon soils (Quesada et al. 2010).

Organic phosphorus can occur as microbial biomass or phosphate adsorbed or directly bound to organic matter (Sanyal and De Datta 1991). Of special importance for organic P cycling is the microbial community, which is fundamental to ecosystem maintenance and nutrient transformations in soils (Chen et al. 2008). Microorganisms are responsible for organic matter decay and consequently release of nutrients to soil and, depending on the relative concentrations of carbon and other nutrients, microorganisms can become a

source or a sink of nutrients in soil, especially P (Richardson 2001; Oberson and Joner 2005; Richardson and Simpson 2011). Extracellular enzymes regulate microbial nutrient acquisition from organic matter decomposition and are usually interpreted as indicators of microbial nutrient demand (Olander and Vitousek 2005; Sinsabaugh et al. 2009; Burns et al. 2013). Microbial extracellular production results from the balance between carbon and nutrient costs and the benefits conferred to soil microorganisms (Burns et al. 2013), with microbial nutrient demand being determined by the elemental stoichiometry of hydrolytic enzymes (Waring et al. 2014; Mori et al. 2018). When compared to roots, soil microbes have faster growth rates and higher substrate affinity, being able to outcompete plants for limiting nutrients and even exacerbate nutrient limitation to plant productivity in tropical forests (Cleveland et al. 2002). Therefore, understanding the role of soil microbes in nutrient cycling is critical for predicting ecosystem responses to climate change and rising atmospheric CO_2 concentrations, since changes in nutrient availability to plants will depend on the balance between microbial nutrient immobilisation and mineralisation via organic matter decomposition (Blagodatskaya et al. 2010).

Since soil microorganisms are part of the trophic base, microbial demand can also be ultimately used to make inferences about other biogeochemical processes, such as organic matter decomposition and carbon (C) storage (Sinsabaugh et al. 2009). For instance, acid phosphomonoesterase (PHOS) activity has been shown to play a major role in P hydrolysis (Turner 2008; Steidinger et al. 2015; Jian et al. 2016; Yokoyama et al. 2017), degrading monoesters into available orthophosphate in a one-step enzymatic reaction. Similarly, β-glucosidase (BG) is involved in the acquisition of C, by degrading cellulose to glucose, while N-acetyl β-glucosaminidase (NAG) is involved in N-cycling, degrading chitin and other glucosamine polymers (Sinsabaugh et al. 2008; Turner and Wright 2014; Jian et al. 2016). Microbial enzyme activities are therefore strongly associated with soil resource availability, and microorganisms are predicted to invest more in enzymes that mineralise resources that are in short supply (Sinsabaugh and Moorhead 1994), suggesting that the mobilisation of C, N and P could actually happen via different mechanisms. Carbon and nitrogen would be stabilised together and released via biological mineralisation. Organic P would cycle independently from the C-cycle (McGill and Cole 1981),

being mainly released via biochemical mineralisation, a process controlled by the supply and demand for P, rather than to meet the demand for C.

Accordingly, in lowland tropical forests in Panama, microbial phosphatase activity was found to be disproportionally higher than C and N enzyme activities, pointing to a strong microbial phosphorus limitation caused by the low P-availability of those soils (Turner and Wright 2014). The hypothesis of P-limitation of the microbial community was confirmed through nutrient manipulation experiments across different terrestrial ecosystems, with phosphatase enzyme activity decreasing with the addition of phosphorus, whilst no effects were detected with the addition of cations (Marklein and Houlton 2012; Turner and Wright 2014). Moreover, because phosphatase is a constitutively N-rich enzyme (about 15%) (Olander and Vitousek 2000; Treseder and Vitousek 2001), N inputs have also been shown to be allocated towards increased phosphatase exudation by plants and soil microorganisms (Marklein and Houlton 2012). Nonetheless, total soil P and cation concentrations in central Amazon forests are much lower than in Panama (~100 *versus* 400 mg P kg^{-1}; <10 *versus* >1,000 mg Ca kg^{-1}) (Vincent et al. 2010; Quesada et al. 2011) which reinforces the hypothesis that plant and microbial limitation by rock-derived nutrients (e.g. P and cations) could be even stronger in the Amazon.

This study aimed to characterise soil microbial nutrient status in a central Amazon forest and test its potential nutrient limitation by measuring the activity of three hydrolytic enzymes after six months of *in situ* phosphorus, nitrogen and cations addition in a fully factorial design. I hypothesised that the soil microbial community is mainly limited by P and that 1) the addition of P would decrease the investment in phosphatase production and therefore 2) C:P and N:P ratios would increase, reflecting the disproportionate effect on phosphatase activity. Moreover, I also hypothesised that 3) the addition of N would increase microbial phosphatase activity, with the additional N being allocated to overcome P limitation. Finally, because I predict that microorganisms in this central Amazon forest are strongly P-limited, I hypothesised that 4) the addition of cations would exacerbate P limitation by altering soil nutrient stoichiometry and therefore greater investments in phosphatase activity would be expected.

5.4. Methods

5.4.1. Site description and experimental design

This research was also part of the AFEX project and a more detailed description of the study site and experimental design are therefore given in Chapter 4.

5.4.2. Soil sampling

All 32 plots were sampled in January, 2018 (wet season) before one of the fertilisation campaigns. Three soil cores (5 cm diameter) were sampled with an auger inside the central 30 m x 30 m of each plot. Soil samples were divided in 0-5, 5-10, 10-20 and 20-30 cm depth and samples were composited by depth and plot (*n*= 4 depths x 32 plots = 128 samples), with roots removed using a 2 mm mesh sieve. Subsamples of fresh soils from superficial soil layers (0-5 and 5-10 cm only) were separated for enzyme activity assays and the remaining soil samples were subsequently dried, sieved or ground and stored for chemical analyses. Soil enzyme activity and microbial biomass were analysed only for superficial soil layers (0-5 and 5-10 cm; *n*= 64) whilst soil fertility analyses were conducted for all four soil layers (0-30 cm; *N*= 128).

5.4.3. Hydrolytic enzyme assays

Potential activities of three soil hydrolytic enzymes involved in the cycling of carbon, nitrogen and phosphorus were measured using microplate fluorimetric assays based on Kaiser et al. (2010). One gram of fresh sieved soil was added to centrifuge tubes containing 100 ml of 100 mM of sodium acetate buffer (pH adjusted to 5.5) and homogenised with an ultrasonicator for 1 min at the lowest power. Two hundred microliters of soil suspension were pipetted into a black 96-well microplate together with 50µl of substrate 4-Methylumbelliferyl-β-D-glucopyranoside (BG), 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide (NAG) and 4-Methylumbelliferyl-phosphate (PHOS) in three analytical replicates.

Standard curves and Methylumbelliferyl (MUF) buffer blanks were also prepared for BG, NAG and PHOS microplate assay for the different soil depths. Samples were incubated for 1 hour at room temperature (~25° C) and fluorescence was read on a fluorometer (Tecan Infinite® 200 PRO, Grödig, Austria), at 365 nm excitation and 450 nm emission. Enzyme activities are presented in nmol MUF g soil dry weight^{-1} hour^{-1}. All laboratorial analyses were conducted at the Soil and Plant Laboratory at INPA in Manaus, Brazil.

5.4.4. Soil chemical analyses

Carbon (C) and nitrogen (N) concentrations were analysed in an automatic CN analyser (Pella 1990). Soil pH was determined as 1:2.5 H_2O extracted with 10 g of fine soil shaken with 25 ml H_2O for 1 h on a circular shaker, rested for 1 h and with the pH of supernatant read using an automatic probe (Quesada et al. 2010).

5.5. Statistical analyses

The effect of each treatment was tested on soil pH, soil C and N concentrations, CN ratios and also hydrolytic enzyme activity and enzyme ratios using linear mixed-effect models using 'lmerTest' package (Kuznetsova et al. 2015) where treatments (n=8) were used as a fixed factor, and blocks (n=4) as a random factor as follows: lmer (soil property~treatment +(1|block). Statistical analyses on soil pH were done using hydrogen ion concentrations ($[H^+] = 10^{-pH}$). Results are shown for the mean 0-10 cm soil depth and also by depth separately (0-5 and 5-10 cm) when indicated (*n*= 4 plots per treatment in each case). Data were checked for normality and the selection for the best model was made based on functions from 'LMERConvenienceFunctions' package (Tremblay and Ransijn 2015). When the model was significant, Dunnett post-hoc test was conducted to detect for differences between treatments and control plots. Paired t-tests were used to detect differences in the variables tested between the two soil depths.

Linear mixed-effect models were also used to test the effect of the interaction between nutrients added using the factorial design N*P*cations as follows: lmer (soil property~N*P*cations +(1|block). When the addition of a single nutrient or the interaction between them were significant, a backward elimination of all effects of the linear mixed model was performed using the *step* function from the 'lmerTest' package and only the significant effects of nutrient additions are reported. When the addition of an element and/or their interaction resulted in significant effects, a post-hoc Tukey pairwise comparison of means between all the contrasts was performed using the package 'emmeans'. All analyses were conducted in R version 3.4.4 (R Core Team 2018).

5.6. Results

5.6.1. Variation in soil chemical properties

The treatments did not significantly affect soil pH in water for the mean 0-10 cm layer ($F_{7,21}$= 1.96, p= 0.11), or when comparing 0-5 cm and 5-10 cm layers separately ($F_{7,21}$= 1.96, p= 0.10; $F_{7,21}$= 1.56, p= 0.19 respectively) with mean soil pH of 4.12 ± 0.03 across all plots. Moreover, no significant differences were found between depths in each of the treatments using paired t-tests (Table 5.1). When testing for the effect of the factorial N*P*cations addition, soil pH for the 0-10 cm soil layer significantly increased with the addition of cations-only ($F_{1,21}$=8.26, p= 0.009; +cations: 4.18 ± 0.04 *versus* -cations: 4.05 ± 0.03), with no effect of the addition of P, N or interaction between nutrients. The effect of cations was also significant for both soil depths separately ($F_{1,21}$=7.61, p=0.01 and $F_{1,21}$=5.14, p=0.03 respectively for 0-5 and 5-10 cm soil layer; Figure 5.1). Only a marginal effect of P-addition ($F_{1,21}$=4.17, p=0.05) was detected in the 0-5 cm soil layer, with no effects of N addition on soil pH (Figure 5.1). Carbon and nitrogen concentrations as well as CN ratios in soils were not significantly different among treatments for the 0-10 cm soil layer ($F_{7,21}$= 0.38, p= 0.90, $F_{7,21}$= 0.73, p= 0.64 and $F_{7,21}$= 0.60, p= 0.74 respectively), nor when analysing each depth separately. Mean carbon concentration for all treatments was 3.12 ± 0.12%, with mean N of 0.215 ± 0.009% and CN ratio of 14.92 ± 0.49. In general, C and N concentrations were higher in the 0-5 cm than in the 5-10 c soil layer (Table 5.1). When analysing the addition of nutrients using the factorial interaction, no significant effects were

detected for C, N and CN ratios with the addition of N, P or cations for the 0-10 cm soil layer (results not shown) nor for 0-5 cm and 5-10 cm layers separately (Figure 5.2).

Table 5.1. Mean soil pH in H_2O, carbon (C) and nitrogen (N) concentrations and CN ratios ± standard errors in eight treatments and two soil depths (0-5 cm and 5-10 cm) in central Amazon. *n*= 4 per treatment per depth.

Treatment	Depth	pH H_2O	C (%)	N (%)	CN
CONTROL	0-5cm	3.96 ± 0.09	4.03 ± 0.64*	0.27 ± 0.04*	14.41 ± 0.38
	5-10cm	4.18 ± 0.11	2.37 ± 0.27	0.163 ± 0.016	14.44 ± 0.36
N	0-5cm	3.99 ± 0.02	3.85 ± 0.19**	0.29 ± 0.01**	13.21 ± 0.27
	5-10cm	4.07 ± 0.06	2.55 ± 0.18	0.185± 0.01	13.76 ± 0.57
P	0-5cm	4.01 ± 0.06	3.78 ± 0.33*	0.24 ± 0.04*	16.79 ± 2.76
	5-10cm	4.07 ± 0.04	2.07 ± 0.05	0.15 ± 0.01	14.35 ± 1.26
CATIONS	0-5cm	4.07 ± 0.06	3.29 ± 0.14**	0.23 ± 0.02***	14.52 ± 1.63
	5-10cm	4.17 ± 0.04	2.13 ± 0.07	0.14 ± 0.02	15.97 ± 2.83
N+P	0-5cm	4.06 ± 0.13	4.39 ± 0.78*	0.30 ± 0.07	15.07 ± 1.44
	5-10cm	4.02 ± 0.07	2.42 ± 0.37	0.17 ± 0.03	14.39 ± 0.90
N+ CATIONS	0-5cm	4.05 ± 0.09	4.21 ± 0.51*	0.28 ± 0.04*	15.62 ± 2.55
	5-10cm	4.10 ± 0.08	2.28 ± 0.27	0.16 ± 0.02	14.84 ± 1.35
P+ CATIONS	0-5cm	4.30 ± 0.12	3.53 ± 0.46*	0.24 ± 0.01	14.68 ± 1.43
	5-10cm	4.31 ± 0.12	2.54 ± 0.41	0.16 ± 0.03	16.63 ± 2.14
N+P+ CATIONS	0-5cm	4.23 ± 0.10	4.17 ± 0.80*	0.28 ± 0.06	15.14 ± 1.14
	5-10cm	4.24 ± 0.03	2.36 ± 0.24	0.16 ± 0.02	14.95 ± 1.20

Differences between soil depths in each treatment were tested and significant effects from t-tests are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

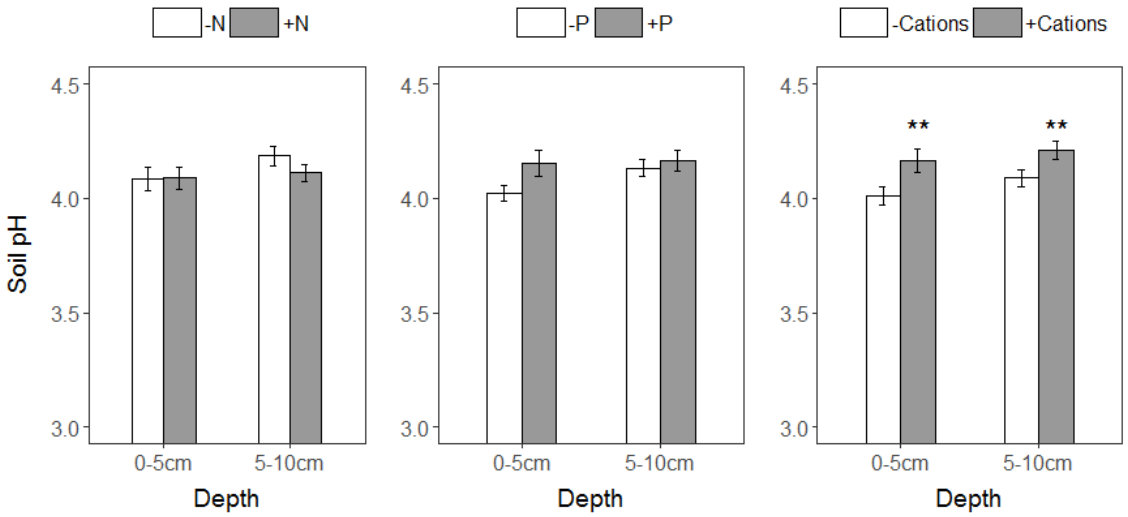

Figure 5.1. Mean soil pH in water in two different depths (0-5 cm and 5-10 cm) in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient per depth. Error bars represent standard errors. Responses were analysed based on $[H^+]$ and significant effects are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

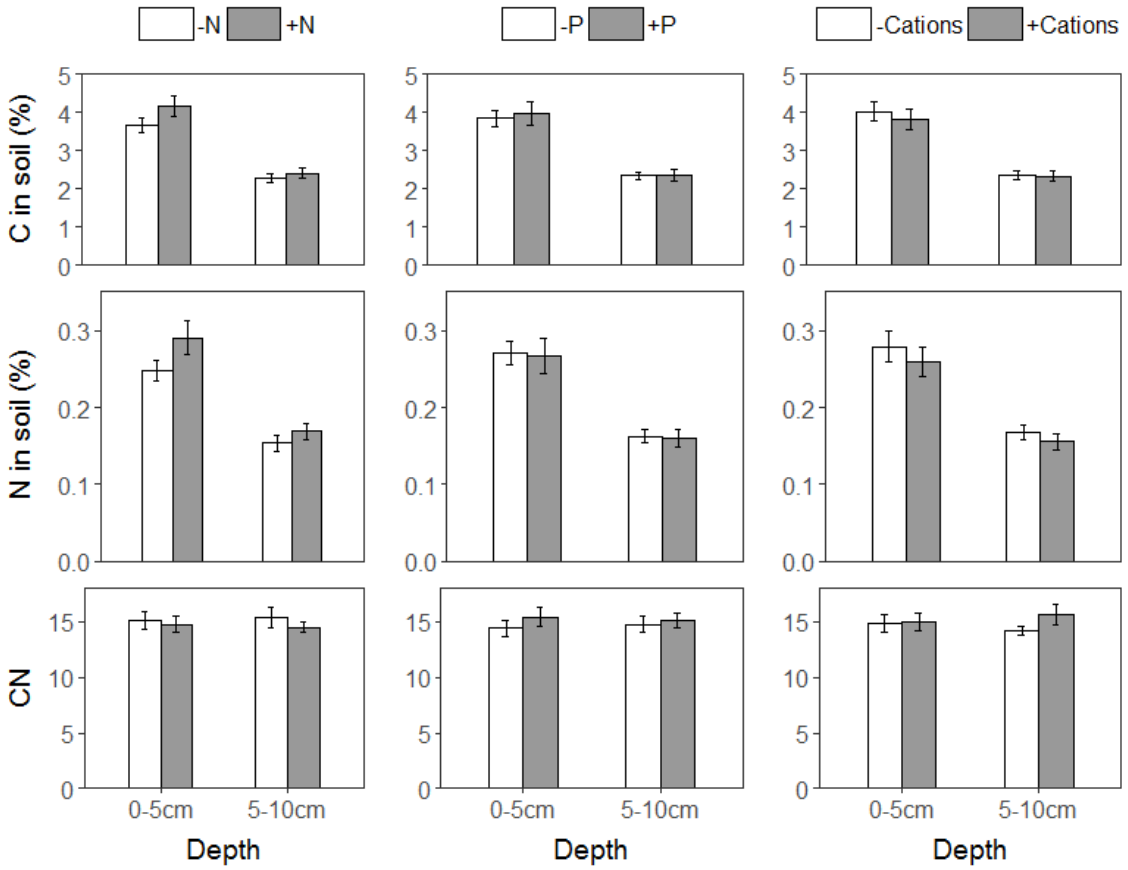

Figure 5.2. Mean soil carbon and nitrogen concentrations and CN ratios in two different depths (0-5 cm and 5-10 cm) in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient per depth. Error bars represent standard errors.

