Will nutrients limit the tropical forest carbon sink?

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The candidate confirms that the work submitted is their own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgement

Everything has an end. It is time to review the journey of my PhD which I spent 4 years completing. Looking back to the last 4 years, many people should be acknowledged.

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This is the end of my PhD but a new start in my career and life. Throughout the rest of my life, the above-mentioned people will be memorized. Inspired by them, I will also do my best to help out in the future.

Abstract

Tropical secondary forests and primary forests play a critical role in the terrestrial carbon sink and can help to slow global warming. However, the carbon sink of tropical forests may be limited by soil nutrients, especially nitrogen and phosphorus which is generally low in tropical soils. To overcome nutrient limitation, trees may adjust above- and belowground carbon allocation and change nutrient composition (ratios of carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorus) and allocations in tissues. Yet, it is still unclear if and how patterns of nutrient limitation (type and strength) on the forest carbon sink shift over tropical forest succession, and how nutrient limitation affects forest dynamics, including tree growth, recruitment, and mortality. In addition, there has been little investigation into whether and to what extent of trees change their allocation of carbon, nutrient composition, and nutrient allocation to address nutrient limitation over the course of tropical forest succession. To fill these knowledge gaps, I used a large-scale, long-term nutrient manipulation experiment across a tropical successional gradient in Panama, including a mature forest and secondary forests aged 0 (newly regenerating forests), 10, and 30 years following deforestation and cattle ranching. I analysed data from multiple censuses in 76 plots totalling 8.56 ha, computed biomass, and analysed the responses of aboveground biomass and its dynamics (growth, recruitment, and mortality) to nutrient addition. I also assessed the effects of nutrient addition on changes in standing fine root biomass, the ratio of fine root biomass to aboveground biomass, nutrient composition, and nutrient content in each tissue type over forest succession. I found that patterns of nutrient limitation on forest aboveground carbon sink shift across forest succession from strong nitrogen limitation in young secondary forests, to phosphorus limitation in middle stage forests, and to no evidence of nutrient limitation in the mature forests. To address nutrient limitation, trees adjust above- and belowground carbon allocation and change nutrient compositions and allocations in tissues. Following addition of limiting nutrients, trees allocate more carbon aboveground to boost aboveground carbon accumulation, decrease the ratio of carbon to nitrogen and/or phosphorus, and allocate more nutrients from leaves and fine roots to wood. These strategies may successfully address weak nutrient limitation on the carbon sink in mature forests. My project is the first to demonstrate how nutrient limitation on the aboveground carbon sink shifts over tropical forest succession and provides insight for policymakers planning to use reforestation to meet carbon targets. It demonstrates that tropical forests can be nitrogen as well as phosphorus limited, challenging the dominant

biogeochemical paradigm. Furthermore, this project will help improve predictions of future forest carbon sinks.

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Aboveground biomass assessments:

Trees: AGB = exp [-1.803 – 0.976E + 0.976 ln (WD) + 2.673 ln (DBH) – 0.0299 [ln(DBH)^2]. This function was formed in Chave et al., (2014), and some parameters were adjusted according to the local site condition (Rutishauser et al., 2020).

Where AGB represents aboveground biomass (kg/ha), E is the local climatic index (Rutishauser et al., 2020), and WD is wood density (g/cm^3). DBH represents the diameter at breast height (cm), the climate index, E=0.05645985 near our study site, stands for the effect of environment on tree height allometry (Rutishauser et al., 2020), and species-specific wood density (in g/cm^3) was estimated from most common species in Agua Salud and Gigante (Rutishauser et al., 2020 and Wright unpublished data).

Lianas: AGB = exp [-0.999 + 2.682 * ln (DBH)]. This function was formed in Schnitzer et al., (2006), and some parameters were adjusted according to the local site condition (Lai et al., 2017).

Palm trees: AGB = 0.0417565 * (DBH) $^{2.7483}$. This function was formed in Goodman et al., (2013), and some parameters were adjusted according to the local site condition (Rutishauser et al., 2020).

Plot weight nutrient concentrations and compositions ((C, N, P, C:N, C:P, or N:P)com):

$$(C, N, P, C\!:\!N, C\!:\!P, or\ N\!:\!P)com = \frac{\sum_{i=1}^{n}([C, N, P, C\!:\!N, C\!:\!P, or\ N\!:\!P]_i * B_i)}{\sum_{i=1}^{n}[B_i]}$$

Where [C, N, P, C:N, C:P, or N:P]$_i$ is the carbon concentration, nitrogen concentration, phosphorus concentration, carbon to nitrogen ratio, nitrogen to phosphorus ratio of the *i*th species, respectively, n is the number of selected species in the community, and B is the biomass of each species.

Plot scale leaf mass per area:

$$(Leaf\ mass\ per\ area)_{plot} = \frac{\sum_{i=1}^{n}([Leaf\ mass\ per\ area]_i * B_i)}{\sum_{i=1}^{n}[B_i]}$$

Where [Leaf mass per area]$_i$ is the leaf mass per area (*see above*) of the *i*th species, n is the number of selected species in the community, and B is the biomass of each selected species.

Plot scale leaf biomass:

$$\text{Plot scale leaf mass} = (\text{Leaf mass per area})_{\text{plot}} * (\text{LAI}_{\text{plot}}),$$

Plot scale wood biomass:

$$(\text{Wood biomass})_{plot} = (\text{Aboveground biomass})_{plot} - (\text{Leaf biomass})_{plot}$$

Plot scale leaf nutrient contents:

$$(C, N, P)_{contents} = Plot\ leaf\ mass * (C, N, P)_{com}$$

Plot scale wood nutrient contents:

$$(C, N, P)_{contents} = (Wood\ biomass)_{plot} * (C, N, P)_{com}$$

Plot scale fine root nutrient contents:

$$(C, N, P)_{contents} = (Fine\ root\ biomass)_{plot} * (C, N, P)_{plot}$$

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Abbreviations

CO_2-Carbon dioxide

C-Control treatment

N-Nitrogen addition treatment

P-Phosphorus addition treatment

NP- Nitrogen plus phosphorus addition treatment

AGB- Aboveground biomass (Mg/ha)

WD-wood density (g/cm^3)

DBH-diameter at breast height (mm)

AIC- Akaike information criterion

LAI-Leaf area index

LMA-Leaf mass per area (g/m^2)

B-Aboveground biomass of each plot (Mg/ha)

Chapter 1

Introduction

Tropical forests play a critical role in mitigating climate change - accounting for nearly 30% of terrestrial net primary productivity, contributing more than 60% of the terrestrial carbon sink over recent decades (Phillips et al., 1998; Pan et al. 2011; Keenan and Williams, 2018; Houghton et al., 2018), and offsetting more than 2 Pg carbon emissions every year (Mitchard, 2018, Phillips and Brienen, 2017). This carbon sink is expected to increase with the regrowth of tropical secondary forests following disturbance (Pan et al., 2011; Houghton et al., 2015; Poorter et al., 2016) alongside the growth of primary forests under a carbon dioxide fertilization effect (an increase in photosynthetic carbon uptake by plants under a higher carbon dioxide) (Fleischer et al., 2019). However, forests do not require only carbon for growth, and thus tropical forest carbon sequestration may be limited by the availability of soil nutrients (Vitousek & Sanford 1986; Vitousek et al., 2010; Wieder et al., 2015; Hedin 2015; Wright, 2019; Terrer et al., 2019), especially nitrogen and phosphorus, which play key roles in supporting primary productivity.

Biogeochemical theory holds that the tropical forest carbon sink may be limited by nitrogen and phosphorus (Vitousek, 1984). This assumption is based on the factors that, first, tropical soils are at the later stage of primary succession and have low phosphorus availability (Vitousek et al., 2010; Reed et al., 2011). During pedogenesis, a large amount of phosphorus is lost due to high weathering and leaching, but a small amount returns from atmosphere and volcanic ash (Walker & Syers 1976; Menge et al., 2012). The median amount of soil total phosphorus in the tropics is less than 300 mg / kg, which is lower than in temperate areas (He et al., 2021, Wright, 2022). Second, at the early stage of secondary succession, nitrogen in tropical forests can easily be lost via leaching and gaseous emission following disturbance, while P remains bound to soil particles tightly (Davidson et al., 2004; Davidson and Howarth, 2007).

The pattern of nutrient limitation on the tropical forest carbon sink may shift along forest succession, from strong nitrogen limitation at the early stage of forest succession to phosphorus limitation or no evidence of nutrient limitation at the late-successional gradient (Nagy et al., 2017). The reason for this pattern is because the amount of nutrient supply and trees' nutrient requirement changes along forest succession. First, trees need more nitrogen than phosphorus to capture per-unit carbon; second, nitrogen-fixing trees become abundant and actively fix nitrogen early in forest succession, building up nitrogen in the soil until they decrease fixation late in succession (Batterman et al., 2013); and third, the forest net biomass accumulation (requirement of nutrients) rate decreases along forest succession (Brown and Lugo, 1990).

Evidence for nutrient limitation on the tropical forest carbon sink derives from studies that use a variety of approaches around the world (Gough et al., 2000; Elser et al., 2007; Aragao et al., 2009; Vitousek et al., 2010; Gerber et al., 2010, 2013; Wright et al., 2011, 2018; Cleveland et al., 2011; Quesada et al., 2012; Fernandez-Martinez et al., 2014; Wieder et al., 2015; Wang et al., 2018; Craine et al., 2018; Fleischer et al., 2019; Terrer et al., 2019; Wright 2019). First, field tracer experiments, which used labelled nitrogen isotopes to simulate nitrogen cycling in tropical ecosystem, showed that nitrogen limits the carbon sink in some Asian tropical forests (Wang et al., 2018). Second, predictions from dynamic global vegetation models (DGVMs) suggest that in tropical forests carbon sink is constrained by soil nutrient availability (Fisher et al., 2012; Wieder et al., 2015; Terrer et al., 2019). Third, a study assembling the 14 terrestrial ecosystem models, which simulating Amazon free-air CO_2 enrichment experiment, demonstrated that available soil phosphorus will limit the response of carbon sink in Amazon forest to atmospheric CO_2 fertilization (Fleischer et al., 2019). Finally, fertilization experiments, which provide a direct test of nutrient limitation patterns (strength and type), show that soil nutrient limitation on tropical successional forest carbon sink exists widely (Wright et al., 2018, Wright, 2019, Waring et al., 2019; Cunha et al., 2022). However, whilst evidence for nutrient limitation in tropical forests is abundant, it remains unclear how nutrient limitation shifts over the course of tropical forest secondary succession and how trees in tropical forests can adapt to nutrient limitation, and whether this will mitigate the effects of limiting nutrients on forest growth and carbon uptake.

Trees may use a variety of strategies to allow them to remain competitive and thrive when facing limiting resources. These strategies may include partnerships with microbes: nitrogen-fixing trees may up-regulate fixation when the nitrogen requirement of tree growth cannot be met by soil supply (Batterman et al., 2013; Zheng et al., 2019), and, for many tree species, extracellular enzymes and/or associations with mycorrhizal fungi will increase access to nutrients by increasing the volume of soil explored (Treseder and Vitousek, 2001). Trees living in phosphorus-limited conditions may increase investment in arbuscular mycorrhizal fungi to improve phosphorus uptake; trees living in nitrogen-limited soils may increase investment in ectomycorrhizal fungi to enhance nitrogen uptake, an uncommon strategy in Neotropical forests (Read and Perez-Moreno, 2003; Averill et al., 2019). They may also include different ways that trees use and allocate nutrients and carbon to different tissues (Bloom et al., 1985; Chapin III et al., 1990; Schonbeck et al., 2021). However, whether a wide variety of tree species can apply common strategies to address nutrient limitation - such as adjusting carbon and nutrient allocations or changing nutrient compositions in tissues – remains unclear. Studies from Free-Air CO_2 Enrichment experiments demonstrated that nitrogen concentration in leaves decreased (Wujeska-Klause et al., 2019) and fine roots biomass increased (Norby et al., 2004) after CO_2 fertilization, suggesting that these strategies may be plastic. Importantly, if trees can adjust biomass and nutrient allocation in tissues to address nutrient limitation, they will be able to grow under apparent nutrient limitation, and so more carbon will be taken up by tropical forest ecosystems under rising atmospheric carbon dioxide.

Studying the pattern of nutrient limitation in tropical forests, and the strategies utilized by tropical trees to address nutrient limitation, will enable us to understand the role of tropical forests in alleviating global climate change. I here review the existing literature concerning tropical forest nutrient addition experiments to evaluate our understanding of ***(1) whether and which soil nutrients will limit the tropical carbon sink**, **and, if so, how these nutrient limitation patterns (strength and type) change along forest succession**, **(2) how trees can adjust their biomass carbon allocation as a strategy to overcome nutrient limitation, and, finally, (3) how trees can adjust their nutrient use and allocation as a strategy to overcome nutrient limitation.*** I then outline a series of projects that will address gaps in the literature and go on to form my PhD dissertation.

1.1 Literature review

1.1.1 Nutrient limitation on the tropical forest carbon sink

Nutrient limitation on the terrestrial carbon sink is typically defined as an increase in some aspect of net primary productivity (stem growth, root growth, reproductive output and litterfall) following the addition of a nutrient (Vitousek, 1982). Due to the difficulty in detecting net primary production, the responses of each tissue biomass, especially forest growth, to nutrient addition have usually been measured instead (Wright, 2019).

The use of nutrient fertilization experiments has been applied widely to demonstrate nutrient limitation patterns (strength and type) in tropical forests. At least 49 nutrient addition experiments have been set up in 36 sites over 14 countries across the American, Asian and African tropical forests (**Figure 1.1**, see 'the summary of nutrient fertilization experiments in tropical forests' in **Supporting document**). These nutrient fertilization experiments show that, in general, early successional forests have stronger nutrient limitation than old-growth forests, which have only moderate responses to nutrient addition (Wright, 2019). This difference in the strength of nutrient limitation between secondary and old growth forests may exist widely. For example, in lowland tropical forests of Costa Rica, nutrient addition increase tree growth in secondary forest (Chou et al., 2018), but not in primary forests (Alvarez-Clare et al., 2013). Yet, there has been no single study demonstrating how nutrient limitation strength changes along forest succession.

Beside the understanding on nutrient limitation strength along succession, it also remains unclear whether the type of nutrient that limits forest growth changes with forest development over secondary succession. In general, nutrient addition experiments are conducted in different sites, and the exact limiting nutrient varies across forests. For example, in a 2-year-old forest in Brazil, tree growth increased after phosphorus addition (Gehring et al., 1999), while in a nearby 6 years old forest tree growth increased after nitrogen addition (Davidson et al., 2004). The main reason for this divergence of nutrient limitation in different forests may be because these ecosystems have different tree communities and parent material (soil ages), which are the two major factors affecting nutrient limitation patterns. A shifting pattern of nutrient limitation has been found across a primary succession gradient in Hawaii tropical forests (Vitousek et al., 1993; Vitousek and Farrington 1997; Ostertag, 2001; Harrington et al., 2001). This primary successional experiment demonstrates that trees living in younger soil grow faster after nitrogen addition, but trees living in old soil sites grow faster after phosphorus addition.