5.6.2. Variation in soil enzyme activity with nutrient addition

Mean ß-glucosidase, N-acetyl ß-glucosaminidase and phosphomonoesterase activities for the 0-10 cm soil layer (n=32) were not significantly affected by the main treatments when compared to control plots ($F_{7,21}$=1.46, p=0.23; $F_{7,21}$=1.24, p=0.32; $F_{7,21}$=2.31, p=0.06 respectively; Figure 5.3). Mean BG, NAG and PHOS activities across all treatments were 24.42 ± 1.80, 124.92 ± 8.35 and 2,330.22 ± 93.60 nmol MUF g dry soil^{-1} hour^{-1}. No differences were found between treatments and control plots when comparing soil depths separately (Table 5.2). Paired t-tests were conducted to detect any differences between mean enzyme activities between soil depths in each of the

treatments (Table 5.2). Higher BG activity was found for 0-5 cm soil layer only in the N treatment (t=3.41 *p*=0.042). NAG activity was significantly higher in the superficial layer only for the cations and N+cations treatments (t = 4.71, *p*= 0.018 and t = 16.16, *p*= 0.0005 respectively). No differences between soil layers were found for PHOS activity in the different treatments.

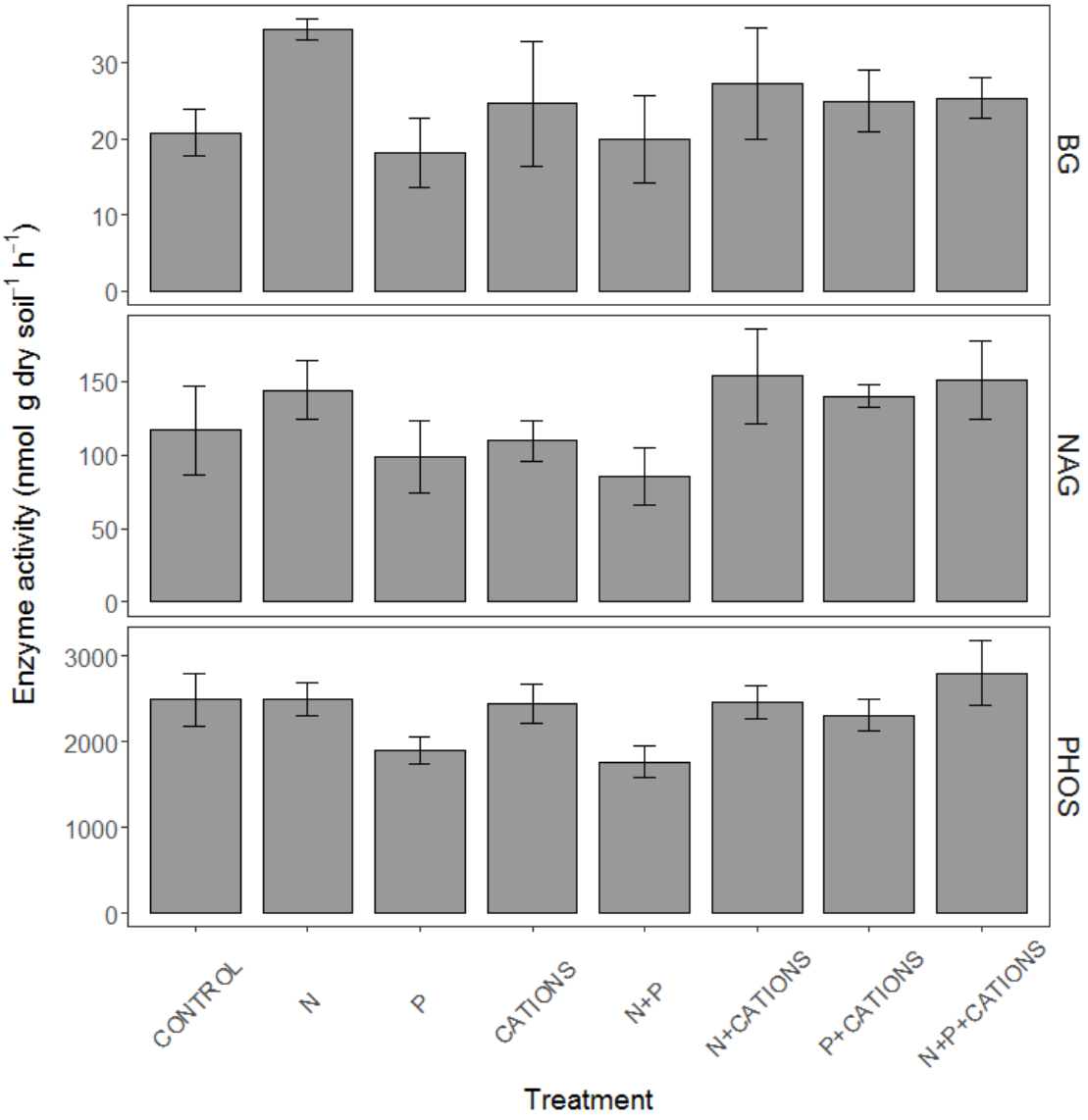

Figure 5.3. Mean BG, NAG and PHOS activity for the 0-10 cm soil depth in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. *n*= 4 per treatment. Error bars represent standard errors. Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}.

Table 5.2. Mean enzyme activities ± standard errors in eight treatments and two soil depths in central Amazon. *n*= 4 per treatment per depth.

Treatment	Depth	BG	NAG	PHOS
CONTROL	0-5cm	21.74 ± 1.94	124.61 ± 30.26	2,516.85 ± 306.31
	5-10cm	19.9 ± 4.22	109.11 ± 30.91	2,455.46 ± 337.56
N	0-5cm	42.93 ± 3.25*	161.3 ± 22.93	2,568.89 ± 191.12
	5-10cm	25.72 ± 2.5	127.17 ± 19.77	2,415.06 ± 217.88
P	0-5cm	19.73 ± 6.14	113.53 ± 37.06	1,870.01 ± 187.52
	5-10cm	16.52 ± 4.19	84.51 ± 13.02	1,929.71 ± 246.31
CATIONS	0-5cm	32.08 ± 11.55	132.11 ± 15.13*	2,652.08 ± 310.24
	5-10cm	17.09 ± 5.18	86.95 ± 14.38	2,223.95 ± 154.95
N+P	0-5cm	20.94 ± 3.86	96.86 ± 25.24	1,730.28 ± 116.10
	5-10cm	18.89 ± 8.72	74.11 ± 21.03	1,795.08 ± 277.44
N+CATIONS	0-5cm	33.09 ± 8.13	187.69 ± 32.81***	2,625.32 ± 98.23
	5-10cm	21.47 ± 7.16	119.36 ± 30.98	2,294.54 ± 330.88
P+CATIONS	0-5cm	24.66 ± 4.63	130.84 ± 25.01	2,153.76 ± 333.51
	5-10cm	25.26 ± 6.55	149.03 ± 36.68	2,454.83 ± 154.48
N+P+CATIONS	0-5cm	31.21 ± 5.65	188.39 ± 41.12	2,751.14 ± 546.26
	5-10cm	19.45 ± 1.09	113.2 ± 17.05	2,846.65 ± 274.55

Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}. BG: ß-glucosidase; NAG: N-acetyl ß-glucosaminidase; PHOS: phosphomonoesterase. Differences between soil layers were tested using t-test and when significant, effects indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

When analysing the interaction between nutrients using the factorial design N*P*cations, the addition of P-only and cations-only affected PHOS activity, although only marginally for P addition ($F_{1,21}$=3.10, p=0.09; $F_{1,21}$=4.65, p=0.04 respectively), with no significant effects found for BG and NAG activities (Figure 5.4). More importantly, a significant interaction between P*cations was detected

when testing the effect of the full factorial model for PHOS activity ($F_{1,21}$=5.81, *p*=0.02; Figure 5.5). PHOS activity only declined significantly with P addition when cations were not also added (Figure 5.5; +P-cations: 1,831.26 ± 114.12 *versus* +P+cations: 2,551.59 ± 217.05 nmol g soil^{-1} hour^{-1}; *p*=0.01). The same trend for the interaction between P and cations was observed for NAG and BG, to a lesser extent, but the interaction terms were not statistically significant (Figure 5.6).

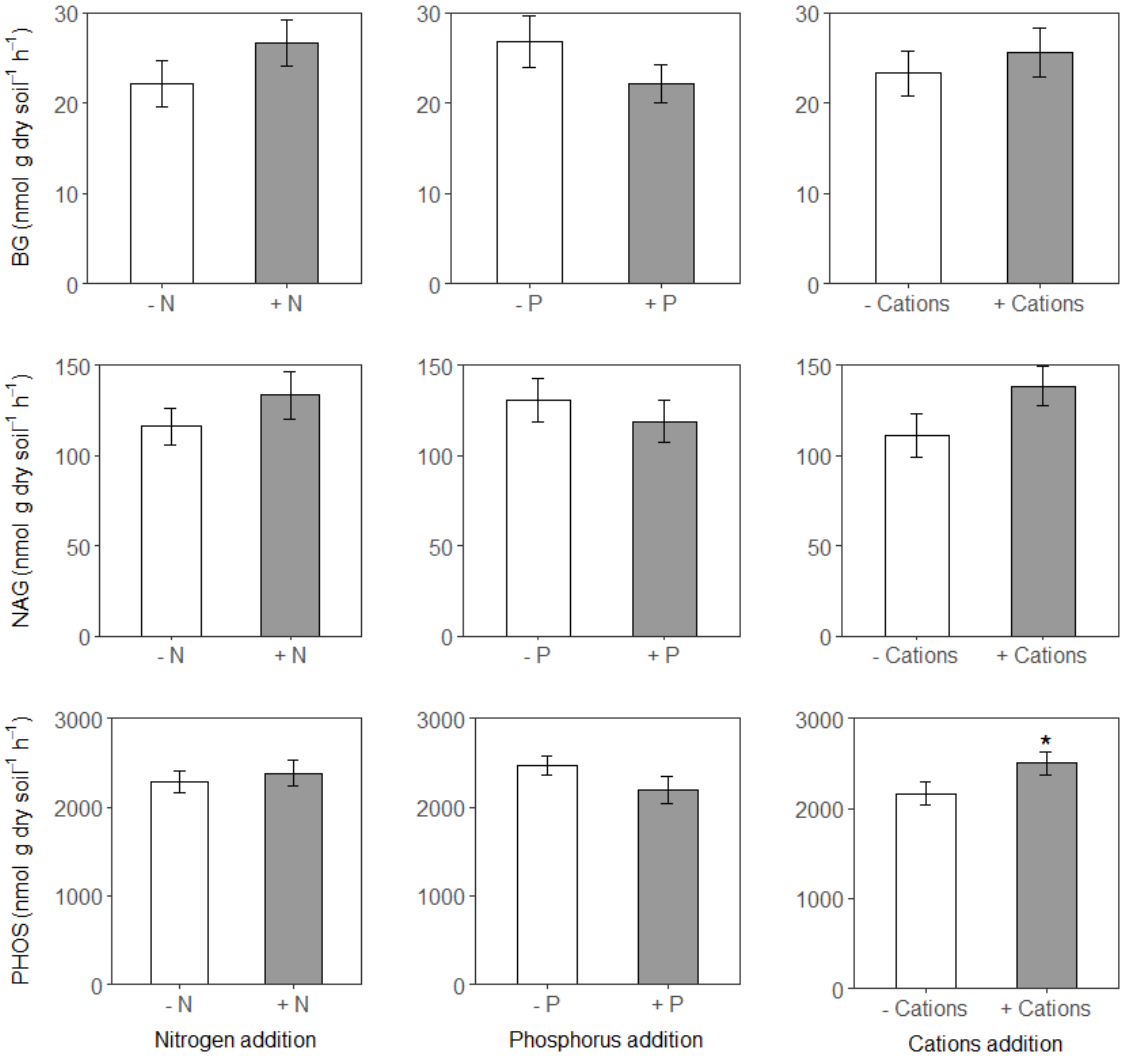

Figure 5.4. Mean BG, NAG and PHOS activity for the 0-10 cm soil depth in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient. Error bars represent standard errors. Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}. Significant effects of the N*P*cations are

indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

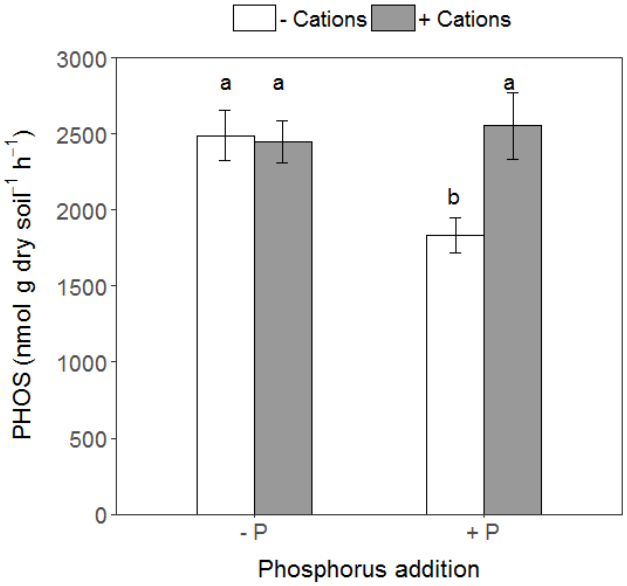

Figure 5.5. Responses of mean phosphatase activity for the 0-10 cm soil layer (*n*=8) with or without the addition of P and cations in a lowland tropical forest in central Amazon, Brazil. Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}. Results from the pairwise comparisons are indicated by letters.

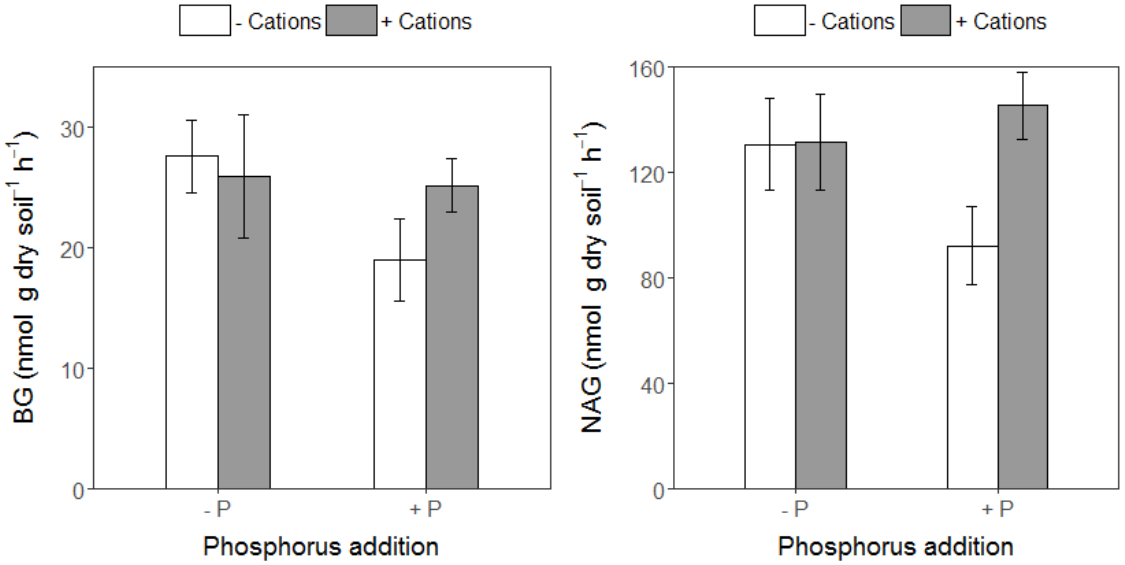

Figure 5.6. Responses of mean BG and NAG activity for the 0-10 cm soil layer (*n*=8) with or without the addition of P and cations in a lowland tropical forest in

central Amazon, Brazil. Error bar are standard errors. Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}.

Changes in enzyme activities, when detected, were mainly restricted to the 0-5 cm soil layer when analysing the full factorial nutrient addition. Significant effects of nutrient addition were detected for PHOS activity (Figure 5.7), which tended to decrease significantly with P addition only in the 0-5 cm layer ($F_{1,21}$=5.08; p=0.034), with no significant effect of cations or nitrogen addition. No significant effects were detected for BG and NAG activities in any soil layer (Figure 5.7). The same pattern of interaction between P and cations addition was found for each soil layer when analysed separately (+P-cations: 1,800.14 ± 105.45 *versus* +P+cations: 2,452.44 ± 317.05 nmol g soil^{-1} hour^{-1} for the 0-5 cm soil layer and +P-cations: 1,862.39 ± 173.61 *versus* +P+cations: 2,650.74 ± 163.55 nmol g soil^{-1} hour^{-1} for the 5-10 cm soil layer).

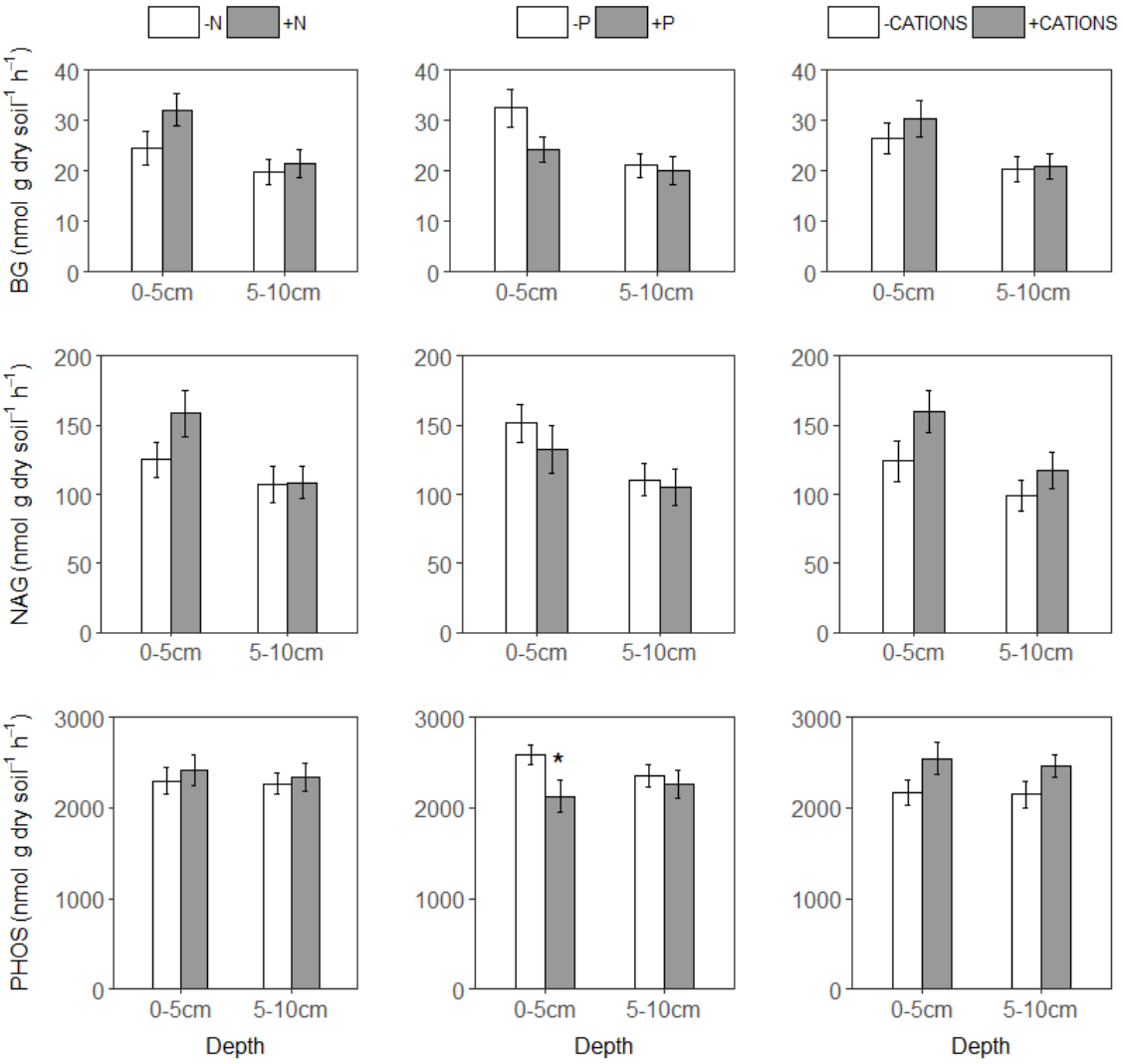

Figure 5.7. Mean BG, NAG and PHOS activity in two different soil depths (0-5 cm and 5-10 cm) in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient per depth. Error bar are standard errors. Enzyme activities are shown in nmol MUF g soil dry weight^{-1} hour^{-1}. Significant effects of the N*P*cations by soil layer are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

5.6.3. Stoichiometric enzyme ratios

The treatments did not affect enzyme ratios significantly for the 0-10 cm soil layer when compared to control plots ($F_{7,21}$=0.74, p=0.64; $F_{7,21}$=1.35, p=0.27 and

$F_{7,21}$=0.73, *p*=0.65 respectively for BG:NAG, BG:PHOS and NAG:PHOS). Mean BG:NAG, BG:PHOS and NAG:PHOS ratios for all treatments were 0.208 ± 0.014, 0.010 ± 0.0007 and 0.053 ± 0.003, respectively. When comparing both 0-5 cm and 5-10 cm depth separately, no significant effects of the treatments were detected (Table 5.3). Paired t-tests were conducted to detect any differences between mean enzyme activity ratios between soil depths in each of the treatments (Table 5.3). No differences between soil depths were detected for BG:NAG ratios in any of the treatments. NAG:PHOS ratios varied significantly by depth when comparing the means from all treatments (0.060 ± 0.004 *versus* 0.046 ± 0.003 for 0-5 cm and 5-10 cm respectively; *p*=0.002) but no differences were detected when analysing each treatment separately by depth. Mean BG:PHOS ratios for all treatments were different among the two soil depths (0.012 ± 0.0008 *versus* 0.009 ± 0.0008 for 0-5 cm and 5-10 cm respectively) and were significantly higher in the 0-5 cm soil layer for the cations (*p*=0.04) and N+cations (*p*=0.04) treatments (Table 5.3).

Table 5.3. Mean enzyme activity ratios ± standard errors in eight treatments and two soil depths in central Amazon. *n*= 4 per treatment per depth.

Treatment	Depth	BG:NAG	BG:PHOS	NAG:PHOS
CONTROL	0-5cm	0.209 ± 0.060	0.0092 ± 0.0017	0.0480 ± 0.0070
	5-10cm	0.204 ± 0.050	0.0083 ± 0.0017	0.0426 ± 0.0062
N	0-5cm	0.276 ± 0.026	0.0171 ± 0.0020	0.0646 ± 0.0115
	5-10cm	0.222 ± 0.044	0.0108 ± 0.0014	0.0549 ± 0.0108
P	0-5cm	0.179 ± 0.030	0.0099 ± 0.0024	0.0584 ± 0.0159
	5-10cm	0.185 ± 0.035	0.0083 ± 0.0018	0.0443 ± 0.0063
CATIONS	0-5cm	0.239 ± 0.068	0.0116 ± 0.0031*	0.0498 ± 0.0021
	5-10cm	0.198 ± 0.059	0.0075 ± 0.0020	0.0387 ± 0.0048
N+P	0-5cm	0.240 ± 0.053	0.0118 ± 0.0018	0.0545 ± 0.0122
	5-10cm	0.250 ± 0.074	0.0099 ± 0.0044	0.0385 ± 0.0084

N+CATIONS	0-5cm	0.191 ± 0.059	0.0123 ± 0.0027*	0.0710 ± 0.0107
	5-10cm	0.199 ± 0.067	0.0092 ± 0.0029	0.0517 ± 0.0115
P+CATIONS	0-5cm	0.209 ± 0.046	0.0119 ± 0.0020	0.0592 ± 0.0036
	5-10cm	0.172 ± 0.024	0.0101 ± 0.0024	0.0604 ± 0.0141
N+P+CATIONS	0-5cm	0.177 ± 0.026	0.0118 ± 0.0014	0.0746 ± 0.0189
	5-10cm	0.184 ± 0.031	0.007 ± 0.0009	0.0394 ± 0.0031

BG: ß-glucosidase; NAG: N-acetyl ß-glucosaminidase; PHOS: phosphomonoesterase. BG:NAG ratios indicate C:N ratios; BG:PHOS indicate C:P ratios and NAG:PHOS indicate N:P ratios. Differences between soil layers were tested using t-test and when significant are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

BG:NAG, BG:PHOS and NAG:PHOS ratios were not significantly affected when analysing the addition of nutrients in the full factorial model for the 0-10 cm soil depth (Figure 5.8) or when analysing 0-5 cm and 5-10 cm soil depths separately.

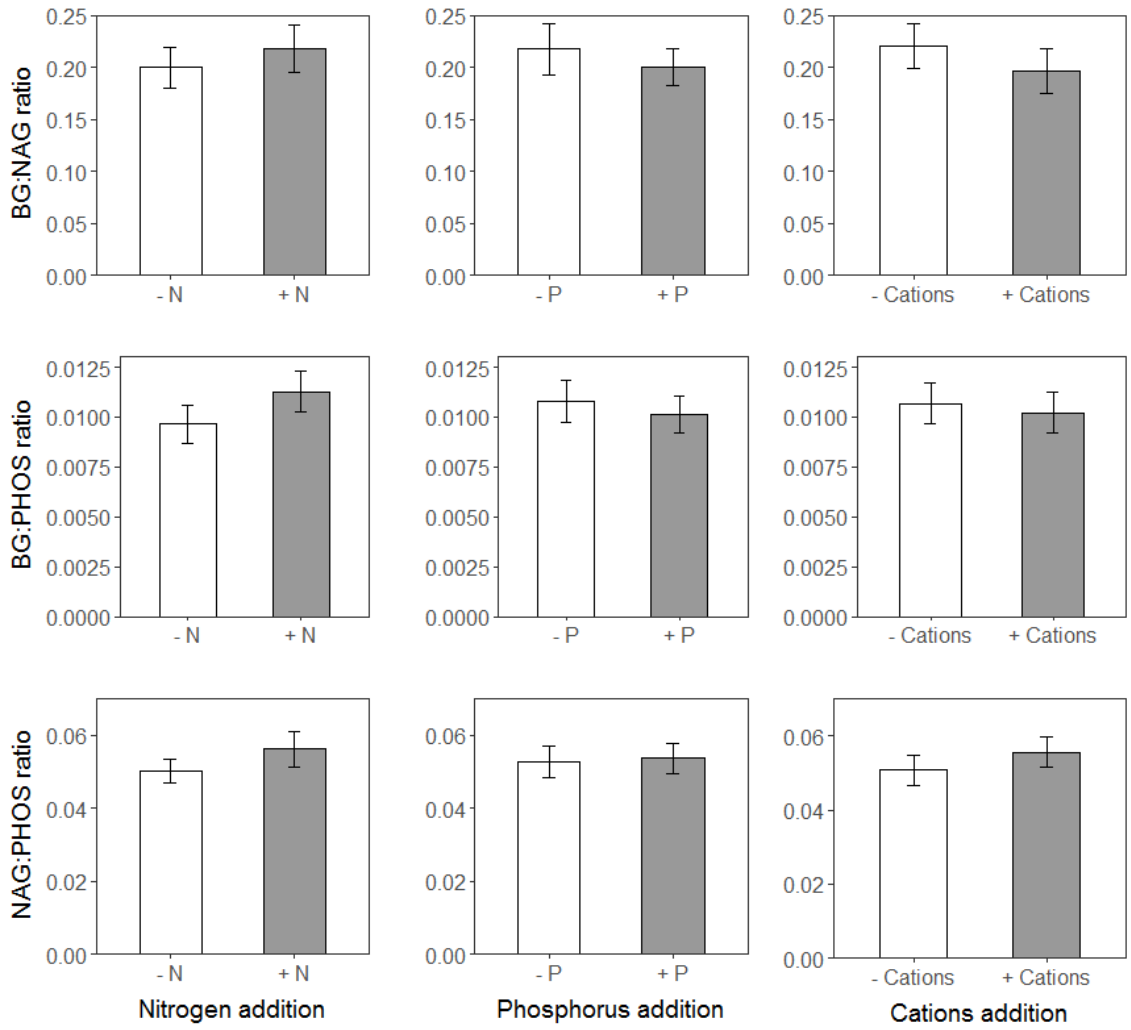

Figure 5.8. Mean BG:NAG, BG:PHOS and NAG:PHOS in response to nutrient fertilisation in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient per depth. Error bars represent standard errors.

5.7. Discussion

5.7.1. Comparison of central Amazon potential microbial enzyme activities with other tropical studies

The activities of ß-glucosidase (BG), N-acetyl ß-glucosaminidase (NAG) and phosphomonoesterase (PHOS) in this study (control plots only) were generally low when compared to data from other tropical forests, especially for BG (Table 5.4). Microbial enzyme activities usually increase as a response to low resource availability (Sinsabaugh and Moorhead 1994), and because soils in central Amazon are, in general, less fertile than other tropical soils, higher absolute

microbial enzyme activity was expected. Since the measurements presented here were conducted during the wet season, the lower enzyme activities in this central Amazon forest could be due the lower litterfall production during this period (Luizao 1989), which would therefore decrease the amount of organic matter substrate available for soil microorganisms. However, Turner and Wright (2014) found the opposite pattern, with lower hydrolytic enzyme activities in the dry season in lowland forests in Panama and they attributed this finding to the death of microorganisms following desiccation.

The low P availability of the majority of tropical soils result in high investments in phosphatase exudation and consequently, BG:PHOS and NAG:PHOS ratios in tropical areas are much lower than global averages (Sinsabaugh et al. 2008; Waring et al. 2014). Although the absolute values for enzyme activity found in this study were generally low, the investment in NAG and PHOS activities relative to BG were much higher than obtained in other tropical studies (Table 5.4). Extracellular enzyme activities are usually described as indicators of microbial nutrient demand (Sinsabaugh et al. 2008). Particularly, inferences about microbial investment in resource acquisition could be made when comparing enzyme stoichiometric ratios and environmental nutrient availability (Sinsabaugh et al. 2009). BG:PHOS enzyme ratios in control plots in central Amazon were at least one order of magnitude lower than in other tropical areas (Table 5.4), and NAG:PHOS enzyme ratios were substantially lower than at all other sites, with the exception of one study in Panama (Turner 2010b), where values approached those observed in this study. These data highlight the potential P limitation of soil microorganisms in central Amazonia and how it results in disproportionate investment in P-cycling enzymes (Turner and Wright 2014).

Table 5.4. ß-glucosidase (BG), N-acetyl ß-glucosaminidase (NAG), phosphomonoesterase (PHOS) activities and BG:NAG, BG:PHOS, NAG:PHOS ratios in different tropical wet forests (extracted from Waring et al. 2014) compared to the activities from central Amazon (this study; mean of control plots only; *n*=4). Soil carbon (C) and nitrogen (N) concentrations are given in percentage; P means total P and is given in mg kg^{-1}; pH means soil pH in water; BG, NAG and PHOS activities are given in nmol g^{-1} dry soil h^{-1}.

Study	Location	Soil order	C	N	P	pH	BG	NAG	PHOS	BG:NAG	BG:PHOS	NAG:PHOS
Acosta 2007	Puerto Rico	Various	2.67	0.24		4.9	162	111	1,415	1.46	0.114	0.0784
Cadwell 1999	Costa Rica	Inceptisol	3.79	0.34		5.5	1,135		11,300		0.100	
Dinesh 2004	India	Inceptisol	1.31	0.13		5.2	3,533		10,050		0.352	
Sjogersten 2010	Panama	Histosol	49.18	2.55	650	3.9	903	671	5,773	1.35	0.156	0.1162
Turner 2010a	Panama	Various	4.89	0.38	490	4.8	324	450	1,659	0.72	0.195	0.2712
Turner 2010b	Panama	Various	3.56	0.31	430	4.9	235	112	2,029	2.10	0.116	0.0552
Turner 2014	Panama	Oxisol			600	5.2	192	360	4,500	0.53	0.043	0.0800
Waldrop 2000	Tahiti	Mollisol	7.81	0.40		4.57	131		571		0.229	
Waring unp.		Ultisol				4.3	1,995		19,312		0.103	0.1196
Weintraub 2012	Costa Rica	Ultisol	5.53	0.46	670	5.2	143	78	652	1.83	0.219	0.1955
Yavitt 2004	Panama	Oxisol							7,700			
This study	**Brazil**	**Oxisol***	**3.20**	**0.22**	**85**	**4.1**	**21**	**117**	**2,486**	**0.18**	**0.008**	**0.0471**

*Oxisols are the American soil classification analogous to Acrisols and are used here to facilitate comparison between studies described in the table.

5.7.2. Effects of nutrient addition on enzyme activity and ratios

The activities of soil hydrolytic enzymes were clearly influenced by the short-term addition of nutrients in this central Amazon. In partial support to my hypotheses, P addition decreased the microbial investments in phosphatase production, whilst cation addition suppressed the reduction on phosphatase activity caused by the addition of P. In other words, the addition of cations eliminated the effect of alleviation of P limitation when added together with P. The addition of N, on the other hand, did not stimulate enzyme production as I hypothesised. Soil phosphatase activity was reduced with P addition for the top soil layer and such reduction was even stronger in plots where P was added without cations. The decrease in phosphatase activity with P addition was demonstrated by earlier studies on fertilisation experiments (Olander and Vitousek 2000; Turner and Wright 2014), meta-analysis (Marklein and Houlton 2012) and also in natural soil fertility gradients (Kitayama 2013), but this is the first time that direct evidence of P-limitation in soil microbes has been detected in central Amazon forests.

Phosphatase activity decreased by 28% (for the 0-10 cm soil layer) after only six months of P addition without cations, suggesting that soil microbes are highly dependent on organic P as a P source (Yokoyama et al. 2017) and can respond rapidly to changes in soil nutrient availability in this tropical forest. Phosphorus constitutes an important part of soil fungi and bacteria metabolism, being found as nucleic acids (30-65% of total microbial biomass), phospholipids, acids, enzymes and coenzymes (Oberson and Joner 2005). Besides the evidence corroborating the predictions that P strongly limits soil microorganisms, the results found in this study also suggest that cation concentrations could directly or indirectly affect enzyme exudation in central Amazon. The interaction between P and cations controlling microbial enzyme activities could also result from the increased competition between roots and soil microorganisms. The addition of cations increased root biomass and root phosphatase activity (Chapter 4), which could therefore benefit roots over soil microorganisms when acquiring nutrients. This way, P limitation from the soil microbial community might have been alleviated only when P was added without cations.

Despite the similar patterns of reduction in BG and NAG activities with P addition, only PHOS activity was affected significantly after 6 months of nutrient addition. The lack of significant changes in BG and NAG activities could indicate that soil microbial community in central Amazon forests might not be as limited by the supply of C and N as it is by the supply of P, but because carbon limitation was not directly tested in this experiment, there is not enough evidence to support this hypothesis. It is more likely that the greater absolute values for PHOS activity when compared to BG and NAG, could help explain why a more significant decrease in PHOS after P addition was detected. Furthermore, because most of the extracellular phosphatases hydrolyse P derived from a wide range of compounds, changes in PHOS potential activity could be easier to capture when compared to N and C-cycling enzymes (McGill and Cole 1981; Sinsabaugh et al. 2008). The general decrease in the activities of all the enzymes studied here in response to P addition, and near constant C:P and N:P ratios (Figure 5.8), therefore do not support the conceptual model of McGill and Cole (1981), in which organic C, N and P would be mineralised through different pathways during organic matter decomposition. In this model, the authors proposed that P cycles independently, via biochemical mineralisation, which is controlled by soil microbial demand for P, rather than energy. For this reason, P acquisition would not be as dependent on C mineralisation as N acquisition is, mainly because organic P can be found as phosphate esters, mineralised independently by phosphatase enzyme activity (McGill and Cole 1981). Contrary to my hypothesis, the results found here support the idea that enzymes related to C, N and P cycles are still produced by soil microorganisms in a consistent stoichiometric ratio even after short-term nutrient addition, reflecting an equilibrium between microbial nutrient content and nutrient assimilation in these soils (Sinsabaugh et al. 2008; Sinsabaugh et al. 2009). The general reduction in the potential activity of all enzymes could therefore be a consequence of an overall reduction in P limitation or decreased P demand by microorganisms, resulting in reduced enzyme exudation of all enzyme involved in organic matter decomposition.

The similar trends of BG, NAG and PHOS activities with the addition of nutrients resulted in similar enzyme ratios across all treatments, with no significant effect of fertilisation detected. The results found here therefore contradict my initial hypothesis that enzyme C:N:P ratios change in the different

treatments, since I predicted stronger effects on PHOS, rather than BG and NAG activities with nutrient addition. For instance, Turner and Wright (2014) found that PHOS and NAG decreased 65% and 24% respectively after 10 years of phosphorus addition in forests in Panama, with no effects on BG, resulting in disproportionally higher BG:NAG and BG:PHOS ratios. The lack of significant differences in enzyme ratios between treatments in the present study could therefore be attributed to 1) the short-term and variable responses detected for enzyme activities under different treatments, in which significant changes in enzyme stoichiometry would be captured only with chronic nutrient addition or 2) the constrained stoichiometry ratios of soil microbial biomass, with enzyme ratios being tied to microbial nutrient demand as proposed by Sinsabaugh et al. (2008). Despite the reduced investment in enzyme production with P addition, the consistency in microbial enzyme ratios found in this study ultimately suggests that microbial C:N:P ratios are constrained, at least in the short-term, and strongly linked to environmental nutrient availability (Cleveland and Liptzin 2007; Sinsabaugh et al. 2008).