However, to date, no fertilization experiment has been carried out across a full secondary succession gradient which have different tree communities but the same soil age.

Nutrient limitation has also been studied in mature forests using nutrient addition experiments, but the nutrient limitation patterns in mature forests remain unclear. A synthesis of eight nutrient (nitrogen and/or phosphorus) fertilization experiments in tropical primary forests that have been conducted for up to 15 years demonstrated that forest growth in primary forests did not increase following nutrient addition (Wright et al., 2018). There are five possible reasons that no growth response was detected. First, mature forests are limited by other nutrients, such as potassium (Wright et al., 2011). Second, forests in late-successional forests are dominated by large trees which may invest more carbon to increase the biomass of other tissues or organs, such as seed or fruit mass for reproduction, rather than enhancing woody growth. Accordingly, nutrient limitation on growth in mature forests may not be detected in fertilization experiments. Third, mature forests often have low growth rates, so it may be difficult to detect the change of big trees following short term nutrient addition. Instead, longer monitoring of fertilization experiments is required to test if growth is limited by soil nutrients in tropical mature forests. Fourth, the detection of forest growth can be affected by other dynamics, especially forest mortality, i.e. the forest growth may be underestimated if some trees died between two census intervals. In spite of this, the effects of nutrient addition (or nutrient limitation) on forest dynamics have been rarely discussed. Fifth, mature forests may have weak nutrient limitation, and trees may apply strategies, such as adjusting carbon and nutrient allocation across tissues, to successfully address the weak nutrient limitation, which is the second part I am going to focus on in my PhD thesis (Chapter 3 and 4).

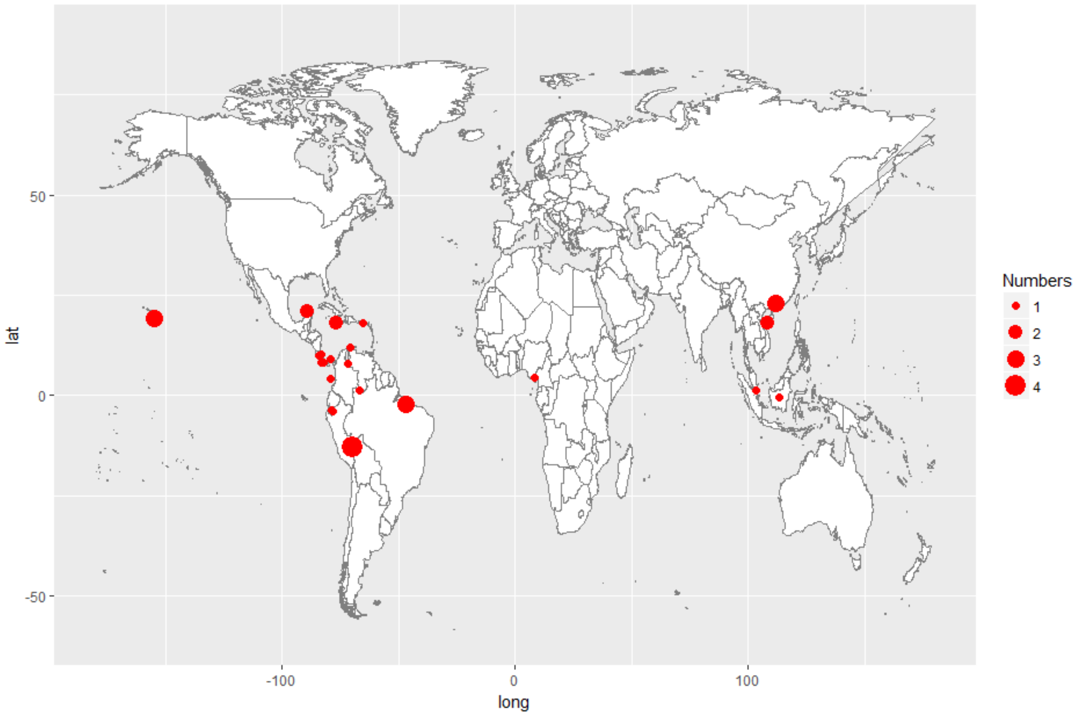

Figure 1.1 | Fertilization experiments in tropical forests across the world.

The size of each point represents the number of forest types at each site where fertilization experiments have been established.

1.1.2 Biomass allocation in tissues to address nutrient constraints

To address nutrient limitation, the optimal partitioning theory suggests that trees may also adjust carbon allocation across tissues (Thornley, 1972; Poorter et al., 2012; Malhi et al., 2011). More carbon will be allocated to the tissue and increase the biomass for increasing limited resource competition. For example, when trees are limited by soil nutrients, more carbon may be allocated to fine roots and increase fine root biomass to enable increased nutrient acquisition from the soil; while when nutrient limitation is relieved, more carbon will be allocated aboveground to enhance light and space competition. This adjustment may be an active process to optimally partition nutrients and maximize growth. It may also be a passive process due to source sink dynamics during elemental cycling.

Carbon allocation is the partitioning of net primary production to different tissues. Only a few nutrient addition experiments to date have tested how net primary production allocation to tissues changes in response to nutrient addition (Alvarez-Clare et al., 2013; Waring et al., 2019; Cunha et al., 2022). However, these studies are difficult to determine if forests can adjust carbon allocation to address nutrient limitation, because these studies ignored the partitioning of net primary production across tissues and also did not measure total net primary production.

Changes in biomass of tissues can be treated as a proxy index to stand for the change in carbon allocation to tissues, so most studies, focused on how biomass comprising different tissues changed in response to nutrient addition. Here, I summarize the change in biomass of each tissue (leaves, wood, or fine roots) in response to nutrient addition in tropical forests instead.

1.1.2.1 The response of fine root biomass to nutrient addition

Fine roots are the locus of nutrient absorption for tropical trees, and the proportion of biomass allocated to fine roots may change in response to nutrient addition. So far, seventeen (of forty-nine) tropical fertilization experiments have reported the response of fine root biomass to nutrient addition (Wright, 2019). These experiments found that fine root biomass may decrease, increase or remain unchanged in response to nutrient addition. For example, in a primary montane forest of Ecuador and a montane primary forest of Hawaii, fine root biomass decreased following the addition of limiting nutrients (Gower and Vitousek, 1989; Homeier et al., 2012). In a primary forest of Panama, fine root biomass showed no evidence of change after nutrient addition (Wurzburger and Wright, 2015). In contrast, in two tropical secondary forests of China which experienced high nitrogen deposition, fine root biomass increased in both nitrogen and phosphorus fertilized forests (Zhu et al., 2013).

This diverse range of responses of fine root biomass to nutrient addition may result from four different mechanisms. First, in non-nutrient limited forests, the turnover rate of fine roots may increase after nutrient addition (Ostertag, 2001), which may result in the moderate decrease of fine root biomass; second, in forests with weak nutrient limitation, both net and relative primary production allocation to fine roots decrease in response to limiting nutrient addition, which may decrease fine root biomass; third, in forests with strong nutrient limitation, relative net primary production allocation to fine roots may decrease but absolute net primary production allocation to fine roots may increase after limited nutrient addition, which may overall increase fine root biomass; fourth, in addition, in phosphorus limited forests, nitrogen addition may enhance photosynthesis and increase the allocation of net primary production to fine roots in order to allow increased fine root biomass exploration for soil phosphorus. These four mechanisms of change in carbon allocation to fine root in response to nutrient addition suggest that fine root biomass may increase, undergo no change, or decrease following nutrient addition. Therefore, changes in fine root biomass alone cannot be used to illustrate whether trees can alter carbon allocation to fine roots as a strategy to address nutrient limitation. To improve the assessment of the change in belowground carbon allocation in response to nutrient availability, it is necessary to simultaneously assess aboveground carbon or biomass change (leaves and wood).

1.1.2.2 The response of leaf biomass to nutrient addition

In theory, the biomass of leaves that are responsible for carbon uptake via photosynthesis should also change in response to nutrient addition. On the one hand, trees need more leaf biomass to provide the photosynthetic potential necessary to increase gross primary production with total biomass increase following nutrient addition; on the other hand, if trees can up-regulate foliar nitrogen concentration and increase photosynthesis via producing more proteins involved in photosynthesis per unit leaf area (*see 1.1.3.1*), then trees can invest less carbon in leaf tissue and therefore decrease leaf biomass. Therefore, it is reasonable to expect a range of leaf biomass responses (increase, decrease or no change) to nutrient addition. However, standing leaf biomass is difficult to measure – with no good method for measuring standing leaf biomass correctly. So far, no research has examined the direct response of canopy leaf biomass to nutrient addition. Fourteen (of forty-nine) studies examine a proxy for leaf biomass: the response of litter fall – including leaf fall and reproduction – to nutrient addition.

Fertilization experiments showed that litter fall biomass can increase or undergo no change in response to nutrient addition caused by different mechanisms. For example, in two secondary forests of Yucatan, Mexico, litter fall increased after both nitrogen and phosphorus addition (Campo & Dirzo 2003; Campo & Vazuez-Yanes 2004; Campo et al., 2007). This increase in litter fall may be because trees grow up and have more canopy leaf biomass following nutrient addition. In addition, in a lowland primary forest of Puerto Rico (Li et al., 2006) and a lowland primary forest of Costa Rica, increase of litter fall was observed for a short period after nutrient addition (Cleveland et al., 2006). These increases in litter fall may be because trees in primary forests need less canopy leaves after nitrogen addition, as photosynthetic rates per leaf increase. Furthermore, dominant trees' leaf biomass may relieve light limitation of under canopy trees, which may increase the leaf biomass of second layer trees and result in no change in litter fall following nutrient addition. No change of litterfall following nitrogen and phosphorus addition has also been observed in a primary forest of China (Mo et al., 2008) and a primary forest of Costa Rica (Alvarez-Clare and Mack 2015). These diverse mechanisms of litter fall biomass change demonstrate that the response of litter fall to nutrient addition may not represent the response of canopy leaf biomass to nutrient addition.

1.1.2.3 The response of wood biomass to nutrient addition

The biomass of wood, which is responsible for increases in height and crown in response to light competition, may also change in response to the addition of the limiting nutrient. The reasons for the change of woody biomass are that both net and relative net primary production allocation to wood may increase when trees are relieved from soil nutrient limitation, but turnover of wood is quite low. Therefore, it is reasonable to expect that wood biomass either increases or undergoes no change in response to nutrient addition. So far, eighteen (of forty-nine) fertilization experiments discuss the response of wood biomass to nutrient addition. These fertilization experiments showed that after nutrient addition, in general, wood biomass increases in secondary forests which have strong nutrient limitation patterns (Uhl 1987; Gehring et al., 1999; Harrington et al., 2001; Campo and Vazquez-Yanes, 2004; Jiang et al., 2018), but have no significant change in primary forests which have moderate or no nutrient limitation pattern (Mirmanto et al., 1999; Newbery et al., 2002; Li et al., 2006; Kaspari et al., 2008; Fisher et al., 2013). These changes in wood biomass in response to nutrient addition do not reveal whether forests adjust carbon allocation to wood and change wood biomass in order to address nutrient limitation.

1.1.2.4 Knowledge gap of biomass allocation strategy to address nutrient limitation

Whilst nutrient fertilization experiments have showed that biomass of fine roots, leaves and wood can change in response to nutrient addition, it remains unclear whether trees can change biomass and /or carbon allocation as a strategy to address nutrient limitation. In addition, if tropical forest trees use a strategy of adjusting biomass carbon allocation to address nutrient limitation, the degree to which this strategy helps tropical forests address nutrient limitation as forests develop from young secondary forests (with high growth rate) to mature forest (with low growth rate) remains unknown.

1.1.3 Nutrient compositions and allocations in tissues change following nutrient addition

Growing in low fertility soils, trees may apply nutrient stoichiometry strategies, such as changing nutrient allocations and compositions (carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorous) in tissues to address nutrient limitations. Nutrients, especially nitrogen and phosphorus, play important roles in photosynthesis, protein synthesis, cell growth, and metabolism (Chapin et al., 2002; Reich et al., 2009; Walker et al., 2014), and the changes in these two nutrient concentrations in tissues and allocations across tissues may be very fast. Previous studies demonstrated that nutrient compositions in tissues can vary with soil fertility. For instance, trees growing across a soil fertility gradient exhibit different tissue nutrient concentrations or compositions (Townsend et al., 2008; Heineman et al., 2016; Tian et al., 2019). However, this difference in nutrient concentrations in tissues of trees living in different fertile soils cannot tell us if trees apply stoichiometric strategies to address nutrient limitations. To test if trees can adjust nutrient allocations and compositions in tissues to address nutrient limitation, we need to measure how nutrient concentrations and pools in tissues (leaves, wood and fine roots) respond to nutrient addition.

1.1.3.1 The response of foliar nutrients to nutrient addition

Nutrient fertilization experiments show that foliar nutrient concentrations (nitrogen and phosphorus) often change in response to nutrient addition. The response of foliar nutrient concentrations to nutrient addition was reported in sixteen (of the forty-nine) tropical forest fertilization experiments (*see above*, Wright et al., 2018; Wright 2019). In general, nitrogen addition increased foliar nitrogen concentrations in nitrogen-limited forests but had no significant effect in forests with ample available soil nitrogen. For example, in the Hawaiian montane nitrogen-limited forest, foliar nitrogen content increases in nitrogen added plots (Vitousek and Farrington, 1997); while in a nitrogen-rich tropical primary forest of China and the non-nitrogen limited primary forest of Panama, nitrogen addition did not significantly change foliar nitrogen content (Mayer et al., 2014; Lu et al., 2018). In contrast, phosphorus addition can increase foliar phosphorus in both phosphorus limited and non-limited forests. For example, in both phosphorus-limited and non-phosphorus limited forests of Hawaii, foliar phosphorus concentrations increased after phosphorus addition (Vitousek and Farrington, 1997). These different response patterns of foliar nitrogen and phosphorus to nutrient addition suggest that trees have different uptake and/or allocation mechanisms for nitrogen and phosphorus in leaves, which may be because that increased nitrogen results in high herbivory (Throop et al., 2004) while high phosphorus may do not.

1.1.3.2 The responses of fine root and wood nutrients to nutrient addition

Fine roots, which are the major tissue responsible for absorbing soil nutrients, are an important source and sink for nutrients (Gordon and Jackson, 2000). The active roles of fine roots in the plant nutrient economy suggest that nutrients in fine roots could also change in response to nutrient addition. Four fertilization experiments in tropical forests reporting fine root nutrient concentration changes following nutrient addition. These experiments show that phosphorus and nitrogen in fine roots have different response patterns to nutrient addition. Phosphorus addition can increase fine root phosphorus in both phosphorus limited (Zhu et al., 2013; Alvarea-Clare and Mack, 2015) and non-limited forests (Wurzburger and Wright, 2015; Mo et al., 2015); while, nitrogen addition increases fine root nitrogen only in low nitrogen forests (Mo et al., 2015) but has no significant effect in non-nitrogen limited forests (Zhu et al., 2013; Alvarea-Clare and Mack, 2015; Wurzburger and Wright, 2015). These observations indicate that trees may stop absorbing nitrogen when they are not limited by nitrogen, but may continue to take up phosphorus even when sufficient soil phosphorus is available.