Central Amazon soils are characterised by very low concentrations of P and cations as a result of the intense weathering process along soil development (Quesada et al. 2011). The strong effect that the interaction between P and cations caused on phosphatase activity could be interpreted as another indication of the very low nutrient status of this forest. In Panama, however, the addition of Ca and Mg together with micronutrients did not affect the soil microbial community after 10 years of nutrient addition (Turner and Wright 2014). The lack of a decrease in soil phosphatase exudation when both P and cations were added together in my study site could be due the formation of Ca phosphates as a result of the strong affinity between these elements, therefore decreasing, rather than increasing P availability for soil microorganisms (Hinsinger 2001). This effect is however not very likely in such acidic soils, since the stronger affinity between phosphorus and cations (mainly Ca) is usually reported in neutral and calcareous soils, and although an increase in soil pH was detected with cations addition in this study, these soils are still considered very acidic. In very low pH soils, as in this study site, phosphates are usually found to be bound to iron and aluminium-minerals rather than Ca-minerals (Hinsinger 2001; Jones and Oburger 2011). However, the increase in soil pH detected here could also 1) impact soil microbial

community by changing its composition which could indirectly influence the rates of hydrolytic enzymes exudation (Waldrop et al. 2000; Allison et al. 2007; Fanin et al. 2015) or 2) influence the conformation of the enzyme and its retention and solubility in soil particles (Turner 2010). Nonetheless, no direct influence of soil pH on phosphatase activity was detected in the present study (Adjusted R^2= -0.011, *p*= 0.42; Supplementary Figure 5.1. in the Appendix 3)

Although the hypothesis of changes in soil microbial community composition and abundance could not be discarded (since such measurements were not performed in this study), another more likely explanation for the strong effect of cations on phosphatase activity could be related to soil nutrient stoichiometry. Based on the P-paradigm, which assumes that P is the most limiting nutrient in tropical forests (Vitousek et al. 2010; Dalling et al. 2016) I hypothesised that soil microorganisms would strongly respond to P addition. However, more recent studies found evidence for multiple nutrients limiting different aspects of ecosystem functioning (Wurzburger and Wright 2015; Kaspari et al. 2017; Sheldrake et al. 2018), dismissing the classic theory of Liebig that proposes that only a single nutrient can limit a specific ecosystem compartment at a time. Following the idea of multiple nutrient limitation found for different ecosystems (Davidson and Howarth 2007; Elser et al. 2007) the results shown here could suggest a similar stoichiometric balance between P and cations in central Amazon forests. The addition of P and cations together could have resulted in a synergistic response in which although P concentrations increased when P was added with cations, P limitation was still not alleviated in relation to cations concentrations. Contrary, when P was added without cations, relative P concentrations increased and as a result soil phosphatase activity decreased, suggesting an alleviation of P limitation only when P was added without cations. The existence of such a mechanism, however, would have to be directly tested by analysing soil nutrient stoichiometry, but these data were not available for my study area.

In previous studies, root and soil phosphatase activities were found to be responsive to the addition of nitrogen, suggesting that plants and microbes could allocate the extra N to enzyme exudation (Cusack et al. 2011; Marklein and Houlton 2012; Jin et al. 2013). However, the addition of nitrogen did not affect phosphatase exudation in this study, with no evidence that the extra N was

assimilated by soil microorganisms after 6 months of N addition, at least not in amounts that could cause significant changes in enzyme exudation. After a decade of nutrient addition, Turner and Wright (2014) only found a marginally significant increase in soil phosphatase activity with N addition in lowland forests in Panama, with no effects of the addition of this nutrient on BG and NAG activities. Moreover, the addition of N also did not affect soil phosphatase activity in a tropical montane rainforest in Ecuador (Dietrich et al. 2016). One explanation for the lack of stimulation of phosphatase activity with N addition could be the already high availability of N in tropical soils (Turner and Wright 2014). The addition of N could also have negative effects on the soil microbial community by decreasing soil pH and by producing more recalcitrant organic matter (Mori et al. 2018) which could therefore stimulate C and N cycling enzymes. However, N addition did not affect soil pH and also did not change C and N-cycling enzymes exudation in the present study.

5.8. Conclusions

Soil microorganisms responded strongly after 6 months of nutrient addition in low-fertility soils in central Amazon, suggesting that the microbial community is able to rapidly change their investments in nutrient acquisition mechanisms in response to changes in nutrient availability. The addition of phosphorus caused a decline in the activity of phosphatase, the enzyme responsible for degrading organic P. Such fast response could also be translated into direct evidence supporting the hypothesis that phosphorus strongly limits soil microorganisms in this central Amazon forest. However, cations were also found to play a role on microbial enzyme exudation by offsetting the decline in phosphatase activity when added together with phosphorus. The strong interaction between cations and phosphorus, could be attributed to indirect changes on soils properties (e.g. soil pH, formation of Ca-phosphates, enzyme solubility) or direct changes in soil microbial community, ultimately affecting phosphatase production and soil microbial nutrient status. The strong and rapid microbial responses to P and cation additions in this central Amazon forest emphasises the importance of understanding the many aspects controlling soil microbial limitation. Since soil microorganisms can act both as a sink or source of nutrients, the evidence found

here supporting alleviation of microbial P limitation would potentially also affect plant productivity by changing P availability in soils. Such information is crucial if we are to better understand how these forests function and how they may respond to global change. Finally, the following chapter explores the implications of the results discussed in the current and previous chapters in different spatial and temporal scales, also suggesting opportunities for moving forward.

Chapter 6 - Conclusions and recommendations

6. Conclusions and recommendations

The final chapter concludes and discusses the implications of the results found in this thesis. I first summarise the findings of each chapter separately, linking the results with the initial specific objectives I aimed to achieve with this work. I then discuss the implications of my findings in different spatial and temporal scales, exploring my results in the context of natural soil development and evolution across the Amazon basin but also the consequences of plant-soil feedbacks in an era of anthropogenic and climatic changes. Finally, based on the questions that still remain to be answered about nutrient limitation in the Amazon, I suggest areas of future research and the challenges involved in deepening our understanding on belowground tropical functioning.

6.1. Key findings from the present work

6.1.1. Objective 1 - Chapter 2: Overview of the mechanisms related to phosphorus cycling in tropical ecosystems, with emphasis in Amazon soils

In this chapter I reviewed the current knowledge on tropical and Amazon forests regarding the causes and consequences of phosphorus limitation shaping forest functioning. I gathered information about the biotic and abiotic processes controlling the fate of P with soil development across the Amazon basin and also evidence from soil fertility gradients in lowland tropical forests that suggests that P is the main nutrient limiting the majority of forests growing in low-fertility soils in the Amazon. Despite the assumption that the majority of tropical forests are P-limited, direct evidence is still lacking from lowland Amazon forests so I reviewed the main findings from nutrient manipulation experiments in other lowland tropical forests and based on those findings I hypothesised what would be expected in low-fertility Amazon soils. Since disentangling the mechanisms by which Amazon forests thrive on such low-fertility soils is crucial to understanding forest functioning under present and future climate change, I also describe what is currently known about experimental CO_2 enrichment affecting nutrient cycling. Based on the evidence pointing to belowground plant and soil processes as important drivers controlling nutrient cycling and limitation in Amazon forests, I reviewed the current state of art of dynamic vegetation models that include P-cycling as a component of these process-based initiatives and the possible

feedbacks that P-cycle would have on tropical forest functioning and growth under future climate.

6.1.2. Objective 2 - Chapter 3: Root strategies used by trees to overcome phosphorus-limitation in natural low-fertility soils in central Amazon

In this chapter, I tested the hypothesis that a trade-off between root morphological traits and P-uptake strategies would occur under natural low-fertility soils in central Amazon forests, with different strategies being associated with roots of different morphologies. In the central Amazon forests studied here, however, plants invested in many P-uptake strategies at a time. Under P-limitation, plants did not show a trade-off in the investment of P-uptake strategies related to P foraging (morphological adaptations and mycorrhizal colonisation) and mining (enzyme exudation). Moreover, since the majority of P in tropical soils is in its organic form and inorganic P is quite immobile in soils, investing in strategies to both mineralise organic P and acquire inorganic P might be crucial to ensure that the plant demand for P is met. Because multiple steps are needed to make P available for plants, roots also display a range of adaptations to enhance P uptake and therefore investing in fine roots that are associated with arbuscular mycorrhizas but also exudate high amounts of phosphatase enzymes could be seen as complementary mechanisms towards maintaining forest functioning under P-limitation.

6.1.3. Objective 3 - Chapter 4: Nutrient addition effects on P-acquisition mechanisms adopted by trees in a central Amazon forest

In this chapter, I tested the hypotheses that the addition of P would result in alleviation of P limitation by reduced investment in root P-uptake strategies, whilst the addition of N and cations would exacerbate root P-limitation. Short-term nutrient addition resulted in changes in the expression of root traits in a central Amazon forest growing on low-P soils. After only six months of P addition, phosphatase activity decreased, pointing to an alleviation of P limitation by roots. I also found some evidence for increased mycorrhizal colonisation with P addition, suggesting a shift from predominantly more mining traits (enzyme

production) to foraging traits (AM association). Such a shift would be mainly due to changes in the abundance of different P forms after nutrient addition, with increased inorganic P concentrations when compared to organic P. Furthermore, the addition of cations increased root productivity by 50%, also increasing mean root diameter, pointing to either cations limiting root functioning or by playing a role in exacerbating P-limitation by roots in central Amazon. Other morphological properties were not affected by short-term nutrient addition, and the addition of nitrogen had no impact on any root traits analysed here. The results from this chapter suggest that the role of rock-derived elements other than P is usually underestimated, and that multiple nutrients are likely to play key roles affecting the expression of root traits in this central Amazon forest.

6.1.4. Objective 4 - Chapter 5: Nutrient addition effects on soil microbial community function

I also hypothesised that the addition of P would result in evidence for P-limitation by the soil microbial community by decreased investments in P-cycle enzyme production, whilst the addition of N and cations would exacerbate microbial P-limitation and therefore increase investment in P-cycle enzymes. Short-term nutrient addition in this central Amazon forest strongly influenced soil microorganisms. The addition of P generally decreased the activity of enzymes related to carbon (β-glucosidase) and nitrogen cycle (N-acetyl β-glucosaminidase) enzymes, with stronger reduction found for phosphatase activity. This response, however, was influenced by the addition of cations, where phosphatase activity only decreased with P addition when cations were not also added. The general decrease in the activity of all enzymes resulted in similar C:N:P ratios across treatments, pointing to an overall constraint on the exudation of enzymes by the microbial community. The strong interaction between P and cations affecting soil microbial enzyme production could be a result of indirect changes in soil chemical and mineral properties but also through shifts in the microbial community composition. Overall, the results from this chapter indicate an alleviation of P limitation to the soil microbial community following P addition, which is ultimately also affected by cations availability.

6.2. Phosphorus limitation in naturally low-fertility soils in Amazon forests

Determining the nutrient status of Amazon forests could add valuable knowledge to what is currently known about the drivers of spatial patterns of productivity and biomass allocation. The majority of forests across the Amazon basin grow on low fertility soils, with particularly low P and cations availability, and plants might use a wide range of above and belowground mechanisms to overcome nutrient limitation. Although much is known about the role of soil fertility affecting aboveground plant traits expression (Baker et al. 2003; Fyllas et al. 2009; Quesada et al. 2012), the belowground mechanisms by which tropical forests thrive under such adverse conditions are poorly known, but are crucial if we aim to understand forest functioning under current and future climate.

6.2.1. Root foraging and mining strategies to overcome nutrient limitation

Among the variety of strategies that plants can use to increase P-uptake efficiency (Raghothama 1999; Richardson and Simpson 2011), they could be broadly classified as ranging from root traits related to P-foraging to traits related to P-mining, depending on soil P availability (Figure 6.1). Since P is very immobile in soils, plants preferentially invest in root total length and surface area to increase P assimilation (Eissenstat 1992; Eissenstat and Yanai 1997) and/or rely on the very fine hyphal network typical of arbuscular mycorrhizas to acquire P away from depleted zones around roots (Hodge 2004; Smith and Read 2010; Eissenstat et al. 2015). Root morphological adaptations and association with arbuscular mycorrhizas towards increasing inorganic P uptake are examples of root P-foraging strategies. Phosphorus in tropical soils, however, is largely bound in organic compounds or occluded in secondary minerals (Cross and Schlesinger 1995) and roots are able to secrete compounds to degrade these compounds prior to P assimilation (Jones and Oburger 2011). The exudation of phosphatase enzymes (Olander and Vitousek 2000; Hinsinger 2001) and carboxylates (Jones and Oburger 2011; Turner et al. 2012; Lambers et al. 2015) are the main mechanisms by which roots mobilise organic and inorganic occluded forms of P

in soils. Such strategies involved in P degradation from organic or immobilised P forms to inorganic P are examples of P-mining mechanisms.

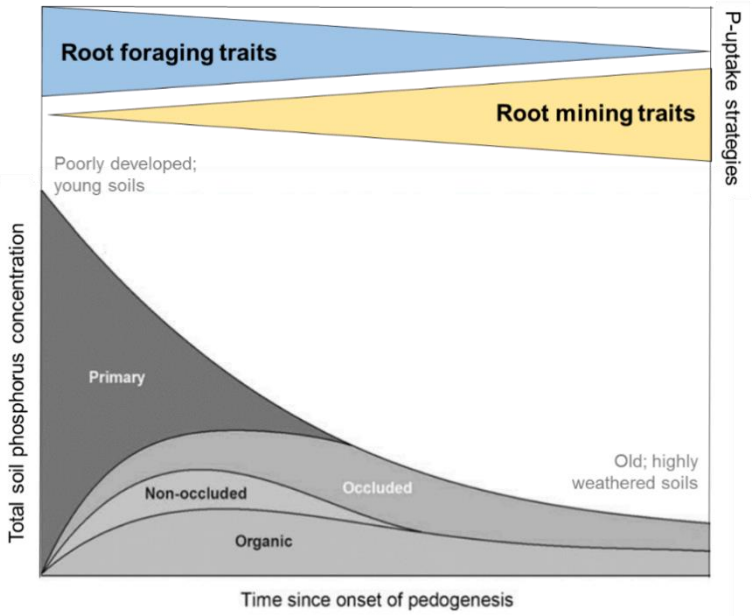

Figure 6.1. Expected root traits associated with soil P-acquisition along a gradient of P availability with soil development (Adapted from Turner and Condron 2013).

Since roots are specialised in different soil P pools (Figure 6.1), it is expected that in young, poorly developed soils with high P availability trees mostly rely in P-foraging strategies whereas in highly weathered soils with low P availability, trees invest in P-mining traits (Ryan et al. 2012; Ushio et al. 2015; Nasto et al. 2017). In addition to specific P forms explored by each root adaptation, the investments in different P-uptake strategies are resource-costly and therefore plants are not expected to invest in multiple strategies but rather display a trade-off between these adaptations. The existence of such a trade-off between the investments in different P-uptake strategies was, however, not found under natural low-P soils in central Amazon forests analysed in this study. Despite the low total soil P concentrations and the fact that the majority of soil P is bound to organic matter in my study site, plants not only invest in the exudation of phosphatase to mine for P but they also display intermediate levels of association with arbuscular mycorrhizas to forage for P. Even though the forests studied here displayed low levels of mycorrhizal colonisation when compared to other tropical

forests (Wurzburger and Wright 2015), all samples showed association with mycorrhizas, pointing to the importance of plant-fungi symbiosis in Amazon forests. Therefore, the lack of trade-offs between P-uptake strategies in the central Amazon forest studied here could reflect the multiple potential roles that mycorrhizas can play regarding uptake of other micronutrients and plant protection against soil pathogens and herbivores. Nonetheless, these results suggest that under P-limitation, plants are able to, or are forced to, invest in many P-uptake strategies at a time despite the potential resource-costs. Due to the very low P availability in these soils, investing in strategies to both mineralise organic P (mining) and acquire inorganic P (foraging) might be crucial to ensure that the plant demand for P is met.

Although soils in central Amazon are considered infertile (total P ~100 mg kg^{-1}) when compared to soils from the western portion of the basin (total P can reach 1,630 mg kg^{-1} in the Andes), they are still not as extremely P-poor as highly weathered soils from white sand forests in Guyana, for example, where total P concentration is <10 mg kg^{-1} (Quesada et al. 2010; Quesada and Lloyd 2016). In such highly weathered soils, nutrients are replenished to soils almost exclusively by organic matter cycling and decomposition. Due to the disproportionally higher concentration of organic P in relation to inorganic P forms, plants in white sand Amazon forests commonly associate with ectomycorrhizas (Roy et al. 2016) that can take up soluble P but more importantly, mine other forms of insoluble P sorbed in organic matter through hydrolysis (Lambers et al. 2008). In that sense, the hyphae network coupled with the ability of hydrolysing organic P, confer plants associated with ectomycorrhizas the ability to both forage and mine for P in these extremely P-poor white sand forests. When compared to the two extremes of soil weathering across the Amazon basin, the intermediate soil fertility of central Amazon forests could therefore explain plant investments in both arbuscular mycorrhizas and phosphatase enzymes aiming to explore different forms of P.

6.3. Belowground plant and soil microorganisms responses to nutrient addition

Determining the direct effect of nutrient limitation on both above and belowground forest functioning can only be achieved experimentally. Nutrient manipulation experiments offer a robust way to test for P-limitation, but up to now, results from tropical forests were inconclusive or pointed to multiple nutrients limiting different ecosystem processes. Although many studies suggest that P-limitation in tropical forests is a widespread phenomenon, the research presented in this thesis represents the first time that direct evidence for P limitation has been demonstrated in Amazon forests. Furthermore, contrary to the idea that because lowland Amazon forests are slow-growing and therefore would not respond to short-term changes in soil nutrient availability, plants and soil microorganisms strongly responded after only 6-7 months of P and cations addition.

6.3.1. Fine root responses to changes in soil nutrient availability

Short-term nutrient addition strongly affected the expression of root traits in a central Amazon forest growing on low-fertility soils, suggesting that these slow-growing trees can be more plastic than previously expected (Chapin III 1980). The decline in root phosphatase activity with P addition is strong evidence that plants growing on low-P availability soils in central Amazon forests are indeed P-limited. The substantial increase (although not statistically significant) in root mycorrhizal colonisation with P addition also suggests a shift from predominantly mining traits (enzyme production) to foraging traits (mycorrhizas; Figure 6.2). Since the main role of phosphatase enzymes is to mineralise organic P into inorganic bioavailable P, the addition of P in its mineral form in my fertilisation treatments could help explain the changes in plant resource allocation from investments in phosphatase exudation to increased association with AM fungi.

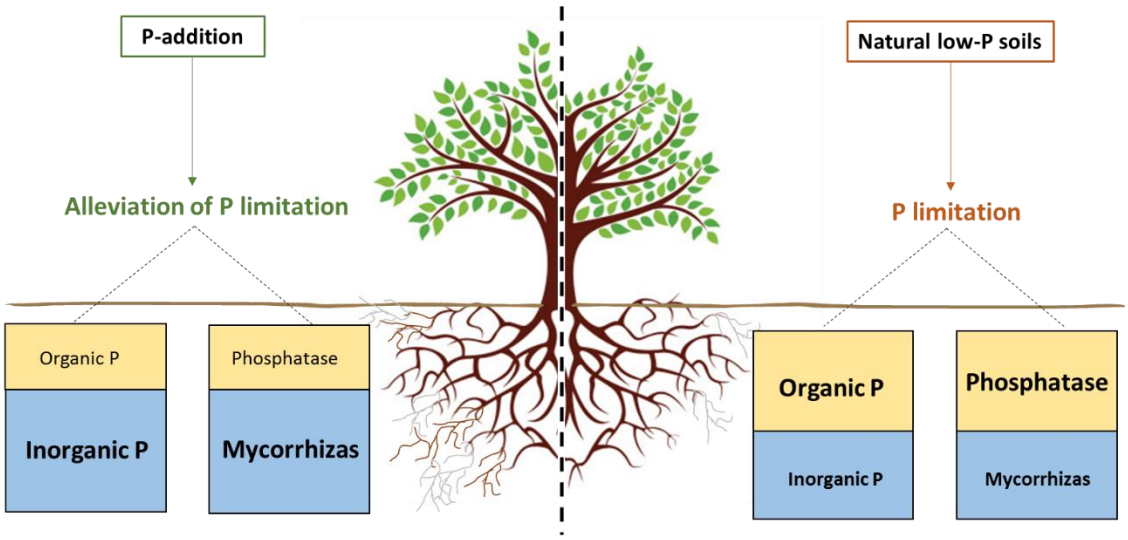

Figure 6.2. Diagram representing some of the results found in this study. With P addition and consequently alleviation of P limitation, plants invest more in strategies associated with P-foraging (e.g. arbuscular mycorrhizas). In natural low-P soils, however, plants invest in root traits associated with increasing P-mining (e.g. exudation of phosphatases) and P-foraging (e.g. arbuscular mycorrhizas).

With chronic P addition, however, the extent to which plants would still rely on AM fungi or only in morphological adaptations (e.g. increased specific root length and area) is still uncertain. Although the balance between root construction and nutrient uptake, as well as association with AM and nutrient foraging are hard to measure, AM still seems like an advantageous mechanism where fungi can directly compete with soil microorganisms. On the other hand, increased root proliferation is not only important towards increasing nutrient uptake, but also for interspecific plant competition (Hodge 2006). The results found in this study, therefore, could change with chronic nutrient addition, since in soils with high P availability, plants would not benefit as much from exudation of enzymes and carboxylates and, could predominantly depend on association with mycorrhizas (as my results indicate) or on fine roots of increasing specific root length and area to acquire P directly from soils. Up to now, in the longest-running nutrient fertilisation experiment in lowland forests in Panama, root mycorrhizal colonisation still increased significantly after 14 years of P addition when compared to control plots (Wurzburger and Wright 2015). Although data

regarding root phosphatase exudation is not available for the Panamanian experiment, the increased association with arbuscular mycorrhiza suggests that plants are still relying on this symbiotic association to meet P-demand, although changes in root morphology towards increased specific root length were also captured.

Other rock-derived elements not commonly studied in large-scale nutrient manipulation experiments appeared to also play important roles, affecting the expression of root traits. In this study, the addition of cations increased root productivity and root diameter, pointing to either cations directly limiting root functioning or by playing a role in exacerbating P-limitation by these roots. Phosphorus availability can be affected by Ca and cation exchange capacity (Malhi et al. 2009) and therefore the role of cations addition exacerbating P limitation is possible. The very low cation availability in soils in central Amazon, particularly Ca and Mg, together with root growth response captured here could also indicate that plants are, to some extent, cation-limited. If it was the latter then I would hypothesise that the initial positive root growth response to cation addition would decline over time with long-term alleviation of cation limitation, as was seen with the decrease in fine root biomass after 10 years of K addition in Panama (Wurzburger and Wright 2015). Determining this, though, would require further study in the future. The exact mechanism by which cations affected root traits is, however, still not clear, and cation-induced changes in soil pH could affect soil mineral configuration and solubility, and also the soil microbial community composition.

6.3.2. Soil microbial community responses to nutrient addition

Short-term nutrient addition in this central Amazon forest also influenced soil microorganisms. The addition of P generally decreased the activity of β-glucosidase and N-acetyl β-glucosaminidase enzymes, with even stronger reductions found for phosphatase activity. This response, however, was directly influenced by the addition of cations, where phosphatase activity only decreased when cations were also not added. The general decrease in the activity of all enzymes resulted in similar C:N:P ratios across treatments, pointing to a coordination in the exudation of microbial enzymes related to C, N and P cycling.

Overall these results suggest an alleviation of P limitation by the soil microbial community, which is ultimately also affected by cations availability. Similarly, the clearest evidence for alleviation of P limitation in forests in Panama comes from the strong reduction in soil microbial phosphatase activity after 10 years of P addition (Turner and Wright 2014). Due to the rapid life-cycle of soil microorganisms, the responses found here could be attributed to shifts in the soil microbial community composition in which microorganisms adapted to low-P soils would lose their competitive ability and be replaced by a community adapted to the new soil conditions. However, to date, there has been no investigation into the effects of the nutrient addition on microbial community composition in the AFEX plots.

6.3.3. Interactions between soil microorganisms and plants responses to changes in soil nutrient availability

Since soil microorganisms can act both as a sink or source of nutrients, the evidence of alleviation of P limitation by the microbial community in this central Amazon forest could also have affected root responses to nutrient addition. Plants and soil microorganisms compete for resources belowground, and in low-fertility soils, nutrient availability to plants is ultimately a product of the balance between microbial nutrient immobilisation and mineralisation through organic matter decomposition (Blagodatskaya et al. 2010). But since root traits in this study site also showed signs of alleviation of P limitation, it is likely that soil microbial nutrient demand was met and they therefore have not simply immobilised all the added nutrients, and are actually acting as source of nutrients to plants. Together, these short-term responses to P addition support the hypothesis that P and cations are, to a certain degree, limiting both plants and soil microorganisms functioning in this central Amazon forest. The fact that rock-derived nutrients are proving more important for both plants and microbes supports the hypotheses of this study, but the role of cations may have been underestimated. The addition of nitrogen had no impact on root traits or soil microbes, suggesting that plants and soil microorganisms are not using the added N towards increasing investment in root traits to increase P or cations acquisition,

and that N addition has not yet resulted in any exacerbation of P or cation limitation.

6.3.4. Experimental evidence for soil nutrient limitation: implications for forests growing across the Amazon basin

Experimental nutrient manipulation not only resulted in direct evidence for nutrient limiting plant and soil microorganisms, but also pointed to the fact that the central Amazon forest studied here can rapidly acclimate to changes in nutrient availability. Across the Amazon basin, the low total P concentrations are found in oldest soils located in central and eastern portions of the basin, whilst high P concentrations are found in young to intermediate age soils located in the west (Quesada and Lloyd 2016). The west-east gradient of soil fertility across the Amazon basin follows the model of soil development proposed by Walker and Syers (1976) and reflects the relationship between soil P availability decreasing along soil development in terrestrial ecosystems (Figure 6.1). Since different root nutrient acquisition strategies are usually associated with different levels of soil P availability, the potential trade-off between root strategies related to foraging and mining found in this study (Figure 6.2) could suggest that: 1) with experimental nutrient manipulation, forests growing in poor soils in central Amazon are functioning similarly to western Amazon forests growing on fertile soils and 2) by increasing nutrient availability, even plants adapted to low-P levels in central Amazon started to function analogously to plants adapted to high-P soils only after 7 months of nutrient addition. Although the results from the present study are based on measurements at the community level, specific species could be responding in different ways, but the signal captured here was still strong enough to indicate alleviation of P limitation. The way plants express root traits was recently found to be less constrained than leaf traits, indicating that a variety of combinations between root traits could be used when plants are exposed to different environmental conditions (Kramer-Walter et al. 2016).

Even though the changes in root traits with nutrient manipulation found in this study are similar to what is expected from forests growing in more fertile soils in the Amazon basin, such results still need to be interpreted with some caution, especially when it refers to aboveground plant compartments. The west-east soil

fertility gradient across Amazonia occurs in paralel with changes in soil physical properties, and together with climate variables shape the distribution of aboveground biomass, wood production and tree turnover rates across the Amazon basin (Quesada et al. 2012). Furthermore, tree species display different levels of soil P-affinity (Condit et al. 2013; Turner et al. 2018): some species occur only on low-P soils (low-P specialists), others only on high-P soils (high-P specialists) whilst some occur across a wide range of P availability (P-generalists). In central Amazon forests, trees are adapted to low-fertility soils and these species may not be able to demonstrate all the belowground traits associated with high-P specialists. The extent to which species composition will change in the plots in the long-term is unknown, but any shifts from low-P specialists to P generalist or high-P species would in turn result in changes in soil-plant feedbacks, such as plant growth rates, wood density, disturbance levels and biomass residence time. Nonetheless, my results highlight the fact that plants can acclimate to environmental changes faster than expected, but whether these belowground changes persist or change over time, and how they affect aboveground forest functioning still remains to be captured by AFEX and other nutrient manipulation experiments in the longer term.

6.3.5. Plant-soil feedbacks under future climate

Free air CO_2 enrichment experiments as well as process-based models are very useful approaches targeting the role of P-limitation affecting plant growth and consequently future carbon assimilation by tropical forests. The actual role of tropical forests as sink or source of carbon under atmospheric CO_2 enrichment will be ultimately controlled by the amount of available nutrients in soil as well as how efficiently plants can acquire and use those nutrients. Empirical data concerning belowground processes in tropical forests are, however, scarce and only recently direct evidence for P limiting tropical forests functioning was found (Turner and Wright 2014; this thesis). The evidence of P-limitation found in this central Amazon forests could imply that with no external input of nutrients, P-limitation could offset or delay the hypothesised stimulation of tropical forest growth under elevated atmospheric CO_2.

The rapid responses captured in my study pointing to changes in resource allocation both in roots and soil microorganisms after nutrient addition are also crucial findings that could contribute to a better representation of belowground traits in dynamic vegetation models. The role of root exudates and association with mycorrhizas are recently gaining more attention, and efforts to include such mechanisms in models have already started. My results highlight the importance of plant belowground carbon allocation towards different nutrient uptake strategies and how the investment in each of those strategies is dependent on soil P availability. The plant's "decision" to invest in different root strategies is closely linked to the distribution of P in pools of different availability, also suggesting that besides the inclusion of P in models, the balance between available and non-available P fractions is ultimately more important in controlling the expression of root traits than total soil P concentrations.

Studies on natural soil fertility gradients as well as nutrient manipulation experiments offer important opportunities to validate the predictive power of C-nutrient interactions in process-based vegetation models. The recent inclusion of biological and biogeochemical processes affecting P availability in a DGVM, lead to a better representation of the shifts in nutrient limitation along a soil chronosequence, from N-limited forests growing in very young soils to P limitation in old and highly weathered soils (Yang et al. 2014). Additionally, the direct link between P availability and NPP resulted in a better representation of aboveground biomass variation along the Amazon basin (Castanho et al. 2013; Yang et al. 2014). Capturing this shift in nutrient limitation and its consequences to NPP in tropical forests is an important first step paving the way to a better representation of C-P feedbacks. Moreover, although the inclusion of interactive C, N and P cycles in models is important to capture the feedbacks between the allocation of these different plant and soil resources, I did not find evidence for N addition significantly affecting root traits and soil microorganisms in the Amazon forest studied here. While N addition is expected to stimulate phosphatase exudation by plants and soil microbes, my results did not support this hypothesis and it is possible that it may be too early to draw conclusions regarding the role of N on plant and soil microbial enzyme exudation, pointing to the importance of long-term nutrient manipulation experiments. The added N could, however, indirectly impact enzyme production by altering soil pH, microbial community and

organic matter decomposition, but no evidence for such changes were captured by the short-term measurements conducted here (no changes in soil pH with N addition) or were not the focus of this study.

6.4. Perspectives and challenges

The results from this thesis provide many avenues for future research but also bring to light some of the challenges in studying plant-soil interactions in tropical forests. Despite the fact that central Amazon forests studied here have very low concentrations of rock-derived nutrients, the seemingly important role that cations played by affecting both roots and soil microorganisms in this central Amazon forest was still somewhat unexpected. The mechanisms behind the effects of cations on changes in root productivity, morphological traits and both soil and root enzymes are still not clear, mainly because the majority of the studies that focused on nutrient limitation targeted N and P, rather than different cations. Therefore, the idea of single nutrient limiting forest functioning and also of a coordination between plant responses above and belowground needs to be revisited, with numerous advantages of including other nutrients, other than N and P in future experiments. Despite the potential importance of other rock-derived nutrients controlling belowground traits in tropical forests, we are still in an embryonic stage of understanding the actual mechanisms by which Ca, Mg and K and perhaps even other macro and micro elements affect forest functioning under current conditions and future CO_2 concentrations and climate.

Time is an important factor that should be taken into account when interpreting the responses captured by this study as well as other nutrient manipulation experiments in tropical forests. Beyond short- and long-term results in relation to the start of nutrient addition, the importance of time determining the presence or absence as well as the direction (positive/negative) of responses with nutrient addition could vary between studied subjects or compartments. For instance, the soil microbial community is expected to respond faster than plants, due to their short life-span and therefore rapid turnover. Similarly, because roots directly interact with soil resources, belowground plant traits are expected to change faster than aboveground traits. Root turnover is generally faster than leaf turnover and leaf morphological traits are more phylogenetically constrained than

the multidimensional expression of root traits in different environmental conditions (Laughlin and Wilson 2014; Kramer-Walter et al. 2016). Finally, the life-span of tropical trees can vary from some decades to centuries and even though short- *versus* long-term studies are currently analysed inside the scope of grant funding and project duration, it is likely that shifts in species composition in changing environments would be only captured 1) in much longer time frames, 2) by studying seedlings, juveniles and plant recruitment and 3) by testing hypotheses through modelling. Meanwhile, some next crucial empirical and modelling steps would be needed to understand the shifts in plant species distribution (changes in the proportion of low- and high-P specialists) and more importantly, the changes in belowground nutrient uptake strategies adopted by different species under different soil conditions.

Digging even deeper, other measurements that were beyond the scope of this study are also of great importance if we are to increase our understanding of the mechanisms behind belowground carbon allocation and how such mechanisms change with soil nutrient availability. Among such measurements, I highlight the significance of traits related to root architecture (root branching and investment in different root orders), root turnover, investment in other exudates such as organic acids and finally, the actual efficiency of different arbuscular mycorrhizal fungi species in acquiring P (costs and benefits to host plants). Such belowground measurements, however, are challenging under field conditions, especially in species-rich forests such as the Amazon. Due to the complexities of studying belowground traits, most of the information we now have regarding the role of roots and soil microorganisms on nutrient cycling comes from less diverse ecosystems (e.g. temperate forests), laboratory experiments in controlled conditions or composite samples like the ones used in this study. Community and ecosystem studies are, nonetheless, a great first step towards understanding large-scale processes and could be used to point the direction in which species-specific responses could be further studied in detail. Such challenges therefore point to the need for developing new sampling and laboratory techniques to assess belowground traits and processes more accurately and efficiently, especially in tropical forests where climatic conditions impose an extra logistic difficulty.

While our empirical understanding of nutrient cycling and plant-soil feedbacks in tropical forests keeps increasing, a challenge remains for dynamic vegetation models regarding model-data integration. The majority of process-based models that include nutrient cycling in terrestrial ecosystem are restricted to C and N cycling. However, N and P terrestrial cycles are very different in regards to their sources and losses, as well as the way they interact and affect C cycling. Attempts to include P cycling in models therefore, should start by the inclusion of new above and belowground compartments along with new parameters representing different P pools in soils and plants. The inclusion of soil P pools that reflect plant and soil microbial demand, rather than total P is of great importance. Because the addition of mineral P affected the expression of root traits and enzyme exudation both from roots and soil microorganisms in this study, the distribution of P in pools of different bioavailability, especially organic and inorganic P, would directly affect belowground resources allocation under current and future climate. Moreover, incorporating soil microbial biomass as a sink or source of nutrients would directly affect the extent of plant-microorganisms competition, a crucial mechanism that could exacerbate or offset nutrient limitation in tropical forests. Since P mineralisation is one of the most important processes by which organic P is broke into inorganic available P, I suggest that the exudation of enzymes related to the decomposition of C, N and P in organic matter, is one important issue that should be considered when including P cycling in vegetation models.

An important conclusion derived from this study that could be applied in the sphere of modelling studies is the fact that plants and soil microorganisms are plastic and therefore sensitive to changes in soil nutrient availability, even in the short-term. The plasticity of C, N and P investments in root morphological traits, enzymes exudation and associations with mycorrhizas seems to be important features to be included in vegetation models, which could directly affect forests C assimilation and growth under elevated atmospheric CO_2 concentrations. Besides including C:N:P ratios from leaves, litter, roots, bulk soil and microbial biomass, it would be of great value to prescribe such parameters by including thresholds in which some processes could be triggered. For instance, parameterising the minimum soil C:N:P ratios that can sustain wood productivity and below which nutrient limitation is exacerbated would directly affect forest C

assimilation and forest growth. Moreover, linking soil nutrient limitation to aboveground C:N:P thresholds that could trigger nutrient resorption from senescing leaves could both change rates of litter decomposition and therefore nutrient cycling but also offset or delay the hypothesised decrease in C assimilation due to soil nutrient limitation.

6.5. Conclusions

This thesis shows that forests growing in Amazon soils of natural low fertility display multiple root strategies to acquire phosphorus. The substantial investment in multiple root P-uptake strategies further emphasises the importance of P availability affecting central Amazon forest functioning. This is the first study to directly find evidence of P limitation in central Amazon forests through experimental nutrient manipulation, also pointing to cation availability as playing an important role in plants and microorganisms functioning. The addition of phosphorus alleviated roots and soil microorganisms P-limitation by reducing their investments in phosphatase exudation. Higher phosphorus availability also seemed to induce a shift in the expression of root traits in this forest, from root traits associated with organic P-mining to root traits more related to foraging for inorganic P. These findings improve our understanding of how the majority of Amazon forests growing on low-fertility soils function and how adaptable belowground compartments could be in changing scenarios of nutrient availability. Such results are particularly important in the context of incorporating belowground traits associated with nutrient cycling into dynamic vegetation models and would directly affect the predicted stimulation of Amazon forests productivity under future climate.

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8. Appendices

Appendix 1. Chapter 3 Supplementary material

Soil sampling and P fractionation

All 32 plots were sampled in June, 2016 as part of AFEX intensive field campaign in the wet-dry seasons transition. Three soil cores (5 cm diameter) were sampled inside the central 20 m x 20 m of each plot, two of them along the following depths: 0-5, 5-10, 10-20 and 20-30 cm and another one at 0-5, 5-10, 10-20, 20-30, 30-50 and 50-100 cm. Samples from each plot were composited in the field camp by depth (total = 6 samples per plot), where roots were removed and soil sieved in a 2 mm mesh sieve. Soil samples were subsequently dried, ground and stored for laboratorial analyses. Phosphorus concentrations were determined in the 0-10 cm soil depth. Hedley sequential fractionation were conducted (Hedley et al. 1982; Quesada et al. 2010), partitioning soil P in eight different fractions, according to their availability to plants and microorganisms (resin P, bicarbonate inorganic, bicarbonate organic, sodium hydroxide inorganic, sodium hydroxide organic, hydrochloric acid, residual and total phosphorus; the residual fraction being determined here as the difference between the total pool and the sum of all other labile fractions). The Hedley method consists in a sequential extraction with reagents of increasing strength, starting with resin extractable P, followed by 0.5M $NaHCO_3$, 0.1M NaOH and 1M HCl. Total phosphorus was determined in a replicate soil sample by acid digestion using concentrated sulphuric acid followed by H_2O_2 as described in Tiessen and Moir (1993).