Alongside leaf and fine root tissues, wood, which includes stems and coarse roots and accounts for the largest part of the biomass of trees (Chave et al., 2009; Schreeg et al., 2014), may act as a nutrient reservoir. It is reasonable to expect that nutrient concentration in wood increases after nutrient addition, as nutrients are responsible for protein synthesis and cell growth (*see above*, Chapin et al., 2002; Reich et al., 2009; Walker et al., 2014). However, so far, only one study carried out in a phosphorus limited tropical mixed forest in China reported the response of wood nutrient concentrations to nutrient addition. This study showed that phosphorus addition increases woody phosphorus concentration, but nitrogen addition has no effect on the wood nitrogen concentration (Mo et al., 2015). More research is required to examine how wood and fine-root nitrogen and phosphorus concentrations change following nutrient addition in both nutrient-limited and non-limited forests.

Plants have different physiological needs for nitrogen and phosphorus, and depending on the relative physiological need, they may shift between nitrogen and phosphorus limitation. For example, nitrogen is primary used for rubisco and photosynthesis in leaves, whereas phosphorus is mainly used for energy production of adenosine triphosphate (ATP) and in the lipids of cell structure across all tissues of trees. There also may be species difference in the traits and physiological uses of nitrogen and phosphorus. Therefore, the degree of nitrogen or phosphorus limitation may shift as physiological demands change and/ or species composition changes.

1.1.3.3 Knowledge gap of nutrient composition and allocation strategies to address nutrient limitation

Whilst nutrient fertilization experiments have demonstrated that nutrient concentrations in tissues can change following nutrient addition, we still do not understand sufficiently whether and how trees can adjust nutrient stoichiometry (nutrient compositions and allocations) to address nutrient limitation, especially across a gradient in net carbon uptake and forest secondary succession. To test if trees have this nutrient allocation strategy, responses of nutrient concentrations and pools in tissues (leaves, wood, and fine roots) should be measured at the same time and in forests with different nutrient limitation patterns. However, so far, only one study has reported nitrogen and phosphorus concentrations in leaves, wood and fine roots tissues at the same time on seven species in one tropical forest in China (Mo et al., 2015), which cannot be used to identify if trees, growing in different fertility soil, can change nutrient allocation in tissues, since this experiment did not include different patterns of nutrient limitation.

1.2 Research questions and hypothesis

In this thesis, I, first, will focus on the shifts in nutrient limitation over tropical forest secondary succession and ask the following questions in the second chapter:

Chapter 2: Do nutrients limit net carbon uptake rates in tropical forests at varying stages of succession? And, if so, how does nutrient limitation shift over tropical forest succession? Do patterns of nutrient limitation on forest biomass emerge from effects on the forest dynamics of tree growth, recruitment and/or mortality?

After that, I will focus on the strategies (nutrient absorption from the soil and nutrient usage in plants) used by trees to address nutrient limitation, and raise two questions in the third and fourth chapters, respectively:

Chapter 3: Do trees adjust above- and belowground carbon allocation and fine root biomass in response to nutrient limitation? If so, how do changes in above- and belowground carbon allocation and fine root biomass in response to nutrient limitation shift along forest succession?

Chapter 4: Do trees adjust nutrient use strategies – including nutrient composition and allocation to different tissues – in response to nutrient limitation? If so, how do the changes in nutrient ratio and allocation in tissues in response to nutrient limitation shift along forest succession.

For each chapter, I have the following hypotheses:

Chapter 2: Forests in different successional stages are limited by different soil nutrients. I expect to find nitrogen limitation in young tropical forests, moving to phosphorus limitation or nitrogen and phosphorus co-limitation in mid-succession, to phosphorus limitation or no evident limitation in mature tropical forests. Nutrient limitation patterns can also affect the tree community composition, tree mortality and recruitment.

Chapter 3: Tropical forests can adjust above-and belowground carbon allocation and change fine root biomass in response to nutrient limitations. The pattern of belowground carbon allocation changes in response to nutrient limitation shifts along forest successional gradient.

Chapter 4: Tropical forests can adjust nutrient compositions and allocations in tissues in response to nutrient limitation. The stoichiometric strategies applied by forests in response to nutrient limitation shift along forest successional gradient.

Chapter 2

Soil nutrients facilitate tropical forest carbon sequestration rates

Contributions:

Wenguang Tang: analyzed the data, drew figures, lead the discussion, and wrote the draft

Jefferson S. Hall and **S. Joseph Wright**: established experiments, collected data, and discussed the results

Oliver L. Phillips and **Roel J.W. Brienen**: analyzed the data, discussed the results, and wrote the draft

Michiel van Breugel: established the experiment and collected the data

Michelle Wong: provided feedback on the manuscripts

Sarah A. Batterman: established the experiment, collected and analyzed the data, discussed the results, and wrote the draft.

Abstract

Identifying the factors that regulate tropical forests' carbon sequestration is important for predicting the future of the tropical carbon sink and its impact on climate change pathways. Soil nutrients, especially nitrogen and phosphorus, theory suggests, can constrain tropical forest carbon sequestration, in particular in regenerating forests which have the strongest sinks per-unit-area. However, it remains unclear whether and how soil nutrients limit the biomass carbon sink into tropical forests over the course of secondary succession, if so, how nutrient limitation will affect forest dynamics (growth, recruitment, and mortality). To address this knowledge gap, we established a large, long-term nutrient manipulation experiment across a tropical successional gradient in Central America, including a mature forest and secondary forests aged 0, 10, and 30 years following deforestation and cattle ranching. We measured and identified all trees for multiple censuses in 76 plots totalling 8.56 ha, estimated biomass, and analysed the responses of aboveground biomass and its dynamics to nutrient addition. We

found that nutrient limitation changes across forest succession and that the addition of the limiting nutrient significantly affects forest biomass accumulation rates. In the youngest forest, the rate of aboveground biomass accumulation during our observation period increased by 95% after nitrogen addition, and by 20% with phosphorus addition. In the 10-year forest, it increased by 47% with nitrogen addition, while in the 30-year forest it increased by 25% with phosphorus. The mature forest showed no consistent effects of nutrient addition on biomass dynamics. Nutrients constrain carbon sequestration by limiting forest growth and/or recruitment. Our experiment is the first to demonstrate that nutrient limitation shifts over tropical forest succession. It provides insights for policymakers planning to use reforestation to meet carbon targets.

2.1 Introduction

Tropical forests play a critical role in the terrestrial carbon sink acting to slow the rate of global climate change (Pan et al., 2011; Houghton et al., 2015). The carbon sink in tropical mature forests is anticipated in some vegetation models to persist for many decades, primarily due to tree growth being boosted by CO_2 fertilization (Cox et al., 2013; Huntingford et al., 2013). Secondary tropical forests offer an even larger potential carbon sink (Pan et al. 2011; Chazdon et al. 2016) since an increasing fraction of tropical forests are secondary, and since they accumulate carbon at a much higher rate (20 times) than mature forests (Pan et al., 2011; Brienen et al., 2015; Bongers et al., 2015). Thus, there is increased global interest in managing degraded tropical land for carbon sequestration to increase terrestrial carbon sinks (Lewis et al., 2019). However, recent observations of a slow-down of the carbon sink in mature tropical forests (Brienen et al. 2015) and wide variation in the rate at which carbon recovers in secondary tropical forests (Poorter et al. 2016) raise the question of whether nutrients limit the tropical carbon sink.

Inference that soil nutrients (nitrogen and phosphorus) limit the tropical forest carbon sink derives from several lines of evidence, including nutrient addition experiments that find nutrients limit tropical tree growth (Vitousek, 1984; LeBauer and Treseder, 2008; Wright et al., 2018; Waring et al., 2019; Wright, 2019), direct natural observations of tropical nutrient cycles becoming more open with forest succession and in fertile environments (Davidson et al., 2007; Batterman et al., 2013; Du et al., 2020), and models that indicate the land carbon sink will be constrained by nitrogen and phosphorus (Thornton et al., 2007; Fisher et al., 2012; Goll et al., 2012; Wieder et al., 2015; Levy-Varon et al., 2019; Fleischer et al., 2019). The high demand for nitrogen and phosphorus of tropical trees, driven by high productivity, may frequently go unmet. On the one hand, most tropical soils are highly leached and have low availability of phosphorus leading to phosphorus limitation (Walker and Syer, 1976; Vitousek et al., 2010). On the other hand, tropical forests that have experienced recent disturbances such as blow-downs, logging and agriculture are likely nitrogen limited since nitrogen is easily lost through leaching and gaseous emissions following disturbance, while phosphorus remains bound to soil particles (Hedin et al., 2003; Menge et al., 2012). In addition, the overall forest growth rate varies over successional time, and therefore its nutrient requirements are expected to similarly vary with succession (Brown and Lugo, 1990; Batterman et al., 2013; Poorter et al., 2016). For these reasons, forests at different successional stages are expected to differ in the degree to which different nutrients limits growth (Davison et al., 2007; Power and Marin-Spiotta, 2017; Sullivan et al. 2019) (**Figure 2.1**). However, it remains unclear if and how patterns of nutrient limitation (type and strength) on net forest carbon uptake shift over tropical forest succession, and how nutrient limitation affects forest dynamics (tree growth, recruitment, and mortality).

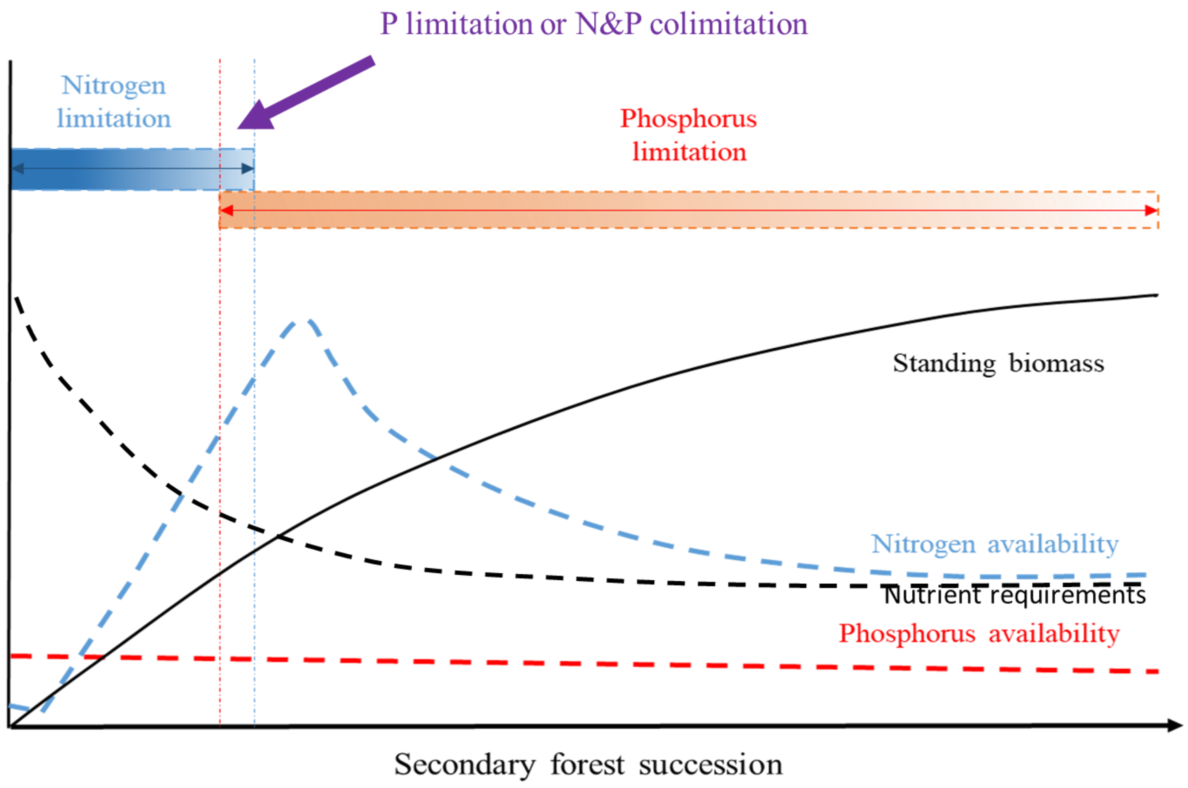

Figure 2.1 | The conceptual model of the nutrient limitation pattern across a secondary forest succession.

The black line curve stands for the standing biomass accumulation along a secondary forest succession, and the black dash line represents the change of nutrient requirement along forest succession (i.e. net nutrient accumulation rate). The blue and red lines represent the nitrogen and phosphorus availabilities (i.e. nutrient supplies) in soil, respectively. The blue and red bars above curves stand for the nitrogen limitation and phosphorus limitation, respectively. In each bar, the color transparency intends to express the strength of limitation. We hypothesis that nutrient limitation pattern (type and strength) shift over succession: the forest following disturbance has strong nitrogen limitation, forests at middle successional stages have phosphorus or nitrogen and phosphorus co-limitation, the forest at the late stage has a slight phosphorus or no evident nutrient limitation.

We established a nitrogen and phosphorus nutrient addition experiment across a secondary successional gradient of lowland tropical forests in Panama and monitored forest changes for up to 21 years following fertilization. We analysed the responses of aboveground biomass and its dynamics to nutrient additions. We hypothesise that the strength of nutrient limitation shifts over succession with the strongest effects at early successional stages. We furthermore hypothesise that the early successional stages are most strongly limited by nitrogen, and that this shifts towards phosphorus or nitrogen and phosphorus co-limitation in middle successional stages, and to phosphorus limitation at the late stage forest.

2.2 Method

2.2.1 Research site

A factorial nitrogen and phosphorus fertilization experiment was established at Agua Salud (9°13’N, 79°47’W, 330 meters above sea level) and Gigante (9°06’31”N, 79°50’37”W, 60 meters above sea level) in the Republic of Panama (**Figure A2.1**). These two sites lie within the Panama Canal watershed and include forests at different stages of succession (from young secondary to mature forest). Both forests are classified as tropical moist forests, receiving similar annual precipitation (~2700 mm) with a dry season (contributing ~ 10% of total rainfall) from December to April, share a similar diverse community of tree species, and the same soil age (Batterman et al., 2013, Ogden et al., 2013, van Breugel et al., 2013, Wright et al., 2018). Soils across all forests are highly weathered and classified as clay-rich Oxisols, with generally low plant-available soil nutrient concentrations (Batterman et al., 2013, Turner et al., 2014,). The soil physical and chemical properties of Agua Salud and Gigante forests can be found in the **Table 2.1**, and the soil properties in Agua Salud fertilization plots can be found in Supporting Document.