Root morphology data from fragments analysed for AM colonisation

Root morphology from the specific fragments subsampled for determining AM colonisation was obtained by scanning the roots from the total sample (from each ingrowth core) twice: first, an image was obtained with all the roots from the sample, subsequently I picked a few root tips (1^{st} to 3^{rd} order roots) to analyse for mycorrhizal colonisation and scanned the sample again, generating then a second image where

root fragments used for the AM analysis were missing. After analysing both images using WinRHIZO, by difference I obtained the actual length, area and volume of those root fragments. Based on the diameter and length of the total sample and the image scanned without some root fragments I estimated the diameter of the roots used for the mycorrhizal analyses, following the formula:

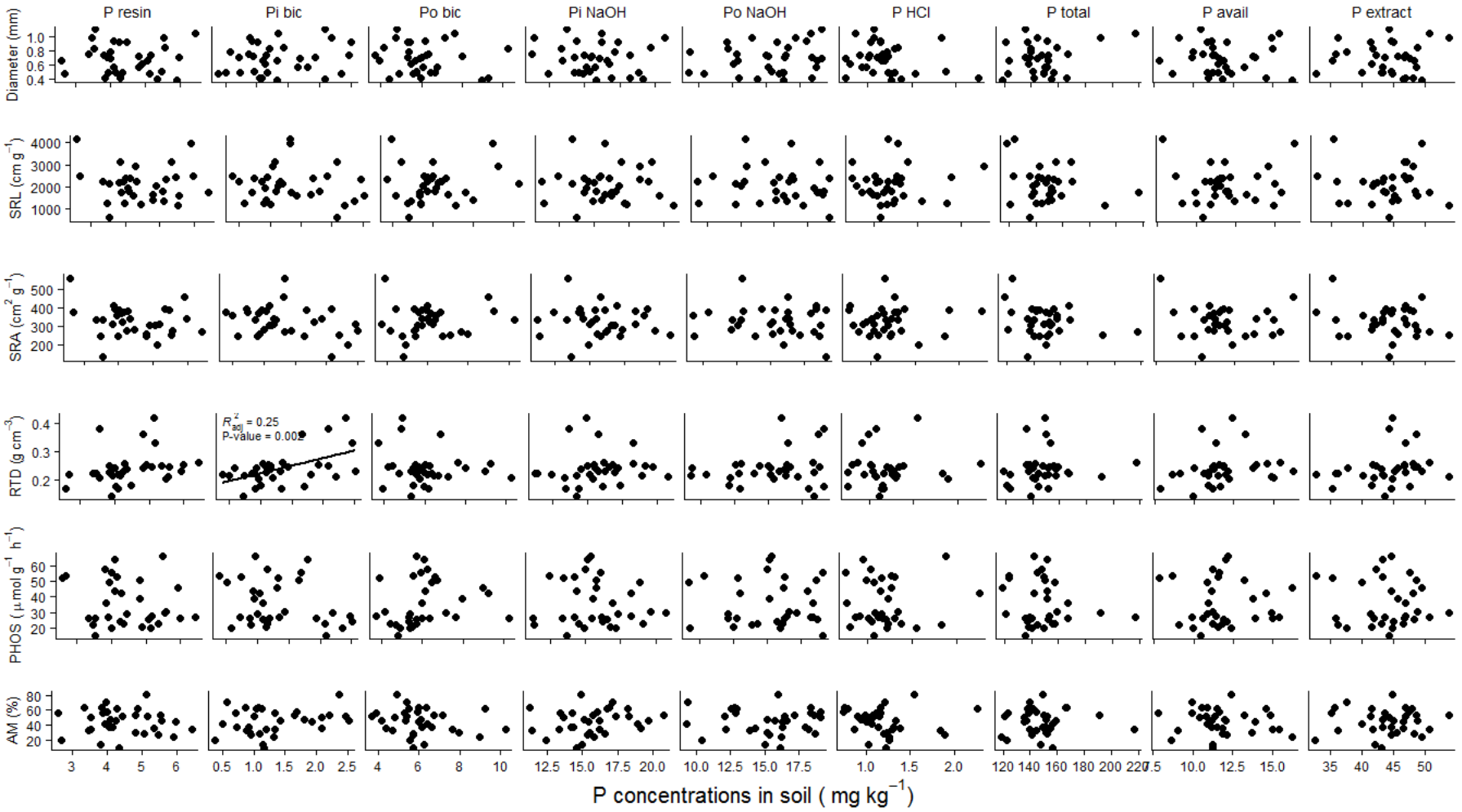
$$diam\ AM\ =\ diam1 - diam2 * \left(\frac{(length2/length1)}{(length\ AM/\ length1)}\right)$$

where diam AM = estimated diameter of root fragments used for AM analysis; diam1 = root diameter obtained from the image of the whole IGC sample; diam2 = root diameter from the image where fragments used for the AM analysis were missing; length2 = root length from the image where fragments used for the AM analysis were missing; length1 = root length obtained from the image of the whole IGC sample; length AM = difference between length 1 and length 2.

In order to calculate SRL, SRA and RTD with these subsamples, I also estimated the dry weight of the root fragments used for AM analysis based on the following formula:

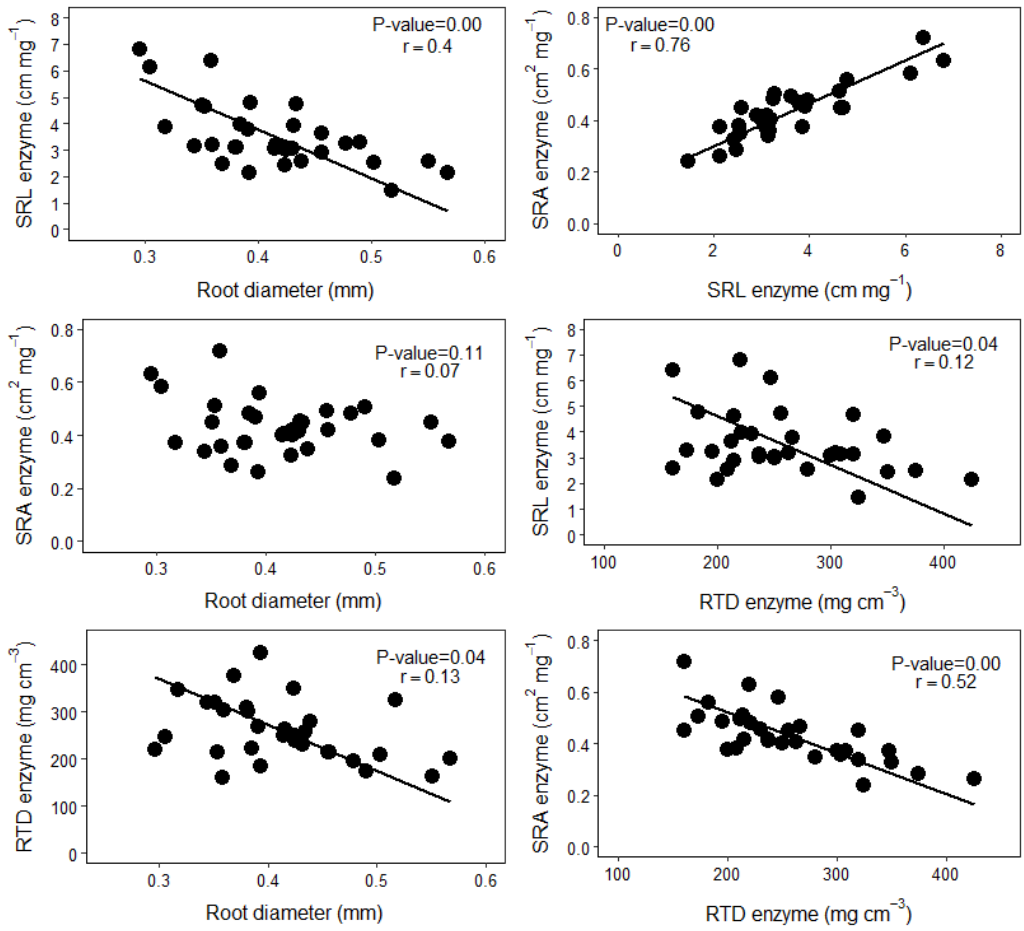
$$weight\ AM = \left(\frac{length1\ \times weight2}{length2}\right) - weight2$$

where weight AM = estimated dry weight of the root fragments used for the AM analysis; length 1 = root length obtained from the image of the whole IGC sample; length2 = root length from the image where fragments used for the AM analysis were missing; weight2 = dry weight of roots remaining after fragments were used for AM analysis.

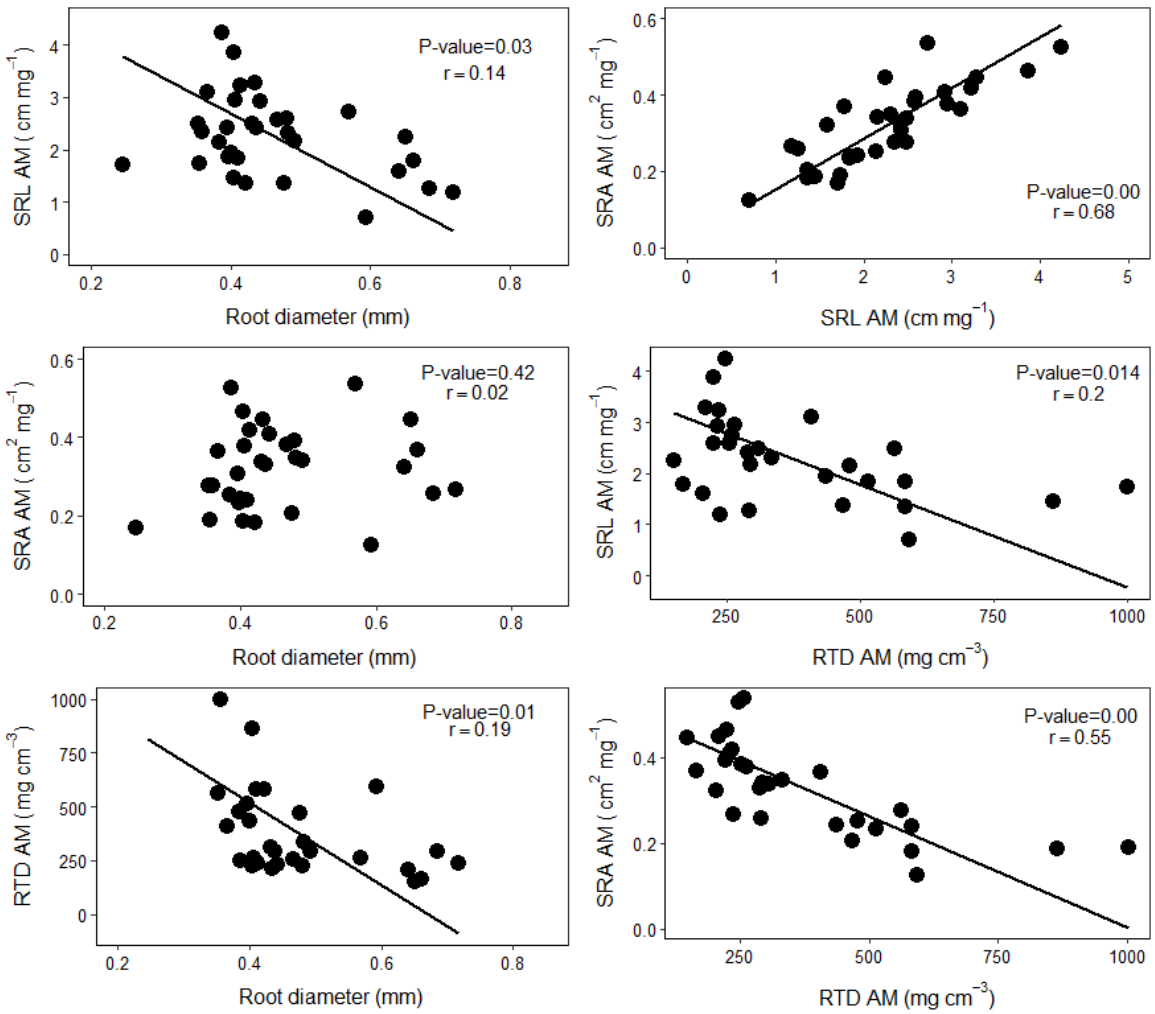


Supplementary Figure 3.1. Linear regression analyses between soil P fractions (x axis) and root traits (y axis) from the whole ingrowth core sample. SRL = specific root length (cm g^{-1}); SRA = specific root area (cm^2 g^{-1}); RTD = root tissue density (g cm^{-3});

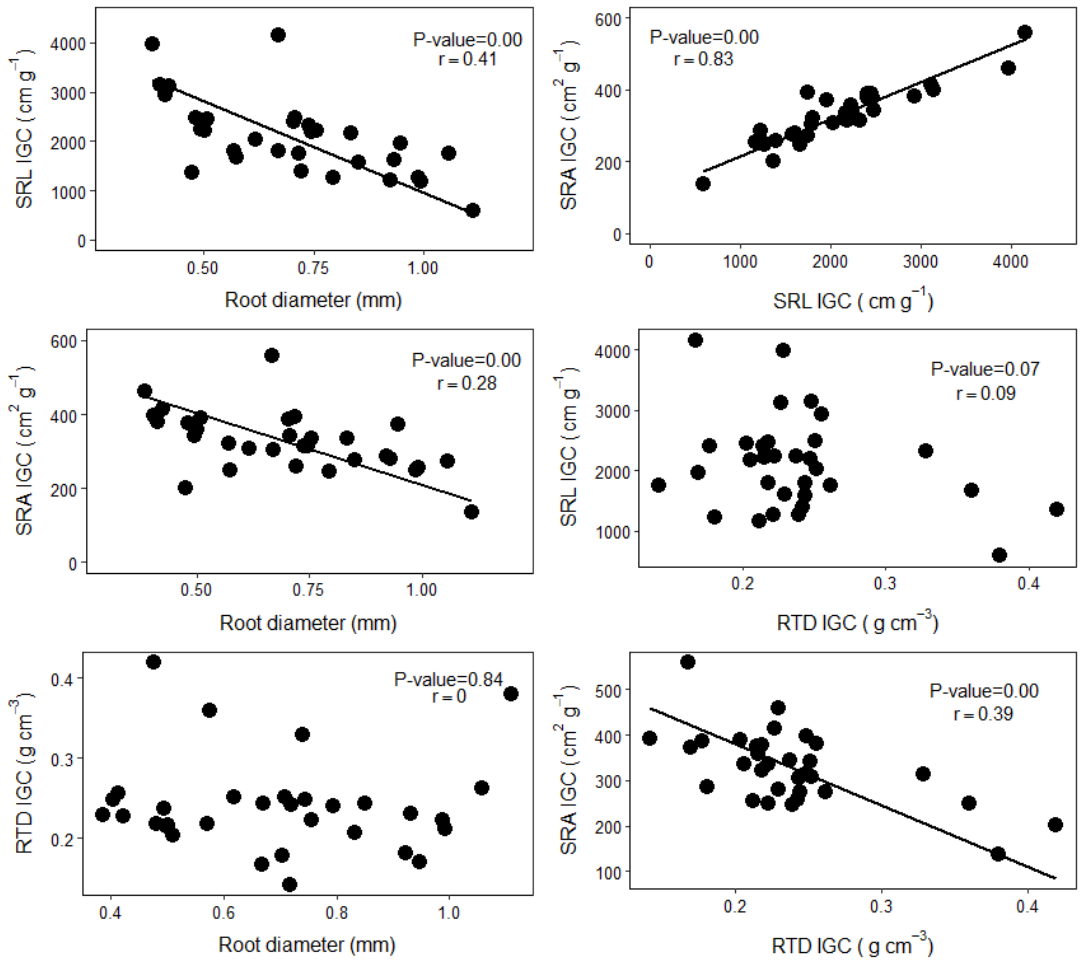
PHOS = root acid phosphatase activity (µmol MUF g^{-1} dry mass h^{-1}); AM = arbuscular mycorrhizal colonisation (%). P fractions are shown in decreasing order of availability, with P resin being the most available fraction and P HCl being the least. P total= sum of all the fractions; P avail= sum of P resin, Pi and Po bicarbonate; P extract= sum of all fractions except P residual. When the relationships are significant ($P<0.05$) P-value, adjusted R^2 and a trend line are shown.



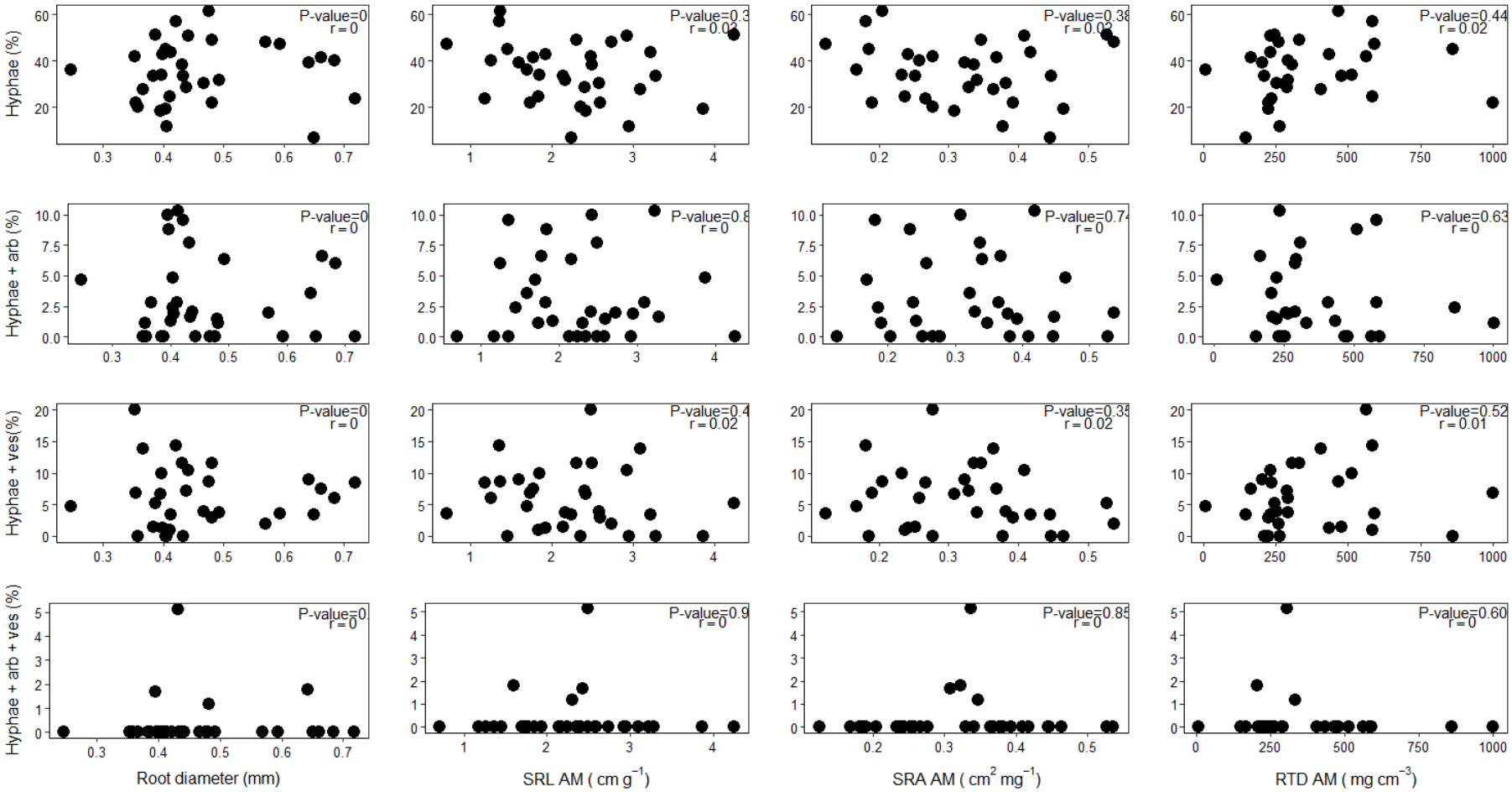
Supplementary Figure 3.2. Standardised major axis (SMA) relationship between root morphological traits from root fragments used for phosphatase activity (PHOS) assay.



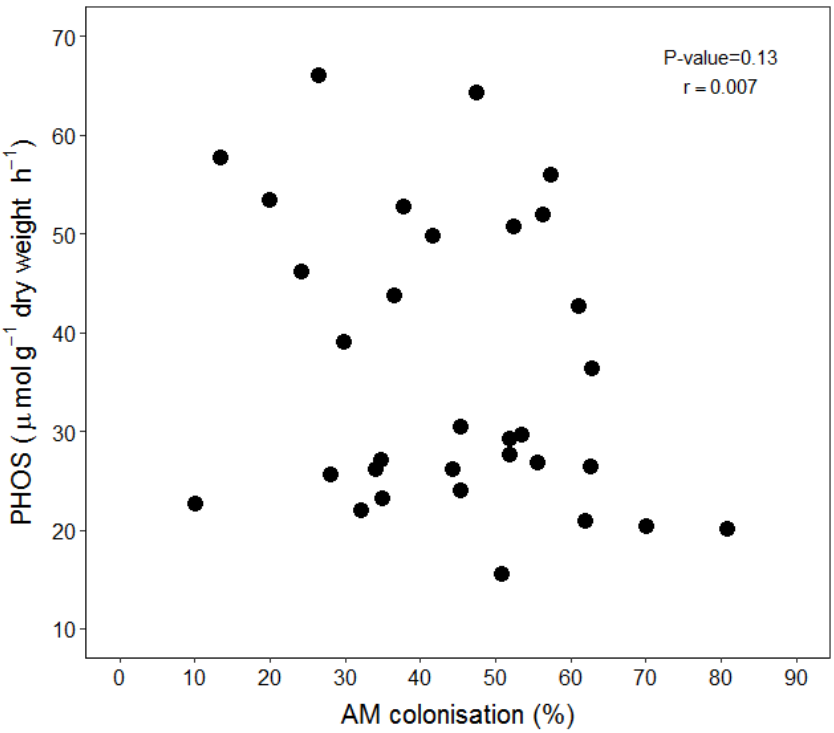
Supplementary Figure 3.3. Standardised major axis (SMA) relationship between root morphological traits from root fragments used for arbuscular mycorrhizal colonisation.



Supplementary Figure 3.4. Standardised major axis (SMA) relationship between root morphological traits from subsamples from the ingrowth cores (IGC).



Supplementary Figure 3.5. Standardised major axis (SMA) relationship between root morphological traits from AM subsamples and AM colonisation expressed as percentage of root colonised by different fungi structures. Root diameter = mean root diameter in mm; SRL = specific root length; SRA = specific root area; RTD = root tissue density. arb = arbuscule; ves = vesicle.



Supplementary Figure 3.6. Standardised major axis (SMA) relationship between root phosphatase activity (PHOS) and mycorrhizal colonisation. Note that PHOS assay and AM colonisation analyses were not carried out on the same subsamples.

Supplementary Table 3.1. Total P and P fractions from 32 plots in central Amazonia. Values represent mean P concentration for 0-10 cm depth.

Plot ID	Resin P (mg/kg)	Bic Pi (mg/kg)	Bic Po (mg/kg)	NaOH Pi (mg/kg)	NaOH Po (mg/kg)	HCl P (mg/kg)	Residual P (mg/kg)	Total P (mg/kg)	Readily available P (mg/kg)	Total extractable P (mg/kg)
P01	3.55	2.09	4.81	13.71	19.08	1.07	91.12	135.43	10.45	44.31
P02	2.59	1.33	3.91	13.43	12.88	1.16	88.27	123.58	7.84	35.30
P03	4.24	2.51	5.34	16.24	15.74	1.04	92.03	137.15	12.10	45.12
P04	6.44	1.34	7.63	16.20	18.25	0.86	166.67	217.40	15.41	50.73
P05	4.34	1.16	5.73	14.57	16.15	1.23	113.74	156.92	11.23	43.18
P06	5.97	1.93	6.00	15.47	16.33	1.01	88.75	135.45	13.90	46.70
P07	5.57	1.43	4.17	19.67	16.61	1.34	110.39	159.19	11.18	48.80
P08	5.55	2.20	7.14	20.73	17.17	1.08	137.53	191.40	14.90	53.87
P09	5.34	2.08	4.41	19.15	14.31	1.38	108.56	155.23	11.83	46.67
P10	5.18	2.47	3.73	18.26	16.44	0.91	105.98	152.97	11.37	46.99
P11	4.90	1.13	5.51	16.78	12.54	0.80	96.04	137.71	11.54	41.67
P12	5.13	2.35	4.91	14.93	15.96	1.55	104.19	149.02	12.39	44.83
P13	3.54	1.19	10.26	13.39	12.15	1.30	98.12	139.97	15.00	41.85
P14	3.48	0.89	4.73	11.41	13.90	1.83	108.25	144.50	9.10	36.24
P15	3.99	0.58	5.41	17.15	9.33	1.22	102.30	139.97	9.98	37.67
P16	4.81	0.94	7.98	15.55	15.15	1.23	106.19	151.84	13.72	45.65
P17	4.29	1.04	9.25	18.25	12.98	2.26	94.15	142.23	14.58	48.08
P18	3.36	0.87	6.35	11.27	12.80	1.17	131.29	167.10	10.58	35.81
P19	5.93	1.32	8.98	15.80	16.25	1.27	68.95	118.50	16.22	49.55

P20	5.48	0.96	5.73	15.37	15.34	1.89	96.89	141.67	12.17	44.77
P21	4.46	1.00	5.41	17.34	12.10	1.15	79.29	120.76	10.87	41.47
P22	2.68	0.39	5.58	12.42	10.39	1.26	90.29	123.02	8.65	32.73
P23	3.94	0.50	6.44	18.88	9.27	1.07	116.82	156.93	10.89	40.11
P24	4.11	1.80	6.10	15.15	15.17	0.94	108.57	151.84	12.01	43.27
P25	3.85	1.08	5.97	16.92	18.38	0.76	119.57	166.53	10.91	46.96
P26	3.89	0.71	5.32	14.26	18.38	1.12	95.16	138.84	9.92	43.68
P27	4.81	1.66	6.75	15.77	18.66	0.99	101.52	150.14	13.21	48.63
P28	4.01	1.69	5.94	16.08	19.09	0.75	96.92	144.49	11.65	47.57
P29	5.08	1.09	5.71	16.57	18.73	1.20	104.03	152.40	11.88	48.38
P30	4.15	0.74	6.64	14.25	16.29	1.31	105.63	149.01	11.53	43.38
P31	3.83	1.15	6.25	14.98	15.00	1.03	105.65	147.88	11.23	42.23
P32	4.08	0.92	6.16	14.40	17.96	1.14	107.16	151.84	11.16	44.67

Supplementary Table 3.2. Root productivity, morphology properties, phosphatase activity and mycorrhizal colonisation in 32 plots in central Amazonia. Diam = mean root diameter in mm; SRL = specific root length; SRA = specific root area; RTD = root tissue density. Root morphological data presented here is from the total ingrowth core root samples, while PHOS and AM colonisation were determined in subsamples from these roots. Phosphatase is shown in 3 different units: mg of dry root, cm of root length and cm^2 of root area. AM colonisation is shown as total (sum of colonisation by all structures) and by fungi structure: hyphae; arbusc = arbuscule; ves = vesicule. Root product = Root productivity in mg of roots produced by day. Note that nmol $mg^{-1}\,h^{-1}$ and µmol $g^{-1}\,h^{-1}$ are equivalent units.

Plot ID	Diam (mm)	SRL (cm mg^{-1})	SRA (cm^2 mg^{-1})	RTD (mg cm^{-3})	PHOS (nmol $mg^{-1}\,h^{-1}$)	PHOS (nmol $cm^{-1}\,h^{-1}$)	PHOS (nmol $cm^{-2}\,h^{-1}$)	Total AM (%)	Hyphae (%)	Hyphae + Arbusc (%)	Hyphae + Ves (%)	Hyphae + Arb + Ves (%)	Root product (mg day^{-1})
P01	1.11	0.59	0.14	380.08	15.46	5.02	38.43	50.88	47.37	0.00	3.51	0.00	8.75
P02	0.67	4.15	0.56	167.32	51.91	8.02	70.81	56.41	51.28	0.00	5.13	0.00	5.09
P03	0.93	1.61	0.28	229.67	23.98	9.46	68.67	45.45	42.86	1.30	1.30	0.00	8.01
P04	1.06	1.75	0.27	261.65	27.07	7.17	72.51	34.85	33.33	0.00	1.52	0.00	8.14
P05	0.49	2.24	0.34	237.28	22.65	7.48	55.55	10.17	6.78	0.00	3.39	0.00	5.97
P06	0.71	2.48	0.34	251.09	26.13	5.65	50.94	44.44	27.78	2.78	13.89	0.00	6.83
P07	0.85	1.58	0.28	243.87	30.36	10.06	75.01	45.35	36.05	4.65	4.65	0.00	6.88
P08	0.99	1.17	0.25	211.47	29.67	11.35	64.07	53.57	39.29	3.57	8.93	1.79	5.04
P09	0.40	3.14	0.40	248.64	23.15	7.36	68.53	35.00	33.33	1.67	0.00	0.00	6.19
P10	0.74	2.32	0.31	328.39	27.57	8.79	77.70	52.00	48.00	2.00	2.00	0.00	6.53
P11	0.62	2.02	0.31	251.67	20.91	6.64	56.72	62.00	42.00	0.00	20.00	0.00	5.90
P12	0.48	1.36	0.20	419.22	20.13	9.38	76.24	80.95	57.14	9.52	14.29	0.00	2.78
P13	0.83	2.16	0.34	205.81	26.11	8.56	72.22	34.21	30.26	0.00	3.95	0.00	10.90
P14	0.99	1.26	0.25	221.95	21.94	5.61	46.62	32.20	23.73	0.00	8.47	0.00	6.51

P15	0.80	1.26	0.25	239.20	20.38	6.89	48.00	70.19	61.54	0.00	8.65	0.00	12.52
P16	0.72	1.39	0.26	242.24	39.05	11.92	75.79	29.89	21.84	1.15	6.90	0.00	10.87
P17	0.41	2.93	0.38	255.41	42.59	9.91	77.63	61.19	50.75	0.00	10.45	0.00	6.22
P18	0.76	2.22	0.34	222.29	26.43	11.20	95.60	62.79	48.84	1.16	11.63	1.16	9.67
P19	0.39	3.97	0.46	228.96	46.15	9.18	88.45	24.19	19.35	4.84	0.00	0.00	7.91
P20	0.51	2.45	0.39	203.20	65.98	23.87	158.00	26.47	22.06	1.47	2.94	0.00	10.04
P21	0.92	1.22	0.29	180.80	29.14	10.53	70.88	52.00	40.00	6.00	6.00	0.00	4.37
P22	0.48	2.46	0.38	218.17	53.32	7.95	84.32	20.00	20.00	0.00	0.00	0.00	12.09
P23	0.50	2.22	0.36	215.52	49.70	15.79	117.70	41.77	31.65	6.33	3.80	0.00	7.10
P24	0.57	1.79	0.32	218.21	64.25	19.29	125.75	47.62	45.24	2.38	0.00	0.00	11.79
P25	0.42	3.11	0.41	227.12	36.37	11.64	87.79	62.82	38.46	7.69	11.54	5.13	8.05
P26	0.72	1.74	0.39	141.78	26.80	12.90	72.17	55.66	41.51	6.60	7.55	0.00	16.01
P27	0.58	1.66	0.25	359.79	50.70	13.10	106.52	52.50	33.75	8.75	10.00	0.00	3.31
P28	0.70	2.41	0.39	177.42	55.93	9.47	96.75	57.47	43.68	10.34	3.45	0.00	9.34
P29	0.67	1.79	0.30	243.53	25.54	17.43	107.20	28.04	24.30	2.80	0.93	0.00	7.24
P30	0.50	2.40	0.38	214.82	52.74	22.68	171.20	37.76	28.57	2.04	7.14	0.00	6.19
P31	0.75	2.19	0.31	247.44	57.71	22.15	139.61	13.46	11.54	1.92	0.00	0.00	7.18
P32	0.95	1.96	0.37	169.16	43.67	13.51	91.22	36.67	18.33	10.00	6.67	1.67	7.79

Appendix 2. Chapter 4 Supplementary material

Supplementary Table 4.1. Mean fine root productivity in Kg ha^{-1} $month^{-1}$, m m^{-2} $month^{-1}$ and m^{-2} m^{-2} $month^{-1}$ ± standard errors in eight treatments and two soil depths (0-5 cm and 5-10 cm). *n*= 4 per treatment per depth.

		Fine root productivity		
Treatment	**Depth**	**Kg ha^{-1} $month^{-1}$**	**m m^{-2} $month^{-1}$**	**m^{-2} m^{-2} $month^{-1}$**
CONTROL	0-10 cm	124.46 ± 23.40*	242.90 ± 52.66	0.492 ± 0.105
	10-30 cm	100.22 ± 27.47	208.80 ± 83.11	0.413 ± 0.162
N	0-10 cm	95.69 ± 15.66	170.82 ± 50.71	0.364 ± 0.102
	10-30 cm	85.96 ± 11.40	140.01 ± 38.52	0.318 ± 0.062
P	0-10 cm	138.04 ± 10.85	247.70 ± 16.34	0.531 ± 0.052
	10-30 cm	108.83 ± 27.03	197.54 ± 63.22	0.413 ± 0.107
CATIONS	0-10 cm	210.47 ± 63.55	328.69 ± 109.12	0.724 ± 0.234
	10-30 cm	119.32 ± 24.15	176.61 ± 37.85	0.401 ± 0.075
N+P	0-10 cm	171.47 ± 27.64*	311.38 ± 59.37*	0.653 ± 0.121
	10-30 cm	103.32 ± 17.79	220.27 ± 49.11	0.450 ± 0.089
N+CATIONS	0-10 cm	189.44 ± 38.38	317.62 ± 51.76	0.670 ± 0.099
	10-30 cm	126.35 ± 28.09	242.07 ± 83.25	0.482 ± 0.137
P+CATIONS	0-10 cm	263.96 ± 33.51	388.71 ± 43.36	0.914 ± 0.080
	10-30 cm	155.77 ± 51.77	287.09 ± 108.03	0.613 ± 0.217
N+P+CATIONS	0-10 cm	216.82 ± 54.81**	467.62 ± 128.28	0.994 ± 0.270
	10-30 cm	134.14 ± 51.84	186.69 ± 75.22	0.432 ± 0.184

Differences between soil depths in each treatment were tested and significant effects from t-tests are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

Supplementary Table 4.2. Mean fine root productivity in Kg ha^{-1} $month^{-1}$, m m^{-2} $month^{-1}$ and m^{-2} m^{-2} $month^{-1}$ ± standard errors with and without the addition of N, P and cations in two soil depths (0-10 cm and 10-30 cm). *n*= 16 per treatment per depth.

		Fine root productivity		
Nutrient	**Depth**	**Kg ha^{-1} $month^{-1}$**	**m m^{-2} $month^{-1}$**	**m^{-2} m^{-2} $month^{-1}$**
-N	0-10 cm	184.23 ± 22.45**	302.00 ± 32.95*	0.665 ± 0.075**
	10-30 cm	121.03 ± 16.35	217.51 ± 36.29	0.460 ± 0.071
+N	0-10 cm	168.34 ± 20.22***	316.86 ± 44.68*	0.670 ± 0.093**

	10-30 cm	112.44 ± 14.84	197.26 ± 30.38	0.420 ± 0.059
-P	0-10 cm	155.02 ± 21.46**	265.01 ± 35.58	0.562 ± 0.075*
	10-30 cm	107.96 ± 11.39	191.87 ± 30.55	0.403 ± 0.054
+P	0-10 cm	197.57 ± 20.01***	353.85 ± 39.50**	0.773 ± 0.085**
	10-30 cm	125.51 ± 18.71	222.90 ± 35.89	0.477 ± 0.074
-Cations	0-10 cm	132.42 ± 11.54**	243.20 ± 24.94*	052510 ± 0.14*
	10-30 cm	99.58 ± 10.07	191.65 ± 28.35	0.398 ± 0.051
+Cations	0-10 cm	220.17 ± 23.05***	375.66 ± 43.39**	0.825 ± 0.091**
	10-30 cm	133.89 ± 18.69	223.11 ± 37.64	0.482 ± 0.076

Differences between soil depths in each treatment were tested and significant effects from t-tests are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

Supplementary Table 4.3. Mean root diameter (mm), SRL (cm g^{-1}), SRA (cm^2 g^{-1}) and RTD (g cm^{-3}) ± standard errors with and without the addition of N, P and cations in two soil depths (0-10 cm and 10-30 cm). *n*= 16 per treatment per depth. No differences between soil layers in each of the nutrient addition treatments were detected using paired t-tests.

Nutrient	Depth	Diameter	SRL	SRA	RTD
-N	0-10 cm	1.05 ± 0.03	1,149.41 ± 56.34	289.95 ± 11.14	0.159 ± 0.005
	10-30 cm	1.06 ± 0.03	1,256.62 ± 68.91	308.22 ± 10.01	0.149 ± 0.004
+N	0-10 cm	1.09 ± 0.03	1,309.20 ± 102.58	319.72 ± 18.68	0.146 ± 0.004
	10-30 cm	1.11 ± 0.03	1,223.96 ± 81.76	307.72 ± 16.19	0.149 ± 0.007
-P	0-10 cm	1.02 ± 0.04	1,252.96 ± 94.23	303.77 ± 15.89	0.157 ± 0.005
	10-30 cm	1.07 ± 0.03	1,221.97 ± 61.09	304.96 ± 10.66	0.148 ± 0.005
+P	0-10 cm	1.11 ± 0.02	1,205.66 ± 74.79	305.91 ± 15.81	0.148 ± 0.005
	10-30 cm	1.09 ± 0.03	1,258.61 ± 87.70	310.48 ± 15.74	0.150 ± 0.006
-Cations	0-10 cm	1.03 ± 0.03	1,256.01 ± 83.27	308.02 ± 14.63	0.152 ± 0.005
	10-30 cm	1.04 ± 0.03	1,330.07 ± 67.93	322.42 ± 13.20	0.146 ± 0.005
+Cations	0-10 cm	1.11 ± 0.03	1,202.60 ± 86.71	301.66 ± 16.95	0.153 ± 0.006
	10-30 cm	1.13 ± 0.03	1,150.51 ± 76.02	293.02 ± 12.61	0.152 ± 0.006

Supplementary Table 4.4. Mean root phosphatase activity in nmol mg^{-1} $hour^{-1}$, nmol cm^{-1} $hour^{-1}$ and nmol cm^{-2} $hour^{-1}$ ± standard errors in eight treatments and two soil depths (0-10 cm and 10-30 cm). *n*= 4 per treatment per depth. No differences between treatments and between soil layers were detected using paired t-tests. (Note that nmol mg^{-1} $hour^{-1}$ = µmol g^{-1} $hour^{-1}$).