In Agua Salud, the landscape consists of cattle pastures and cultivated fields, fallows, plantations, and different age secondary forests which recovered naturally following cattle ranching and small-scale clear-cutting (van Breugel et al., 2013, Batterman et al., 2013). Topography in this area varies, consisting of narrow streams and steep but short slopes (van Breugel et al., 2013). In Gigante, which is a part of the Barro Colorado Nature Monument, the land is covered by a well-protected mature forest on relatively flat terrain (Yavitt et al., 2011).

Table 2.1 Soil physical and chemical properties of our soil from our forest plots before the start of the experiment.

Forest age	Sand (%)	Silt (%)	Clay (%)	pH (H_2O)	pH ($CaCl_2$)	NH_4^+ (mg/kg)	NO_3^- (mg/kg)	Resin P (mg/kg)	Total C (%)	Total N (%)	Total P (mg/kg)	Total K (mg/kg)
0	10.78 ± 1.33	36.07 ± 1.44	53.15 ± 1.06	5.45 ± 0.08	4.68 ± 0.08	11.62 ± 2.78	0.8 ± 0.26	0.86 ± 0.09	3.76 ± 0.08	0.3 ± 0.01	293.85 ± 13.99	501.75 ± 36.85
10	10.30 ± 1.04	33.73 ± 2.02	55.97 ± 2.36	5.25 ± 0.06	4.41 ± 0.07	5.02 ± 0.51	0.12 ± 0.04	0.55 ± 0.06	3.88 ± 0.1	0.28 ± 0.01	255.4 ± 10.49	729.15 ± 114.99
30	13.02 ± 1.99	30.12 ± 1.75	56.86 ± 2.41	5.30 ± 0.09	4.42 ± 0.1	6.88 ± 0.63	0.23 ± 0.08	0.76 ± 0.16	4.17 ± 0.1	0.32 ± 0.01	319.55 ± 15.67	360.25 ± 25.15
300	-	-	-	5.3	4.5	-	-	1-2	-	-	400	-

Note: in Agua Salud forests, these soil properties (mean ± stand error) were measured before 2015 (pre-fertilization); in Gigante forest, the values were got by personal communication from Joe Wright and Yavitt et al (2011). ‘-’ means no data.

2.2.2 Experimental design

The Gigante fertilization experiment on mature forest at least 300-years-old started in 1997. It consists of four nutrient addition treatments (control, nitrogen, phosphorus, and nitrogen plus phosphorus, respectively) with each replicated four times (1 forest age × 4 treatments × 4 replicates, see Wright et al., 2018 for details). The design of the plots in Agua Salud consists of experimental plots at three different successional stages, a very young secondary forest established immediately after clear-cutting (named “0-year-old forest”), and two middle-age secondary forests (named “10-year-old forest” and “30-year-old forest”, respectively). The fertilization started from 2015 with same nutrient addition treatments as the mature forest but with five replicates per treatment (3 forest ages×4 treatments×5 replicates). In both sites, within each replicate, we blocked the control, nitrogen, phosphorus, and nitrogen plus phosphorus plots within sites on the landscape to minimize the effects of small-scale variations in climate, soils, and tree composition. The minimum distance between plots is 40 m. The fertilization area of every plot is 0.16 ha (40×40 m). In every Agua Salud fertilization plot, trees were monitored only within the inner 0.1 ha leaving a buffer zone on four sides.

Fertilizers were added as coated urea ($(NH_2)_2CO$) and triple superphosphate ($Ca(H_2PO_4)_2 \cdot H_2O$) in nitrogen and phosphorus treated plots, respectively. Annual doses were 125 kg $N \cdot ha^{-1} \cdot yr^{-1}$ and 50 kg $P \cdot ha^{-1} \cdot yr^{-1}$, and these fertilizers were added by hand in four equal doses (15-30 May, 1-15 July, 1-15 September, and 15-30 October, Wright et al., 2011).

We also measured the annual rainfall when the Agua Salud fertilization experiment was established (see the annual rainfall variation in the **Figure A2.2**).

2.2.3 Forest inventory

We monitored all 76 plots since the start of the nutrient fertilization (i.e. 2015 in Agua

Salud forest and 1997 in Gigante forest). All free-standing woody plants (trees, palms, and lianas) within the plots were identified, but the monitoring protocols differed slightly between the two sites. In Agua Salud, in the center 0.1 ha of every plot, all stems of trees and palms with diameters at breast height ≥ 5 cm and all lianas with diameter at breast height ≥ 1 cm were measured, as well as 50% of all tree and palm stems with diameter at breast height between 1 and 5 cm. In Gigante, trees with diameter ≥ 10 cm were measured in the 40×40m plots, and trees with diameter between 1 and 10 cm were measured in the central 20×30m subplots. For the large trees, diameters were measured above any buttresses or other deformities of the lower trunk (Wright et al., 2018).

In Agua Salud, fertilization plots were censused every year between 2015 and 2019, but due to site access issues, trees in one replicate of both 10 and 30-year-old forests were not recorded in 2015. In Gigante, fertilization plots were censused in the year 1997, 1998, 1999, 2000, 2001, 2003, 2008, 2013, and 2018.

All Agua Salud data collected annually between 2015 and 2019 were applied to our analysis of net AGB changes and biomass dynamics in each census interval. For the Gigante experiment, considering the significance of data before nutrient addition, a comparable climatic condition as Agua Salud census data, and to eliminate the effect of census interval on dynamic calculations, we applied the inventory data of the years 1997, 2003, 2008, 2013, and 2018 to our analysis of net AGB changes and biomass dynamics between years 1997 and 2013, and between years 2013 and 2018. We considered the full fertilization period (1997-2013) in order to look at the effect of nutrients over the full length of the experiment. We also considered the most recent census interval (2013-2018) that corresponded most closely to the experiment period of Agua Salud forests (2015-2019) to control for environmental variation such as climate. Unfortunately, we did not have a census interval that corresponded for the exact years as the Agua Salud experiment.

2.2.4 Aboveground biomass assessment

We estimated the above-ground biomass (kg/ha) of all recorded plants in Agua Salud and Gigante plots. Stand-level above-ground biomass was calculated by applying different allometric functions to estimate the above-ground biomass of each liana, tree, and palm. For trees, we estimated the above-ground biomass of each stem using the allometric function of Chave et al. (2014) (see 'The allometric function selection' in **Supporting document**):

$$AGB = \exp [-1.803 - 0.976E + 0.976 \ln (WD) + 2.673 \ln (DBH) - 0.0299 [\ln(DBH)^2].$$

where AGB represents aboveground biomass (kg/ha), E is the local climatic index (Rutishauser et al., 2020), and WD is wood density (g/cm^3). DBH represents the diameter at breast height (cm), the climate index, E=0.05645985 near our study site, stands for the effect of environment on tree height allometry (Rutishauser et al., 2020), and species-specific wood density (in g/cm^3) was estimated from most common species in Agua Salud and Gigante (Rutishauser et al., 2020 and Wright unpublished data).

For lianas, the above-ground biomass of each stem was calculated using a liana allometric equation from Schnitzer et al., 2006 (*see* Lai et al., 2017):

$$AGB = \exp [-0.999 + 2.682 * \ln (DBH)].$$

For palms, we calculated the above-ground biomass using an improved palm-specific allometric equation based on DBH from Goodman et al., 2013 (*see* Rutishauser et al., 2020):

$$AGB = 0.0417565 * (DBH)^{2.7483}.$$

2.2.5 Net change of aboveground biomass and forest dynamic calculations

We first calculated the annual net change of AGB for each census interval ((AGB_2016-AGB_2015, AGB_2017-AGB_2016, AGB_2018-AGB_2017 and AGB_2019-AGB_2018 for Agua Salud plots, and (AGB_2013-AGB_1997)/16 and (AGB_2018-AGB_2013)/5)) for Gigante plots. We further calculated the annual net change of aboveground biomass between before fertilization and every later census ((AGB_2019-AGB_2015)/4, (AGB_2018-AGB_2015)/3, (AGB_2017-AGB_2015)/2, and (AGB_2016-AGB_2015)/1 for Agua Salud plots, and (AGB_2018-AGB_1997)/21 and (AGB_2013-AGB_1997)/16 for Gigante plots.

We calculated stand-level AGB dynamics (growth, recruitment, and mortality) for each census interval in Agua Salud plots (i.e., between 2015 and 2016, between 2016 and 2017, between 2017 and 2018), and for longer intervals in the Gigante plots (i.e., between 1997 and 2003, between 2003 and 2008, between 2008 and 2013, between 2013 and 2018). For Gigante, the average of dynamics between 1997 and 2003, between 2003 and 2008, and between 2008 and 2013 were treated as the dynamics between 1997 and 2013. Growth was calculated as the gains of the trees recorded in the first census year which survived until the second census, divided by the time between the two censuses in years (Δt), e.g., stand-level aboveground biomass growth between the year 2015 and 2016 = (aboveground biomass_2016 – aboveground biomass_2015)/ Δt. Recruitment was calculated as the gains of trees which were recorded in the second census but not in the first, divided by the time between the two censuses in years (Δt). Mortality was calculated from the trees which were recorded in the first year but died in the second census year divided by the period between the two censuses in years (Δt).

2.2.6 Statistical analysis

We used linear mixed-effects models (‘lmer’ function in “lme4” package) to test for the effects of nutrient addition on AGB net change, growth, recruitment, and mortality across all censuses in all forest ages. The mixed-effects models included nitrogen, phosphorus, forest age, census interval, and their interactions as fixed effects, with the block as a random effect. In each model, all fixed effects were treated in categorical way. (see the models and results **Table A2.1** and **A2.2**, respectively).

After that post-hoc tests, using the ‘emmeans’ function from the ‘emmeans’ package in R, were applied to test the effect of nutrient addition on AGB net change and dynamics among forest ages and across censuses. For every model, we used natural or log-transformation of the response variable to meet the model’s assumptions, and we checked the residual and Q-Q plots to evaluate model quality. All these analyses were performed in the RStudio (4.0.2).

2.3 Results

The rate of AGB accumulation was affected by soil nutrient addition (**Figure 2.2 and 2.3,** and **Figure A2.3**), age, and census interval (**Table A2.2**), but the effects varied with forest age. We find the largest effect of nutrient addition early in succession (0-year-old forest) where nitrogen (p<0.0001) has a large positive effect on AGB accumulation. Later in succession, the effect of nutrients weakens or disappears. In the 10-year-old forest, we find an effect of nitrogen on AGB net change (p<0.05), and in the 30-year-old forest only a slight effect of phosphorus (p<0.1) (**Table 2.2**, **Figure 2.3, and Figure A2.3**). No effects were evident in the mature forests. Below we now describe for each forest age, in detail the results of the effects of nutrient treatments on AGB net change and its components (growth, recruitment and mort).

In the 0-year forests, we find that there was a strong nitrogen effect (p<0.0001) on net AGB change (**Figure 2.3**, **Figure A2.3** and **A2.4**, and **Table 2.2**). During the post-fertilization period from 2015-2019, net AGB change in nitrogen treated plots (4.67 ± 0.35 $Mg \cdot ha^{-1} \cdot yr^{-1}$, n=10) was almost twice that in the non-nitrogen treated plots (2.40 ± 0.28 $Mg \cdot ha^{-1} \cdot yr^{-1}$, n=10), and net AGB change in phosphorus treated plots was about 20% higher than that in the non-phosphorus treated plots (**Figure 2.3** and **Figure A2.3** and **A2.4**). Nutrient addition stimulated net AGB change primarily through its effects on recruitment and growth. We find a significant effect of both nitrogen and phosphorus on growth and recruitment (**Table 2.2, Figure A2.4**). Nutrient addition also affected mortality leading to significant mortality increases for all treatments (**Figure 2.3** and **Table 2.2**). The largest increase in mortality was found for the nitrogen plus phosphorus treatment, and smaller still significant increases in the separate treatments (**Figure 2.3**).

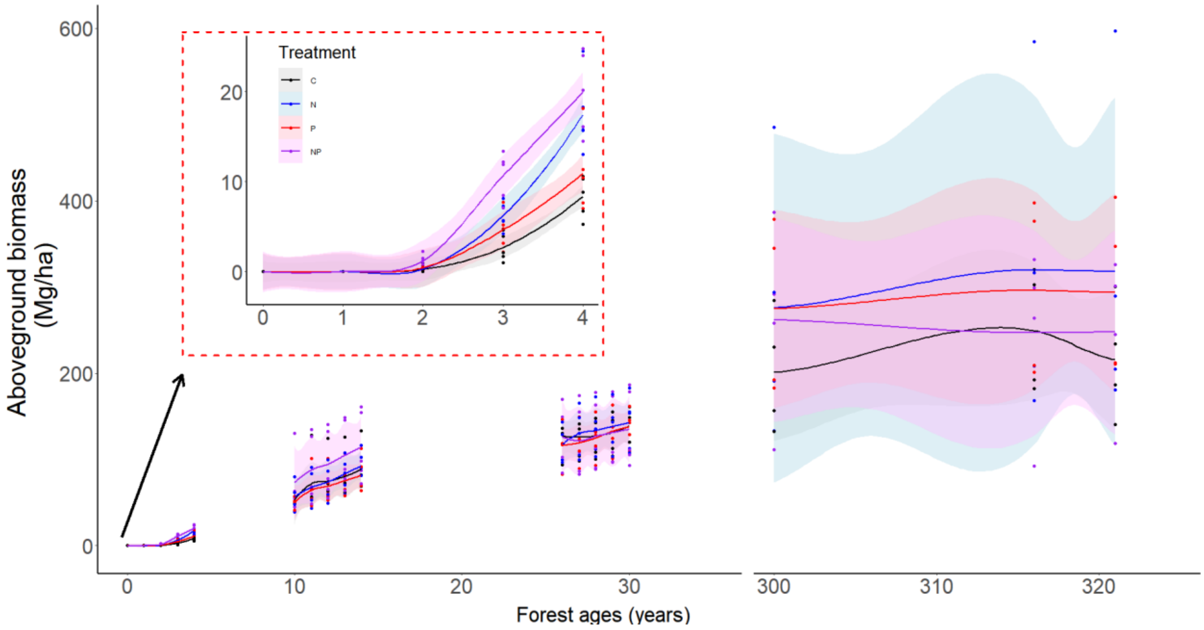

Figure 2.2 | Accumulated aboveground biomass (Mg/ha) and its response to nutrient additions in different stage forests.
Different treatments are represented by different colors. Black dots and lines represent the control treatment (no nutrient addition); blue dots and lines, the nitrogen addition treatment; red dots and lines, the phosphorus addition treatment; purple dots and lines, the nitrogen plus phosphorus treatment. The lines with 95% confidence intervals are fitted using the 'loess' method in RStudio (4.0.2), indicating aboveground biomass over time in each treatment for each forest stage. The black arrow to a red dotted line means the zoom of aboveground biomass change in the newly regenerate forest.

In the 10-year-old forest, we find a strong nitrogen limitation ($p<0.05$) but no phosphorus effect (**Figure 2.3** and **Table 2.2**) on AGB net change. During the fertilization period, the AGB accumulation rate in nitrogen-treated plots (7.83 ± 0.34 Mg·ha^{-1}·yr^{-1}, n=10) was 47.5% higher than in plots without nitrogen addition (5.31 ± 0.62 Mg·ha^{-1}·yr^{-1}, n=10) (**Figure 2.3**). The effect of nitrogen on AGB accumulation rates shifted over the different censuses (**Figure A2.5** and **Table A2.2**). Nitrogen limited AGB accumulation by constraining growth, which increased after nitrogen addition ($p<0.0001$) (**Figure 2.3** and **Table 2.2**).

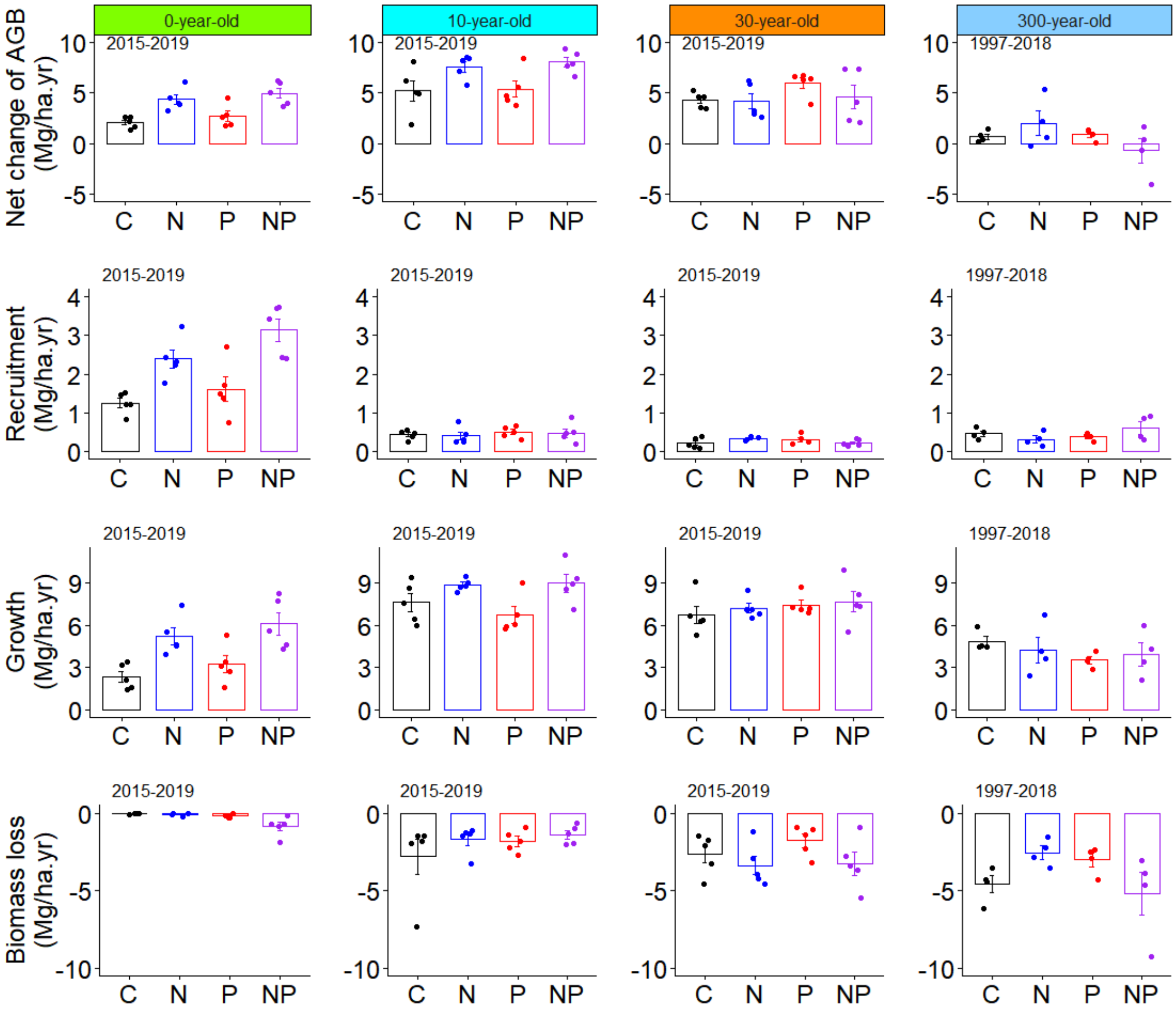

Figure 2.3 | The effect of nutrient addition on aboveground biomass (AGB) and its dynamics (recruitment, growth, and mortality) for each forest across the whole nutrient addition period. Different treatments are represented by different colors. Black columns and dots, control treatment (no nutrient addition); blue columns and dots, nitrogen addition treatment; red columns and dots, phosphorus addition treatment; purple columns and dots, nitrogen plus phosphorus treatment. See the statistical results in **Table 2.1**.

The 30-year-old forest showed weak evidence of phosphorus effect on AGB net change (p<0.1, **Figure 2.3**, **Table 2.2**). During the fertilization period, the net AGB accumulation rate in phosphorus treated plots (5.31±0.64 Mg·ha^{-1}·yr^{-1}, n=10) was 25% higher than in plots without phosphorus addition (4.24±0.40 Mg·ha^{-1}·yr^{-1}, n=10) (**Figure 2.3**). The underlying biomass dynamics of these forests nevertheless showed less clear responses to nutrient additions (**Figure 2.3**, **Table 2.2**). During the fertilization period, phosphorus addition had a weak negative impact on recruitment, with the effect changing across censuses. During our observation period, phosphorus addition appeared to increase growth, but only in a few censuses (**Figure A2.6**), and the growth rate in plots with phosphorus treatment was matched by growth in nitrogen-treated and control plots in later censuses (**Figure A2.6**). Phosphorus addition slightly reduced mortality (17.4%), especially in phosphorus plots, while in nitrogen-treated plots, mortality increased (**Figure 2.3**).

In the 300-year-old forest, we did not find nutrient effect on AGB net change, even after 21 years of nutrient addition (**Figure 2.3**, **Table 2.2**). Whilst the net AGB change in nitrogen plus phosphorus added plots showed some decrease (**Figure 2.2** and **2.3**), this was mainly caused by high mortality in one plot (**Figure 2.3**). During the whole fertilization period, nutrient addition also had no significant effect on growth or mortality, but nutrient, especially phosphorus, did have a positive effect on recruitment (*p*<0.05, **Figure 2.3**, **Table 2.2**). The apparent effect of nutrient addition on forest recruitment changed across census intervals (**Figure A2.7**), with forest recruitment in the second interval increasing following nitrogen and phosphorus addition. This is likely due to the higher mortality in the phosphorus and nitrogen plus phosphorus plots in the first census interval (**Figure A2.7**).

Table 2.2 | Results from post-hoc analysis testing the nutrient effects on response variables across all census intervals.

	0-year-old		10-year-old		30-year-old		300-year-old	
	N	P	N	P	N	P	N	P
Net change of aboveground biomass	<0.0001	n.s.	<0.05	n.s.	n.s.	*P*<0.1	n.s.	n.s.
Recruitment	<0.0001	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05
Growth	<0.0001	<0.1	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.
Mortality	<0.05	<0.001	n.s.	<0.1	n.s.	n.s.	n.s.	n.s.

Note: in this table, ‘N’ signifies the effect of nitrogen addition on the variables (net change of aboveground biomass, recruitment, growth, and mortality); ‘P’ the effect of phosphorus addition on the variables. ‘n.s.’, not significant (*p*>0.1).

2.4 Discussion

Our large-scale ecosystem manipulation experiment indicated that carbon sequestration in recovering tropical forests is limited by soil nutrients. The results support the hypothesis that the type and strength of nutrient limitation on AGB accumulation change over successional time. We found evidence of strong nitrogen limitation in young secondary forests of 0 to 10 years old shifting to some phosphorus limitation in the middle stage, 30-year-old forest, and little evidence for any type of limitation in the mature forest. We found no evidence of co-limitation in any forest ages. The observed effects of nitrogen limitation in the youngest forests are consistent with previous findings indicating impacts of nitrogen availability on forest growth rates across a successional gradient (Batterman et al., 2013; Levy-Varon et al., 2019).

The underlying processes of nutrient limitation in forest succession should, in principle, be similar in other tropical forests undergoing secondary succession. However, in practice, the pattern of nutrient limitation can be affected by many factors, including soil fertility (Du et al., 2020), rainfall (Poorter et al., 2016), atmospheric nitrogen deposition (Reay et al., 2018), land use history (Nagy et al., 2017), species composition (Ter Steege et al., 2006), and light condition (Guariguata and Ostertag, 2001). For instance, in forests with high abundance of nitrogen fixers in the community, phosphorus limitation may exist in young forest at early successional stage, as nitrogen fixers can increase the nitrogen availability in the ecosystem; in high abundance of arbuscular mycorrhizal fungi associated with tree species ecosystem, nitrogen limitation may exist in old forest at late successional stage, as arbuscular mycorrhizal fungi associated with tree species can buffer phosphorus limitation, which shifts ecosystem to nitrogen limitation. Thus, changes in community composition may buffer nutrient limitation over secondary succession. In addition, as the canopy close during the early stage of succession, the forest community may shift from plenty of light and belowground resource limitation to aboveground recourses limitation by light, depending on the level of nutrient supply. Therefore, the patterns of nutrient limitation with forest succession may vary across regions and locations. For example, a new regenerated forest (0-year-old) following clear-cutting and burning in the eastern Amazon, where the soil phosphorus concentration was < 10 mg kg^{-1}, showed phosphorus limitation (Gehring et al., 1999), while a nearby 6-year-old forest, in which the soil phosphorus concentration was about 360 mg kg^{-1}, showed some limitation by nitrogen (Davidson et al., 2004). The soil total phosphorus concentration at our successional sites (200-260 mg kg^{-1}) is close to the median values of total soil phosphorus found in the tropics globally (290 mg kg^{-1}, He et al., 2021). In comparison with the Amazon our values fall between the phosphorus poor eastern Amazon (< 200mg kg^{-1}, Quesada et al., 2010) and the relatively rich western of Amazon forest soils (> 300 mg kg^{-1}, Quesada et al., 2010).

Across different successional stages, forest AGB accumulation was regulated by the responses of the underlying dynamic processes (recruitment, growth, and mortality) to nutrient addition. Forest growth increase in response to nutrient addition was found in all secondary forests, but increases in recruitment were only detected in the newly regenerated forest (0-year-old forest) which recovered from clear-cutting land. This result indicates that the response of recruitment to nutrient addition may be associated with light availability (Hubbell et al., 1999), such that tree recruitment into high light conditions of a recent pasture may be affected by nutrients, while that in a closed-canopy forest may not. In the 0-year-old forest, we saw an increase in mortality with the addition of nutrients, which is likely caused by increases in growth and recruitment resulting in strong competition of trees for light and accelerated forest self-thinning. We did not see an effect of nutrient availability on mortality across the old forest ages, and this may be because mortality is mainly caused by other factors, such as drought and lightning (Gora and Esquivel-Muelbert, 2021).

By contrast, our results do not show clear evidence that nutrient addition affected the net change of AGB of the 300-year-old forest, even after 21 years of fertilization. Similarly, no significant increase in AGB following nutrient addition was detected in most tropical mature forest experiments (Wright, 2019). The lack of a response in net AGB change to nutrient addition in our and other tropical mature forests may be due to four reasons. First, any possible increase in net primary production due to fertilization may not be allocated to stem growth, which contributes most to AGB increase in the forest, but other tree components, such as reproduction, fine roots or leaves. For example, in our research site previous studies found that reproduction in some species and litterfall increased in phosphorus treated plots (Kaspari et al., 2008; Wright et al., 2018; Fortier and Wright, 2021), suggesting greater investment in these tissues. Second, the growth of large trees may be mainly limited by drought (Ryan et al., 2006). Due to hydraulic limitation, some studies indicate old forests decline in productivity and may not be able to respond to nutrient addition (Binkley et al., 2002), even large trees still have strong growth ability (Stephenson et al., 2014). Third, tree mortality in long census intervals may hinder the detection of effects of nutrient addition on net AGB change. This is because the mortality of large trees may lead to an underestimation of the growth rate between two long censuses. In addition, mortality is a highly stochastic process that can drive large changes in stand biomass in small experimental plots. For example, the high recruitment increases in the mature plots with nitrogen and phosphorus addition in the second interval were likely driven by high mortality in these plots in the first interval (see **Figure A2.7**), resulting in forest gaps creation. Finally, our lack response could be due to flat topography relative to our younger forests which may lead to higher nutrient availability (Weintraub et al., 2015). Indeed, the Gigante sites have higher total phosphorus (400 mg/kg) compare to the young forest sites (less than 300 mg/kg, *see* **Table 2.1**).

Although our results provide little direct support for the hypothesis that the slowdown of biomass productivity and the increased mortality in Pan-Amazon mature forests is caused by nutrient limitation (Hedin, 2015), it does suggest that there may be nutrient limitation following small-scale disturbances within mature forests such as following canopy gap formation. Collectively, nutrient limitation on rapid carbon recovery in mature forest gaps may result in nutrient limitation on the mature forest carbon sink, even if it is heterogeneously distributed. Our experimental plots were not large enough to capture these gap dynamics and the emergent consequence for the mature forest carbon cycle, but this should be explored further in the future. Forest dynamics may also be affected by other factors, such as disturbance frequency and size, tree size, species composition, climate, and herbivory, pest or pathogen load (McDowell et al., 2020). Therefore, to determine which factor dominates the decrease of carbon sink in Amazon mature forests, more in situ work is required to disentangle the relationship between soil fertility and dynamics.

Our experimental results showed that soil nutrients facilitate secondary forest recovery and AGB accumulation. Our findings may help guide policymakers as they seek policies to manage and restore tropical secondary forests as a natural carbon solution to tackle global warming (Lewis et al., 2019; Girardin et al., 2021). Many global reforestation schemes, such as Bonn Challenge, the Convention on Biological Diversity Aichi Targets, and the New York Declaration on Forest have been launched. When policymakers are considering where to restore the forests and how to recover the land, such as by natural recovery or plantation, taking nutrient limitations into account will help forests capture more carbon in a limited time. Most broadly, findings from our large-scale ecosystem manipulation experiment reveal the fundamental role of nutrients in constraining the tropical carbon sink in forests recovering from disturbance.

Chapter 3

Tropical forests adjust above- and belowground carbon allocation to address nutrient limitation

Contributions:

Wenguang Tang: lead the discussion, designed and did the field work, analysed samples, made the figures, analysed the data and wrote the draft,

Oliver L. Phillips and **Roel J.W. Brienen**: designed the field work, discussed the results and wrote the draft,

Jefferson S. Hall, **S. Joseph Wright**, and **Michiel van Breugel**: designed and monitored the fertilization plots,

Michelle Wong: provided feedback on the manuscripts

Sarah A. Batterman: designed the field work, designed the fertilization plots, discussed the results, and wrote the draft.