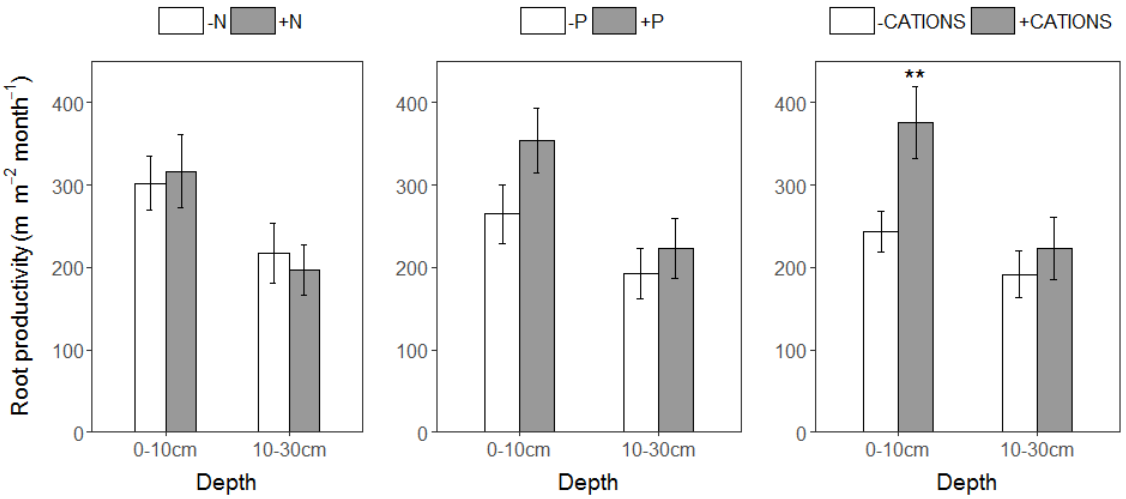
		Root phosphatase activity		
Treatment	**Depth**	**nmol mg^{-1} $hour^{-1}$**	**nmol cm^{-1} $hour^{-1}$**	**nmol cm^{-2} $hour^{-1}$**
CONTROL	0-10 cm	42.50 ± 8.09	13.91 ± 4.16	95.52 ± 26.42
	10-30 cm	39.11 ± 5.93	8.70 ± 1.00	73.36 ± 8.62
N	0-10 cm	42.83 ± 2.03	12.47 ± 2.80	94.64 ± 11.73
	10-30 cm	32.15 ± 4.47	11.20 ± 1.74	86.29 ± 15.81
P	0-10 cm	35.94 ± 5.71	9.99 ± 1.80	78.75 ± 10.39
	10-30 cm	39.64 ± 6.52	12.06 ± 2.59	85.96 ± 17.61
CATIONS	0-10 cm	45.23 ± 7.19	16.03 ± 4.02	11.57 ± 16.39
	10-30 cm	36.38 ± 5.06	13.40 ± 2.61	90.99 ± 11.15
N+P	0-10 cm	34.95 ± 5.23	9.71 ± 1.69	72.80 ± 10.68
	10-30 cm	35.35 ± 11.33	10.98 ± 3.22	84.70 ± 22.42
N+CATIONS	0-10 cm	36.79 ± 3.50	11.02 ± 1.05	80.41 ± 8.78
	10-30 cm	36.23 ± 10.49	13.67 ± 2.29	86.94 ± 8.23
P+CATIONS	0-10 cm	30.74 ± 8.74	11.54 ± 1.50	81.66 ± 14.40
	10-30 cm	33.17 ± 6.64	11.00 ± 3.24	74.17 ± 14.19
N+P+CATIONS	0-10 cm	26.23 ± 4.29	8.54 ± 1.44	57.40 ± 8.33
	10-30 cm	29.60 ± 3.82	9.05 ± 1.98	61.95 ± 10.29

Supplementary Table 4.5. Mean root phosphatase activity in nmol mg^{-1} $hour^{-1}$, nmol cm^{-1} $hour^{-1}$ and nmol cm^{-2} $hour^{-1}$ ± standard errors with and without the addition of N, P and cations in two soil depths (0-10 cm and 10-30 cm). *n*= 16 per treatment per depth. No differences between soil layers in each of the nutrient addition treatments were detected using paired t-tests. (Note that nmol mg^{-1} $hour^{-1}$ = µmol g^{-1} $hour^{-1}$).

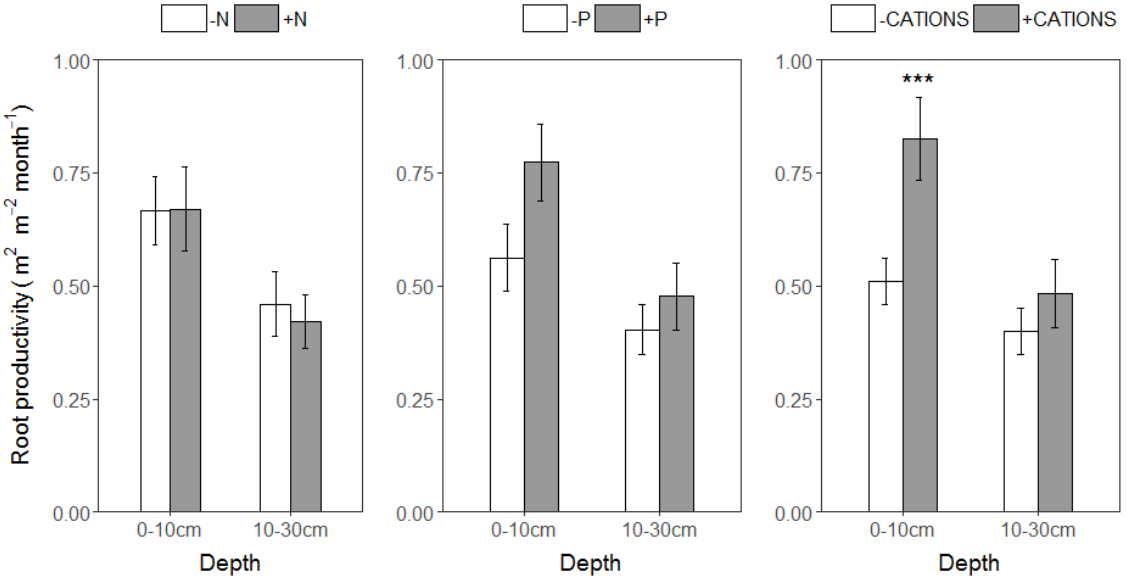
		Root phosphatase activity		
Nutrient	**Depth**	**nmol mg^{-1} $hour^{-1}$**	**nmol cm^{-1} $hour^{-1}$**	**nmol cm^{-2} $hour^{-1}$**
-N	0-10 cm	38.60 ± 3.67	12.87 ± 1.52	91.87 ± 8.68
	10-30 cm	37.08 ± 2.79	11.29 ± 1.20	81.12 ± 6.27
+N	0-10 cm	35.20 ± 2.34	10.44 ± 0.91	76.32 ± 5.65
	10-30 cm	33.33 ± 3.76	11.23 ± 1.14	79.97 ± 7.32
-P	0-10 cm	41.84 ± 2.70	13.36 ± 1.53	95.54 ± 8.20
	10-30 cm	35.97 ± 3.15	11.74 ± 1.03	84.40 ± 5.36
+P	0-10 cm	31.97 ± 2.95	9.95 ± 0.77	72.65 ± 5.55
	10-30 cm	34.44 ± 3.52	10.77 ± 1.29	76.69 ± 7.89
-Cations	0-10 cm	39.06 ± 2.71	11.52 ± 1.33	85.43 ± 7.71
	10-30 cm	36.56 ± 3.45	10.74 ± 1.08	82.58 ± 7.67
+Cations	0-10 cm	34.75 ± 3.36	11.78 ± 1.25	82.76 ± 7.47
	10-30 cm	33.85 ± 3.20	11.78 ± 1.25	78.51 ± 5.80

Supplementary Table 4.6. Mean soil total and available elements, sum of bases (SB), pH in water and soil texture for the 0-30 cm soil depth in each treatment (*n*=4) before nutrient addition in a lowland forest in central Amazon (total 32 plots distributed among 8 treatments). Means are followed by standard errors. TRT= treatments, N= nitrogen, P= phosphorus and CAT= cations.

	Mean total elements concentration ± se					Mean available elements concentration ± se							
	Mg	K	P	C	N	Ca	Mg	K	SB	pH	Clay	Silt	Sand
TRT	$cmol_c\ kg^{-1}$		$mg\ kg^{-1}$		%	$cmol_c\ kg^{-1}$				in water		%	
CTRL	0.339 ± 0.024	0.174 ± 0.007	87.46 ± 1.84	2.25 ± 0.18	0.185 ± 0.011	0.034 ± 0.008	0.055 ± 0.009	0.066 ± 0.006	0.155 ± 0.021	4.25 ± 0.05	77.55	14.75	7.69
N	0.348 ± 0.011	0.202 ± 0.020	85.67 ± 0.71	2.16 ± 0.25	0.179 ± 0.016	0.033 ± 0.004	0.050 ± 0.005	0.071 ± 0.007	0.154 ± 0.012	4.21 ± 0.02	78.61	13.77	7.63
P	0.374 ± 0.025	0.181 ± 0.021	81.28 ± 3.57	2.56 ± 0.17	0.201 ± 0.012	0.032 ± 0.003	0.063 ± 0.004	0.078 ± 0.011	0.174 ± 0.015	4.09 ± 0.13	70.01	15.28	14.70
CAT	0.360 ± 0.018	0.185 ± 0.012	83.47 ± 0.93	2.28 ± 0.08	0.182 ± 0.004	0.032 ± 0.008	0.064 ± 0.011	0.102 ± 0.030	0.197 ± 0.048	4.35 ± 0.07	78.33	13.60	8.08
N+P	0.381 ± 0.020	0.194 ± 0.017	87.88 ± 3.79	2.24 ± 0.12	0.180 ± 0.007	0.029 ± 0.008	0.055 ± 0.005	0.074 ± 0.010	0.158 ± 0.020	4.08 ± 0.10	75.40	15.84	8.75
N+CAT	0.358 ± 0.042	0.213 ± 0.026	85.17 ± 3.23	2.23 ± 0.17	0.179 ± 0.010	0.039 ± 0.006	0.052 ± 0.009	0.080 ± 0.016	0.171 ± 0.030	4.22 ± 0.08	76.47	13.44	10.09
P+CAT	0.383 ± 0.014	0.188 ± 0.016	87.67 ± 2.26	2.93 ± 0.08	0.226 ± 0.004	0.031 ± 0.006	0.071 ± 0.007	0.098 ± 0.011	0.200 ± 0.022	4.20 ± 0.03	77.35	14.08	8.63
N+P+CAT	0.376 ± 0.016	0.178 ± 0.017	86.09 ± 2.76	2.57 ± 0.13	0.223 ± 0.025	0.028 ± 0.005	0.062 ± 0.004	0.080 ± 0.004	0.169 ± 0.012	4.17 ± 0.17	74.31	15.21	10.48

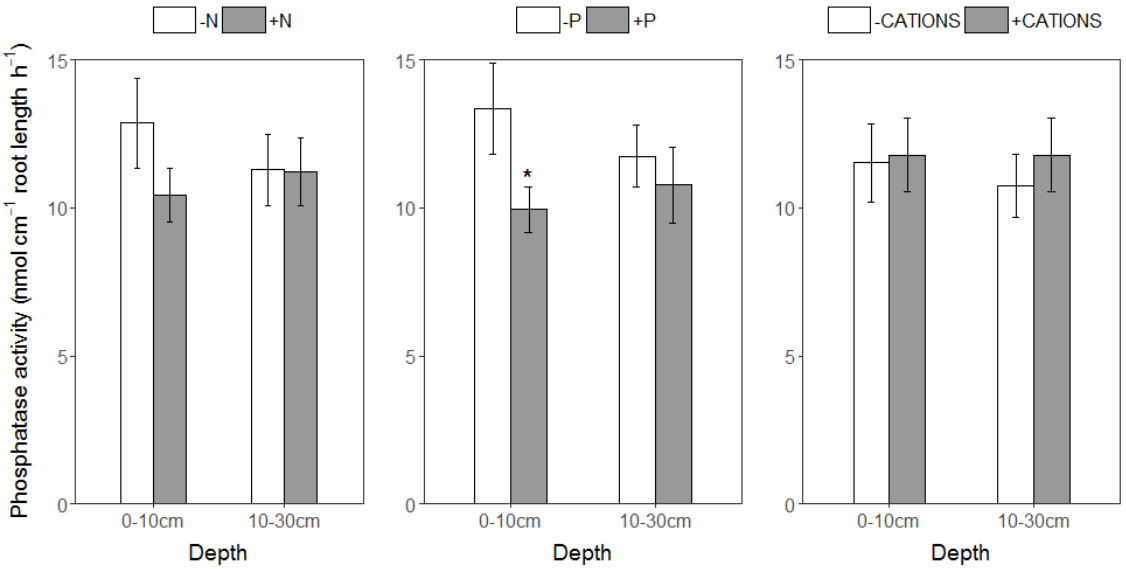


Supplementary Figure 4.1. Fine root productivity in m m^{-2} $month^{-1}$ in two soil layers (0-10 and 10-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

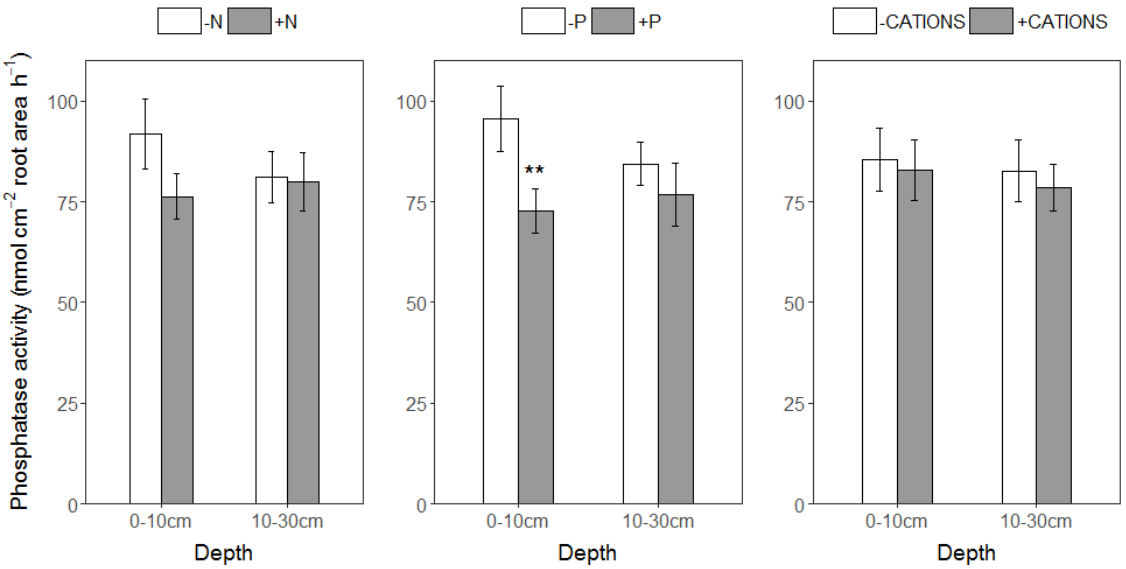


Supplementary Figure 4.2. Fine root productivity in m^2 m^{-2} $month^{-1}$ in two soil layers (0-10 and 10-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient in each depth. Error bars represent

standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

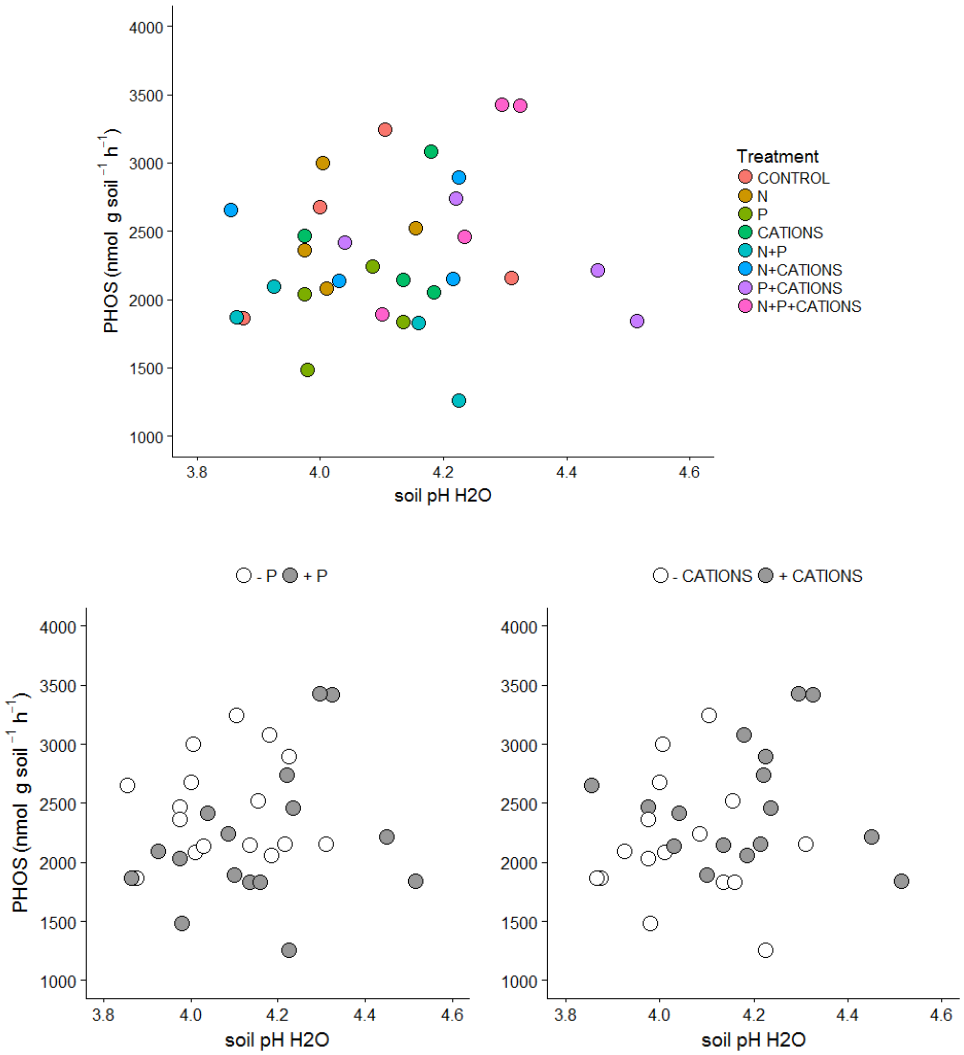


Supplementary Figure 4.3. Mean root phosphatase activity in nmol cm^{-1} root length $hour^{-1}$ in two soil layers (0-10 and 10-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient in each depth. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.



Supplementary Figure 4.4. Mean root phosphatase activity in nmol cm^{-2} root area $hour^{-1}$ in two soil layers (0-10 and 10-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of each nutrient in each depth. Error bars represent standard errors. Significant effects of the N*P*cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

Appendix 3. Chapter 5 Supplementary material



Supplementary Figure 5.1. Correlation (lm) between soil pH (in water) and soil phosphatase activity. Points are means for 0-10 cm soil layer per plot (*n*=32). Upper figure show eight treatments indicated by different colours. Bottom figures

show plots with and without the addition of P on the left and with and without the addition of cations on the right. Adjusted R^2 = -0.011, p=0.42.