Abstract

The carbon sink in tropical primary and secondary forests may be constrained by soil nutrients, and theory suggests trees can adjust above- and belowground carbon allocation to address nutrient limitation. Yet, there has been little investigation into whether forests change their allocation of carbon in response to nutrient limitation over the course of vegetation succession. To address this knowledge gap, we established a nutrient fertilisation experiment across a full successional gradient in central Panama, including naturally regenerating forests of 0, 10, and 30 years old, and a mature forest. We analysed the effects of nutrient addition on fine root biomass and the ratio of fine root to aboveground biomass in the different aged forests. We found a decline in fine root biomass and fine root biomass allocation in response to nutrient

fertilisation, and markedly different responses to nitrogen and phosphorus addition with plot age. For the earliest successional stages (0 and 10 year old vegetation), nitrogen addition affected patterns of fine root biomass or allocation, while for the middle stage (30 year old forest) phosphorus had a detectable effect, and for the late stage (mature forest) no effect of nutrient addition on fine root biomass and allocation change was detected. These results demonstrate that tropical forests can adjust above- and belowground carbon investments in response to nutrient limitation. Adjusting carbon allocation to support nutrient uptake may constitute a mechanism not only to mitigate shifting nutrient limitations during succession, but also to address the challenge of increasing nutrient limitation as atmospheric carbon dioxide becomes more plentiful.

3.1 Introduction

Tropical primary forest growth and secondary forest recovery contribute around one-third of the terrestrial carbon sink (Pan et al., 2011) and play an important role in slowing global warming (Griscom et al., 2017, Lewis et al., 2019). Tropical trees need to invest carbon belowground to take up both water and nutrients – especially nitrogen and phosphorus – from soils. It has been suggested that a shortage of soil nutrients can constrain the carbon sink potential of tropical forests (Wright, 2019, Tang et al., Chapter 2), but to address key uncertainties about how these constraints may operate we need to understand better where, when and how trees are able to adjust carbon allocation to above vs. belowground tissue in order to take up limiting nutrients and thereby overcome nutrient limitation. An ability to adjust carbon allocation may be especially important in forests recovering from disturbance, and as potential growth rates – and therefore nutrient demand – of trees in both secondary and mature forests increase with rising atmospheric CO_2. Allocation to belowground carbon may also shift in response to the changing patterns of nutrient limitation over the course of forest succession (Tang et al., Chapter 2).

There are two alternative hypotheses concerning above- and belowground carbon allocation responses to nutrient limitation (**Figure 3.1**). The first hypothesis suggests that trees can adjust carbon allocation above- and belowground to address nutrient limitation (Hypothesis 1 in **Figure 3.1**; Bloom et al., 1985, Hermans et al., 2006, Poorter et al., 2012, Oldroyd and Leyser, 2020). When nutrients are limiting, trees will allocate more carbon belowground to increase fine root biomass and increase the absorption of limiting nutrients; when nutrients are abundant, trees will allocate more carbon aboveground to enhance space and light competition.

This hypothesis is supported by some nutrient manipulation experiments both in the field and greenhouse, which found that fine root biomass decreased and/or aboveground biomass increased following limiting nutrient addition (Yuan and Chen, 2012, Wright, 2019, Freschet et al., 2021). However, it is also possible (Hypothesis 2 in **Figure 3.1**) that trees cannot adjust carbon allocation above- and belowground to address nutrient limitation (Hungate et al., 2003). Trees may increase fine root biomass, but that would increase in step with aboveground biomass change along forest succession. This hypothesis is supported by natural observations across mature forests in the Amazon which find that the allocation of net primary production to below- and aboveground has no clear relationship with soil fertility (Aragao et al., 2009), and by some nutrient addition experiments which found fine root biomass increased or had no change following nutrient addition (Wright, 2019). Finally, a secondary succession gradient is an ideal place to test whether plant can adjust carbon allocation since the type and strength of nutrient limitation may change along forest succession. Therefore, it is still unclear (1) whether and when tropical trees adjust belowground fine root carbon allocation to address nutrient limitation, if so (2) how carbon allocation changes across a secondary succession gradient, where nutrient limitation patterns shift and aboveground biomass changes (Tang., Chapter 2).

Nutrient manipulation experiments that span successional gradients in tropical forests provide a helpful way to separate out the effects of nutrient limitation and allometric variation on changes in fine root biomass and belowground carbon allocation. To test the below- and aboveground carbon allocation hypotheses and answer the above questions, we conducted a factorial nitrogen and phosphorus manipulation experiment across a tropical forest succession gradient in Panama. In these forests nutrient limitation shifts from strong nitrogen limitation with some phosphorus limitation in young secondary forests, to phosphorus limitation in the middle stage forest, to little indication of nutrient limitation in the mature forest (Tang et al., Chapter 2). We assessed how fine root biomass and the ratio of fine root to aboveground biomass changes across a successional gradient following four years of nutrient addition in the young forests and 21 years of nutrient addition in the mature forest.

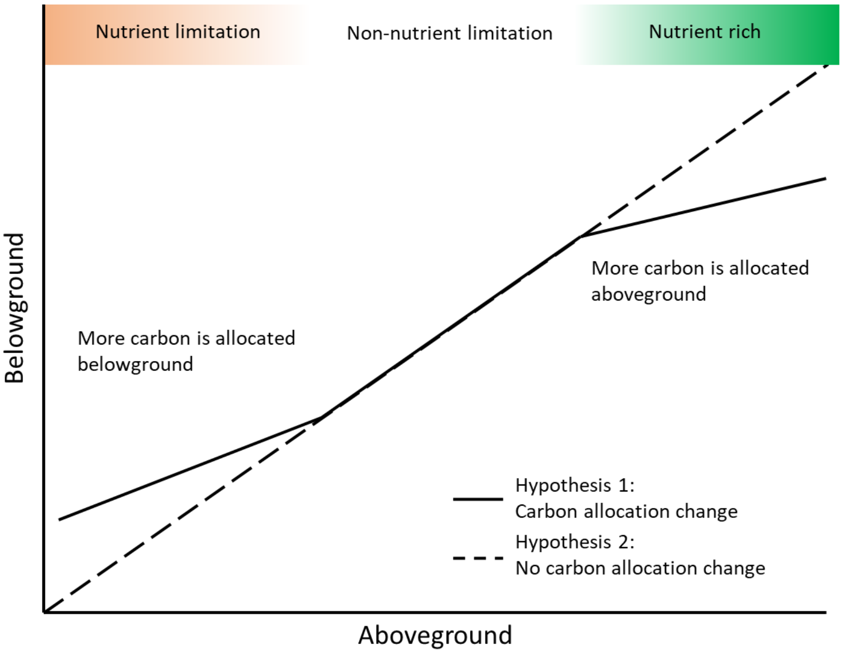

Figure 3.1 | Conceptual model of carbon allocation above- and belowground change in response to nutrient addition.

The solid black line stands for hypothesis 1 which suggests trees change carbon allocation above- and belowground to address nutrient limitation. When nutrients are limiting, more carbon is allocated belowground to increase fine root biomass; when nutrients are abundant, more carbon is allocated aboveground to increase aboveground biomass. The dash black line represents hypothesis 2 which suggests that trees cannot adjust carbon allocation to address nutrient limitation.

3.2 Methods

3.2.1 Research site

We established a factorial nitrogen and phosphorus fertilization experiment spanning a successional gradient of lowland tropical moist forest in Agua Salud (9°13’N, 79°47’W, 330 meters above sea level) and Gigante (9°06’31’’N, 79°50’37’’W, 60 meter above sea level) areas in central Panama. These two research sites are located closely within the Panama Canal Watershed. In Agua Salud, hills with small steep slopes and small streams dominate, and the landscape consists of cattle pastures, plantations, fallows, and different-aged secondary forests which naturally recovered after abandonment following cattle ranching and clear-cutting (Bretfeld et al., 2018). The topography in Gigante is characterized by flat terrain with some small streams, and the land is covered by well-protected mature forest (> 300 years old) (Yavitt et al., 2011).

Both sites receive around 2700 mm rainfall on average per year, about 90% of which falls during a marked wet season from early May to middle of December (Ogden et al., 2013). The forests grow upon infertile, highly weathered, and drained soils which are classified as Oxisols (Turner and Wright, 2014; Lai et al., 2017).

3.2.2 Experimental design

Our nutrient manipulation experiment includes four age classes of forests. We selected three different age forests in Agua Salud – a newly regenerating forest (our '0-year-old forest'), a young secondary forest ('10-year-old forest'), and a middle stage forest ('30-year-old forest') – as well as a mature forest in Gigante ('300-year-old forest'). In each forest age, the tree species composition is similar across plots. All forests have the same nutrient addition treatments: a control treatment, a nitrogen fertilization treatment, a phosphorus fertilization treatment, and a nitrogen plus phosphorus fertilization treatment. Each treatment in the Agua Salud forests are replicated five times (3 forest ages × 4 treatments × 5 replicates) and treatments in the Gigante forest are replicated four times (1 forest age × 4 treatments × 4 replicates). To minimize the effects of micro-climate and soil properties on our results, in every forest, four different treatment plots were set closely together as a block. The minimum distance among plots is 40 m. The experimental setup is further described in Tang et al. (Chapter 2).

We established the Gigante experiment in 1997 and the Agua Salud fertilization experiment in 2015. Twenty kg nitrogen as coated urea ($(NH_2)_2CO$) and 8 kg phosphorus as triple superphosphate ($Ca(H_2PO_4)_2.H_2O$) were hand-applied to every 0.16 ha (40 × 40 m) nitrogen and phosphorus treated plot, respectively, every year in four equal doses (late May, early July, early September, and late October, Wright et al., 2011). The nitrogen plus phosphorus treated plots received both nutrients in the same amount.

For the details of plot monitoring, please see the second chapter.

3.3.3 Fine root biomass assessments

To minimize the effect of fine root turnover, in the middle of the rainy season (early August to middle September) of 2019, we collected fine root samples and assessed fine root biomass in each plot. For each treatment, we randomly selected four replicates in Agua Salud forests and four replicates in the Gigante forest. In each of the 64 plots (four treatments × four replicates × four age forests), five soil cores (6 cm in diameter to 10 cm depth) were sampled in the inner 25 × 25 m subplot and were mixed into one sample (Yavitt et al., 2011). Soil samples were stored in a 4 ^{o}C fridge and were processed within 48 hours of collection.

Live fine roots <2 mm were manually removed by hand from soil samples (see 'Time efficiency in searching fine roots in tropical forests' **Supporting document** for detailed methods), washed, dried at 65 °C for more than 72 hours and weighed for dry biomass. We took the total weights of the 5 cores per plot to scale up fine root biomass to the plot and hectare scales.

3.3.4 Ratio of belowground to aboveground biomass estimation

We calculated the aboveground biomass in our 64 focal plots, using our latest tree census data (in 2019 for Agua Salud plots and 2018 for Gigante plots). The aboveground biomass of each tree was estimated using a global allometric function incorporating the species-specific wood densities collected in our research sites (Rutishauser et al., 2020 and Wright unpublished data): aboveground biomass = exp[-1.803 - 0.976E + 0.976 ln (wood density) + 2.673 ln (diameter at breast height) – 0.0299[ln(diameter at breast height)$^{\wedge 2}$] (Chave et al., 2014, see Tang et al, Chapter 2). The aboveground biomass of each palm was estimated using a palm-specific allometric equation based on diameter at breast height: aboveground biomass = 0.0417565 * (diameter at breast height)$^{\wedge 2.7483}$ (Goodman et al., 2013, see Rutishauser et al., 2020). The aboveground biomass of lianas was calculated using a liana-specific allometric function: aboveground biomass = exp[-0.999 + 2.682 * ln (diameter at breast height)] (Schnitzer et al., 2006, see Lai et al., 2017).

Aboveground biomass was calculated at the stem scale and scaled up to the plot and hectare. We calculated the ratio of fine root biomass to aboveground biomass in each plot.

3.3.5 Statistical analysis

First, we used mixed-effects models to test the effects of nutrient addition on fine root biomass and the ratio of fine root biomass to aboveground biomass across all forests (Zuur et al., 2009). The mixed-effects models included nitrogen, phosphorus, forest age, and their interactions as fixed effects, with the block as a random effect. In each model, we treated all fixed effects in categorical way. Post-hoc tests, applying the 'emmeans' function from the 'emmeans' package in R, were used to test the effect of nutrient addition on changes in fine root biomass and the ratio of fine root biomass to aboveground in each forest.

For every model, log-transformation or non-transformation of the response variables was conducted to meet the model's assumptions of normality, and residual and Q-Q plots were used to evaluate the model's quality. All analyses were performed in RStudio (4.0.2).

3.4 Results

Fine root biomass increased with forest age ($p<0.05$, **Figure 3.2** and **Table A3.1**), while the ratio of fine root biomass to aboveground biomass decreased with forest age ($p<0.0001$, **Figure 3.3** and **Table A3.1**). Across forest succession, the effects of nutrient addition on fine root biomass and the ratio of fine root biomass to aboveground biomass shifted (**Figures 3.2** and **3.3** and **Table A3.1**). In the 0-year-old forest, nitrogen addition decreased both fine root biomass ($p<0.05$, **Figure 3.2** and **Table 3.1**) and the ratio of fine root biomass to aboveground biomass ($p<0.0001$, **Figure 3.3** and **Table 3.1**). In the 10-year-old forest, nitrogen, not phosphorus, addition decreased fine root biomass ($p<0.05$, **Figure 3.2** and **Table 3.1**) and the ratio of fine root biomass to aboveground biomass ($p<0.05$, **Figure 3.3** and **Table 3.1**). In the 30-year-old forest, phosphorus, not nitrogen, addition decreased fine root biomass ($p<0.05$, **Figure 3.2** and **Table 3.1**) and the ratio of fine root biomass to aboveground biomass ($p<0.05$, **Figure 3.3** and **Table 3.1**). In the 300-year-old forest, nutrient addition had no significant effect on changes in fine root biomass and the ratio of fine root biomass to aboveground biomass (**Figure 3.3**).

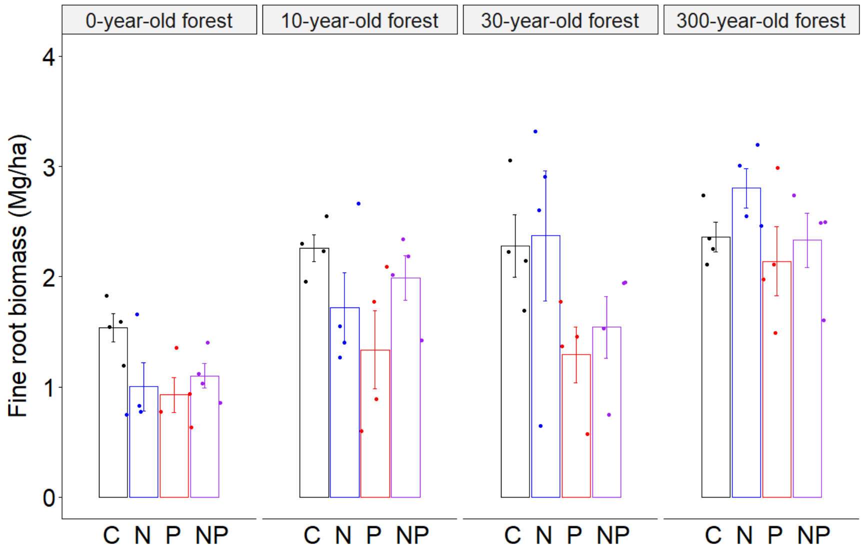

Figure 3.2 | The responses of fine root biomass (Mg/ha) to nutrient addition over tropical forest secondary succession.

Bars represent the mean (+/- standard error) fine root biomass across plots that are either treated as a control (C, black), or with nitrogen (N, blue), phosphorus (P, red) or nitrogen plus phosphorus (NP, purple). Points represent the fine root biomass for each plot. N=4 for each treatment in each forest age. See the statistical results in the **Table 3.1** and **Table A3.1**.

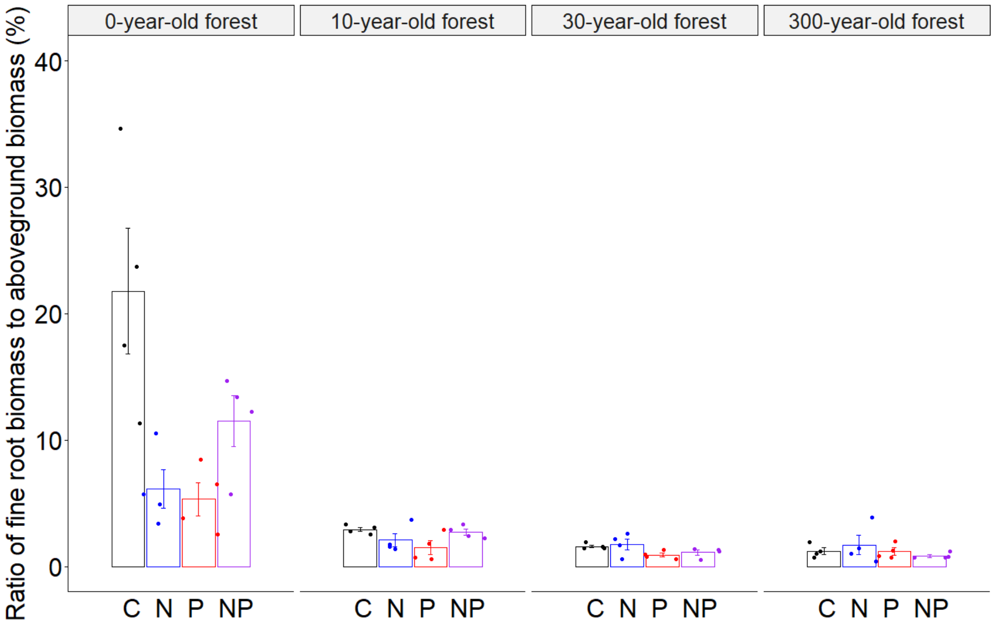

Figure 3.3 | The responses of the ratio of fine root biomass to aboveground biomass to nutrient addition over tropical forest secondary succession.

In the figure, bars represent the mean (+/- standard error) ratio of fine root biomass to aboveground biomass across plots that are either treated as a control (C, black), or with nitrogen (N, blue), phosphorus (P, red) or nitrogen plus phosphorus (NP, purple). Points represent the ratio for individual plots. N=4 for each treatment in each forest age. See the statistical results in the **Table 3.1** and **Table A3.1**.

Table 3.1 | The statistical results of the effects of nutrient addition on fine root biomass and the ratio of fine root biomass to aboveground biomass in each forest age.

	Variables	Forest ages			
		0-year-old forest	10-year-old forest	30-year-old forest	300-year-old forest
Fine root biomass	factor(N)	<0.05	<0.05	n.s.	n.s.
	factor(P)	n.s.	<0.1	<0.05	n.s.
The ratio of fine root biomass to aboveground biomass	factor(N)	<0.0001	<0.05	n.s.	n.s.
	factor(P)	<0.1	n.s.	<0.05	n.s.

Note: in this table, N means nitrogen addition treatment; P means phosphorus addition treatment; n.s. stands for no significant difference (*p*>0.1).

3.5 Discussion

Our study examines the flexibility of belowground (mainly fine roots) carbon allocation by assessing the standing fine root biomass and its changes relative to aboveground biomass across nutrient treatments and over successional time. Although we did not measure fine root autotrophic respiration, which also accounts for part of belowground carbon allocation (Litton et al., 2007), elsewhere they are closely correlated (Feng and Zhu, 2019), so we assume that the fine root autotrophic respiration responds similarly to fine root biomass in response to limiting nutrient addition. This is supported by experimental evidence, for example in a nitrogen addition experiment in a southern Chinese tropical forest, fine root biomass and autotrophic respiration together declined following nitrogen addition (Mo et al., 2008). In addition, we did not measure carbon allocation to coarse roots as well, because coarse roots contribute a small partition of carbon allocation (Chen et al., 2004; Litton et al., 2007) which is difficult to determine. Therefore, we here interpret fine root biomass changes to represent change in belowground carbon allocation in response to nutrient addition.

Our results support the Hypothesis 1 that trees adjust above- and belowground carbon allocation and change fine root biomass to address nutrient limitation. We find that less carbon is invested to fine roots in response to the addition of the limiting nutrient. In addition, the pattern of belowground carbon investment in response to nutrient addition shifts with nutrient limitation across forest succession. In the nitrogen limited forests at early successional stages, nitrogen addition decreased belowground carbon allocation; and, in the 30-year-old forests even through, we did not find strong support for the idea that AGB net change is limited by phosphorus (Chapter 2), phosphorus addition decreased belowground carbon allocation (**Table 3.1**), suggesting that the trees adjust carbon allocation to address and almost overcome phosphorus limitation. With belowground carbon investment decreased, more carbon can be invested to aboveground and accelerate the accumulation of aboveground biomass (Tang et al., Chapter 2). These results indicate that trees can adjust belowground carbon allocation as a strategy to address nutrient limitation and accelerate biomass accumulation across forest succession. Thus, we find no support of Hypothesis 2 that trees do not adjust nutrient addition to address nutrient limitation.

Our results are inconsistent with some results from fertilization experiments in secondary forests which found that the fine root biomass increased or had no change following the addition of the limiting nutrient (Zhu et al., 2013; Wright, 2019). This difference may be because the belowground carbon allocation and fine root biomass in response to nutrient availability are controlled by two mechanisms. First, following the addition of the limiting nutrient, trees need less investment in carbon belowground to meet their nutrient requirements, so fine root biomass will decrease. Second, increased nutrient availability may increase net primary production allowing more investment in belowground carbon, which would result in increased fine root biomass. When the second mechanism overwhelms the first mechanism, the fine root biomass will increase following limiting nutrient addition, even if trees decrease relative carbon allocation to belowground.

The mature forest also showed some above- and belowground carbon allocation changes in response to nutrient addition. In our mature forest, the ratio of fine root biomass to aboveground biomass showed some decrease following phosphorus addition (**Figure 3.3**), and the carbon was allocated aboveground to increase reproduction (Kaspari et al., 2008; Fortier and Wright, 2021). These results indicate that the carbon sink in our mature forest may be phosphorus limited. However, the effect of phosphorus addition on fine root biomass change is limited (**Figure 3.2**), the aboveground biomass did not have any evident difference between phosphorus treated and other plots (Chapter 2). This lack of evident responses could be caused by two reasons. First, phosphorus limitation in the mature forest is not evident (Tang et al., Chapter 2), and carbon allocation change between fine roots and reproduction can successfully address the light nutrient limitation, so the aboveground biomass did not have evident changes following phosphorus addition. Second, the light phosphorus limitation may be eliminated with increased nitrogen input, which could support higher photosynthetic rates (Liang et al., 2020) and, in turn, an increased carbon allocation belowground and to fine root biomass (**Figure 3.2**). This would allow trees to absorb more phosphorus which bonds to soil tightly (Pregitzer et al., 1995, Norby et al., 2004, Drake et al., 2011). This nutrient trading strategy may successfully address light phosphorus limitation in our mature forest, as the Panamanian mature forest may be nitrogen rich due to a legacy of nitrogen fixation (Batterman et al., 2013) and anthropogenic nitrogen deposition (Hietz et al., 2011).

Our research demonstrates that tropical forests adjust below- and aboveground carbon and biomass to address nutrient limitations over the course of vegetation succession. These results, firstly, help to improve the estimation of biomass accumulation rate with succession. Most studies estimated biomass accumulation rates with succession by only focusing on changes in aboveground biomass (Poorter et al., 2016; Poorter et al., 2021; Tang et al., Chapter 2) but ignore belowground biomass. If one uses a fixed ratio of below to above-ground biomass, then these studies may underestimate total biomass accumulation during early successional stages, as our results show that forests in younger stages invest more in belowground fine root biomass relative to aboveground biomass than forests in late stages (**Figure 3.3**). In Panamanian lowland tropical forests, fine root biomass comprises about 20% of aboveground biomass in newly regenerated forests (4-year-old forests) but only about 1% of aboveground biomass in mature forests. In addition, our results suggest that plasticity of above- and belowground biomass allocation over time and in response to nutrient limitation should both be considered within model structures when simulating carbon stocks of forests growing across different soil fertilities. This may markedly change the ratio of aboveground to belowground biomass across soil fertilities but has been usually ignored in both models and empirical studies (Hungate et al., 2003; Poorter et al., 2016; Poorter et al., 2021). In sum, to improve the estimation of tropical forest biomass in both observational and modelling studies, the changes in above- vs belowground biomass along succession and across soil fertility should be taken into account.

Our study also has implications for improving the prediction of the future carbon sink in tropical forests, especially as atmospheric CO_2 concentrations rise. With CO_2 fertilization, the carbon sink in tropical primary forests is predicted to continue for decades more (Cox et al., 2013; Huntingford et al., 2013), a model outcome that may be at odds with the observational finding that the net sink in some structurally-intact forests has been decreasing, in part because of an apparent saturation of growth rates (Brienen et al., 2015). The decreasing carbon sink in mature forests may thus be partly caused by nutrient limitation (Hedin, 2015), and many studies project that nutrient limitation on tropical primary forest carbon sink will intensify in the future (Fisher et al., 2012; Wieder et al., 2015; Fleischer et al., 2019; Wright, 2019; Du et al., 2020). However, our findings suggest that nutrient limitation on mature forest biomass gains are in fact very little (Chapter 2) and can be successfully addressed by trees adjustments in carbon allocation. This is consistent with the recent observational finding that in many African tropical mature forests growth has continued to increase long-term (Hubau et al. 2020), and experimental insight that growth of some mature forests is unresponsive to nutrient addition (Wright, 2019). Therefore, if tropical trees adjust belowground carbon allocation to address increasing nutrient limitation this century as our results suggest, we can expect greater inputs into below-ground biomass. The impact could be substantial, given that currently around 30% of net primary production is allocated belowground (Jackson et al., 1997, Malhi et al., 2011). Whether such a shift would also increase soil carbon storage is unclear given the countervailing expectations of enhanced respiration due to soil warming (Nottingham et al., 2020). Nevertheless, the potential changes in tree carbon allocation to address nutrient limitation raise the strong possibility of widely enhanced tropical carbon sequestration below-ground.

Chapter 4

Tropical forests adjust nutrient stoichiometry to address changing nutrient limitation over secondary succession

Contributions:

Wenguang Tang: lead the discussion, designed and did the field work, analysed samples, drew figures, analysed the data and wrote the draft,

Oliver L. Phillips and **Roel J.W. Brienen**: designed the field work, discussed the results and wrote the draft,

Jefferson S. Hall, **S. Joseph Wright**, and **Michiel van Breugel**: designed and monitored the fertilization plots,

Michelle Wong: discussed the results and wrote the draft

Sarah A. Batterman: designed the field work, designed the fertilization plots, discussed the results, and wrote the draft.

Abstract

Tropical forests play an important role in slowing global warming, contributing approximately one-third of the terrestrial carbon sink. While there is evidence that this may be limited by soil nutrients, it remains unclear if trees can adjust nutrient concentration and allocation to address limitations, and, if so, how changes in nutrient concentrations in tissues impact forest growth. These responses may be especially important over the course of secondary succession, since both nutrient limitation and net carbon accumulation change greatly with succession. To address these gaps, we used a large-scale ecosystem nutrient manipulation experiment in Panama in which we have found marked shifts in nutrient limitation on growth over a successional gradient from new to mature tropical forest stands. Here, we assess the long-term impact of nutrient addition on nutrient ratios and total nutrient allocation to tissues. We also evaluate the relationships between tissue nutrient concentrations

and forest growth rates over succession. We find that plant nutrient composition and allocation both change in response to nutrient addition, and that these responses in turn change with forest succession. The ratio of carbon to nitrogen declined following nitrogen addition in nitrogen-limited forests, and the ratio of carbon to phosphorus declined following phosphorus addition in all forests. In each forest, with the addition of the limiting nutrient, more nutrients were allocated from leaves and fine roots to wood. The changes in both nitrogen and phosphorus concentrations are not related to forest growth changes. These results demonstrate that tropical forests dynamically adjust nutrient stoichiometry to address ecosystem nutrient limitation, but these stoichiometric adjustments were not alone sufficient to influence growth. Taking account of these dynamic responses to the nutrient environment in global terrestrial models may help improve prediction of the future evolution of the carbon sink in tropical forests.

4.1 Introduction

Tropical mature and secondary forests combined contribute about one-third of its terrestrial sink (Pan et al., 2011; Mitchard, 2018), playing an important role in slowing the rate of growth in atmospheric CO_2 and global warming. This substantial carbon sink in tropical forests may be partly limited by soil nutrients (Wright, 2019; Tang et al., Chapter 2), especially nitrogen and phosphorus. In addition, patterns of limitation on carbon sequestration for both these nutrients shift over the course of forest succession (Tang et al., Chapter 2). Global vegetation models suggest that net primary production in tropical forests will not increase as strongly in response to rising CO_2 in the future as previously predicted because of limitation by nutrients (Fisher et al., 2012; Wieder et al., 2015; Fleischer et al, 2019). To address nutrient limitation, we have found that trees can invest more carbon belowground and increase fine root biomass, which is the key tissue for taking up nutrients from the soil (Tang et al., Chapter 3), to increase nutrient absorption. Yet, it is still unclear whether forests can adjust a set of strategies relating to nutrient stoichiometry to address nutrient limitation, including adjusting nutrient composition and allocation of nitrogen and phosphorus to different tissues.

There are two pairs of alternative hypotheses proposing to explain how trees may strategically utilize nutrients in tissues to address nutrient limitation (**Figure 4.1**). In the first pair of hypotheses, the first hypothesis (**Figure 4.1A**) proposes that plants hold elemental compositions in tissues constant across gradients in nutrient availability (stoichiometric homeostasis, Sterner and Elser, 2017). On the one hand, when trees are limited by nutrients, trees may apply some strategies, such as increasing belowground carbon allocation to absorb nutrients (Tang et al., Chapter 3; Hermans et al., 2006) and increasing nutrient resorption (Reed et al., 2012), to keep nutrient stable in tissues; on the other hand, when the nutrient is rich in soil, trees cannot continue to absorb nutrients in tissues. The second hypothesis (**Figure 4.1A**) holds that nutrient compositions are plastic in response to nutrient availability (stoichiometric flexibility; Agren, 2008; Elser et al., 2010). When soil nutrients are limiting, trees can increase the ratios of carbon to nitrogen and/or phosphorus. When nutrients are rich, trees decrease the ratios of carbon to nitrogen and/or phosphorus. This would allow trees to have higher photosynthetic rates and/or nutrient reserves in case of future nutrient hardships. Fertilization experiments in tropical forests found that nutrient concentrations in tissues (especially leaves and fine roots) can both increase or have no change in response to nutrient addition (Wright, 2019). In the second pair of hypotheses, the third hypothesis (**Figure 4.1B**) suggests that trees adjust nutrient allocation between tissues to maximize resource capture (light versus nutrients or water) (Chapin III et al., 1990; Dybzinski et al., 2015). When tree growth is nutrient limited, trees will allocate more nutrients to metabolic tissues (i.e. leaves and fine roots); while when nutrient availability is high, trees may store nutrients in some tissues, such as wood (Chapin III et al., 1990). This strategy would allow trees to hold constant nutrient compositions in tissue (first hypothesis in **Figure 4.1A**) or could be done in concert with shifting nutrient compositions (second hypothesis in **Figure 4.1A**). The fourth hypothesis (**Figure 4.1B**), in contrast, suggests that trees have less ability to adjust nutrient allocation among tissues to address nutrient limitation (Hungate et al., 2003; Kerkhoff et al., 2006; Agren et al., 2008; Heineman et al., 2016).

The type of strategy that tropical trees utilize will likely impact tree growth, as both nitrogen and phosphorus are key nutrients in regulating photosynthesis, protein synthesis, cell growth, and metabolism (Chapin III et al., 2011; Reich et al., 2009; Walker et al., 2014). The growth of trees that use inflexible strategies may be more restricted than trees that use flexible strategies as nutrient availability decreases or increases. We may expect higher leaf nitrogen concentrations to increase forest growth through an increase in photosynthetic apparatus and therefore carbon assimilation. We may also expect higher phosphorus in tissues to increase forest growth, as foliar phosphorus has positive relationship with annual net primary production (Cleveland et al., 2011). Previously, natural observations (Townsend et al., 2008; Fyllas et al. 2017; Cleveland et al., 2011; Heineman et al., 2016) and nutrient addition experiments (Wright, 2019, Waring et al., 2019) have found that nutrient compositions in plant tissues vary with soil nutrient availability, with nutrient concentrations remaining constant or increasing with nutrient availability. In addition, forest growth rates increase or do not change in high fertile soils (Wright, 2019). However, no study has examined nutrient composition and allocation to leaves, fine roots and wood change in response to nutrient variations across an entire tropical forest successional gradient. Furthermore, whether or not any changes in nutrient concentrations in response to nutrient availability influence growth would impact how we conceptualize and model the impact of the nutrient use strategy. It therefore remains unclear (1) whether tropical forest trees can adjust nutrient compositions in tissues and nutrient allocation among tissues to address nutrient limitation across a successional gradient; and, (2) if changes in tissues nutrient concentration are associated with tree growth.

To answer these questions and test the nutrient use and allocation hypotheses described above, we used our factorial nitrogen and phosphorus nutrient addition experiment that spans a tropical forest successional gradient in Panama. Across this gradient, we have already found that the pattern of nutrient limitation on carbon accumulation shifts from strong nitrogen limitation in young secondary forests to some phosphorus limitation in the intermediate stage forest, and finally to no evidence of nutrient limitation in late-successional forest (Tang et al., Chapter 2). Furthermore, our experiment represents a gradient in net carbon uptake into biomass – from very high rates in the youngest forests to low rates in the mature forest. Thus, it offers a valuable setting to test whether trees use a strategy of adjusting nutrient use and allocation to address nutrient limitation of net carbon uptake by forests.

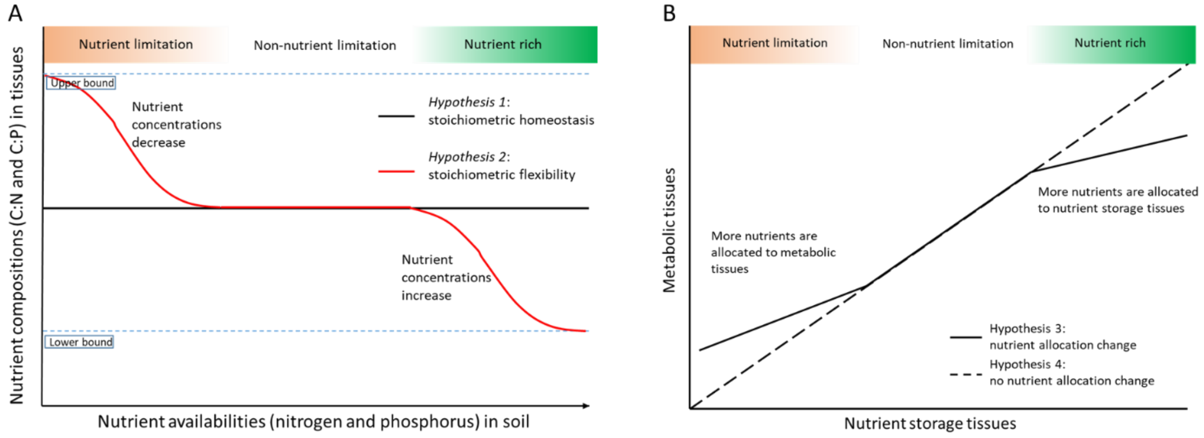

Figure 4.1 | Conceptual models of two pairs of contrasting hypotheses for how trees adjust nutrient concentration (A) and allocation to tissues (B) to address nutrient limitation.

Hypothesis 1 (in **A**): stoichiometric homeostasis hypothesis. This suggests that the ratios between carbon, nitrogen and phosphorus will not change with soil nutrient availabilities, because nutrient concentrations in tissues will hold constant with nutrient availability varies. Hypothesis 2 (in **A**): stoichiometric flexibility hypothesis. This hypothesis suggests that plants flexibly adjust their nutrient compositions as nutrient availability changes. If Hypothesis 2 pertains over at least some of the nutrient availability space, then when nutrients limit forest growth, the ratios of carbon to nitrogen (C: N in the figure) and carbon to phosphorus (C:P in the figure) increase; when nutrients are rich, the ratios of carbon to nitrogen and carbon to phosphorus decrease. It is also possible that these Hypothesis 1 and Hypothesis 2 combine, if trees can adjust carbon allocation to tissues to address nutrient limitation (Hypothesis 3, **B**), so that homeostasis dominates for some of the nutrient availability space, but that stoichiometry varies flexibly at higher and lower extremes if availability. Hypothesis 3 (nutrient allocation change) suggests that, if trees are limited by soil nutrients, more nutrients will be allocated to metabolic tissues (like leaves and fine roots) from storage tissues (such as wood), *vice versa*. Hypothesis 4 (**B**) suggests that trees cannot adjust nutrient allocation to address nutrient limitation.

4.2 Methods

4.2.1 Research site

The factorial nitrogen and phosphorus fertilization experiment is established in Agua Salud (9^{o}13’N, 79^{o}47’W, 330 masl) and Gigante (9^{o}06’31’’N, 79^{o}50’37’’W, 60 masl) research sites which are located close together in central Panama. In Gigante, some small streams traverse flat terrain, and the land is covered by well-protected mature forest (> 300 years old) (Yavitt et al., 2011). In Agua Salud, small streams and small hills with steep slopes predominate, and the landscape is made up of fallow sites, plantations, cattle pastures, and secondary forests of varying ages that are naturally recovering following disturbances such as clear-cutting and cattle ranching (Bretfeld et al., 2018).

These two sites together encompass forest ages that provide a successional gradient. These tropical moist forests receive around 2,700 mm rainfall annually. About 90% of precipitation occurs in the wet season between early May and middle of December (Ogden et al., 2013). The forests grow on highly weathered, drained, and infertile soils which are classified as Oxisols (Turner et al., 2014; Lai et al., 2017).

For details of fertilization experimental design, fertilizer type, plot monitoring, etc., please see the Chapter 2.

4.2.2 Sample collection

For each treatment, we randomly selected four replicates in each forest - 64 plots (four treatments × four replicates × four age forests). In every plot, we collected leaf, wood, and fine root samples.

Fine roots

Fine roots (<2mm) were collected in the middle of the rainy season (early August to middle September) in 2019. Because the majority of fine root biomass at our research site (90%) is in the top 10 cm soil (Yavitt et al., 2011), in each plot five soil cores (6 cm in diameter to 10 cm depth) were sampled in the inner 25 × 25 m subplot. The five soil cores were mixed into one sample for every plot. Soil samples were stored in a 4°C fridge and were processed within 48 hours of collection. We removed the fine root from soils, dried them at 65°C for more than 72 hours and weighed per forest area biomass. More information about the fine root biomass assessment is described in Chapter 3.

Leaves and wood

During the dry season (January to March 2020), we collected leaf and wood samples from target trees in each plot. Before selecting the target trees, we estimated the canopy status of each tree in our research sites based on personal observations. In the 0-year-old forest, all trees are exposed to sunlight with foliage in the top canopy layer. For the 10 and 30-year-old forests, we defined the upper canopy layer as trees with diameters at breast height > 5 cm (all trees measured), and defined as the sub-canopy layer as trees with diameters at breast height < 5cm (half tree were measured, *see above*). In the top canopy of each plot, 9-13 of the species with the greatest abundance (ranked by aboveground biomass) were selected. We then chose the biggest individual of each species from which to collect our tissue samples. Thus, our focal species represented 81-93% of total aboveground biomass in our plots (**Table A4.1).** For each target tree, 5-10 (depend on leaf size) expanding and sun exposed leaves were collected by climbing trees, using pole-pruners and a sling shot (Youngentob et al., 2016), and 1cm depth wood (without bark) was collected using a chisel. Due to logistical difficulties imposed by the COVID-19 pandemic, we did not collect leaf and wood samples from the 300-year-old forest. In total, 1,100 leaf and wood samples from 550 trees were collected.

In the lab, leaves were cleaned, scanned for leaf area, and dried at 65 °C for more than 72 hours along with wood samples. We weighed the dried mass of scanned leaves and calculated the index of leaf mass per area of each selected tree.

4.2.3 Nutrient concentrations

Samples of leaves, wood, and fine roots were ground and analysed for total carbon and nitrogen with elemental analysis and total nitrogen and phosphorus using sulphuric acid (H_2SO_4) digestion at the University of Leeds, the UK. We compared nitrogen concentrations determined by both elemental analysis and digestion methods to ensure consistency of methods. We used the nitrogen concentrations determined by elemental analysis for statistical analysis.

4.2.4 Community level nutrient concentrations and ratios

For leaf and wood tissues, we calculated the community-weighted nutrient concentrations (carbon, nitrogen, and phosphorus) and compositions (ratios of carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorus) in each plot. The community weighted nutrient concentrations and compositions were calculated based on the nutrient concentrations and compositions of leaf and wood tissues of each selected species and the relative aboveground biomass of each species. For leaf and wood tissues, the community weighted nutrient concentrations and compositions ((C, N, P, C:N, C:P, or N:P)com) were calculated as follows:

$$(C, N, P, C{:}N, C{:}P, or\ N{:}P)com = \frac{\sum_{i=1}^{n}([C, N, P, C{:}N, C{:}P, or\ N{:}P]_i * B_i)}{\sum_{i=1}^{n}[B_i]}$$

Where [C, N, P, C:N, C:P, or N:P]$_i$ is the carbon concentration, nitrogen concentration, phosphorus concentration, carbon to nitrogen ratio, nitrogen to phosphorus ratio of the *i*th species, respectively, n is the number of selected species in the community, and B is the biomass of each species.

For fine root tissue, the nutrient concentrations and ratios of each sample were identified as the community level nutrient concentrations and ratios, because the fine root samples were collected at the plot level (*see above*).

4.2.5 Nutrient content in tissues

Plot level leaf nutrient contents were estimated from the plot total leaf biomass and the community weighted leaf nutrient concentrations. Plot total leaf biomass was estimated based on the plot level leaf area index (LAI) and leaf mass per area (LMA). Canopy leaf area index was estimated by taking hemispherical photos using a Nikon Coolpix E4500 camera in each plot in August and September 2019. Following the hemispherical photography protocol (https://www.schleppi.ch/patrick/hemisfer/help.php?t=photo), five photos in each plot (10 photos in the 0-year-old forest) were taken under the canopy and 1m above the ground either in the very early morning (before 7:00 am) or on very cloudy days. The leaf area index of each photo was analysed using the Hemisfer software (Swiss), LAI-2000 method (Thimonier et al., 2010). The leaf area index of each plot (LAIplot) was calculated as the mean of the 5 (in 10-year- old and 30-year-old forests) or 10 (in the 0-year-old forest) photos.

The plot scale leaf mass per area was calculated according to each species' leaf mass per area and the relative aboveground biomass. We calculated the plot scale leaf mass per area using the following function:

$$(Leaf\ mass\ per\ area)_{plot} = \frac{\sum_{i=1}^{n}([Leaf\ mass\ per\ area]_i * B_i)}{\sum_{i=1}^{n}[B_i]}$$

Where [Leaf mass per area]$_i$ is the leaf mass per area (*see above*) of the *i*th species, n is the number of selected species in the community, and B is the biomass of each selected species.

We approximated the plot scale leaf biomass using the following equation:

$$\text{Plot leaf mass} = (\text{Leaf mass per area})_{\text{plot}} * (\text{LAI}_{\text{plot}}),$$

and we calculated the plot scale leaf nutrient contents as follows:

$$(C, N, P)_{contents} = Plot\ leaf\ mass * (C, N, P)_{com}$$

The plot level wood nutrient contents were calculated based on the plot wood biomass and mean nutrient concentrations. We first calculated the aboveground biomass in each plot (please find the details in Chapter 2). After that, we calculated plot-level wood biomass and wood nutrient contents as follows:

$$(\text{Wood biomass})_{plot} = (\text{Aboveground biomass})_{plot} - (\text{Leaf biomass})_{plot}$$

$$(C, N, P)_{contents} = (Wood\ biomass)_{plot} * (C, N, P)_{com}$$

The plot scale nutrient contents in fine roots were calculated according to the plot scale fine root biomass, as measured in Chapter 3, and nutrient concentrations. Because both fine root biomass and nutrient concentrations were assessed on the plot scale, we calculated fine root nutrient contents as follows:

$$(C, N, P)_{contents} = (Fine\ root\ biomass)_{plot} * (C, N, P)_{plot}$$

4.2.6 Forest growth

We calculated the forest growth between the two latest censuses (between census 2018 and 2019 in Agua Salud plots and between 2013 and 2018 in Gigante plots). Growth was calculated as the gains of the trees which were recorded in the first census year and survived until the second census year divided by the period between the two censuses in years (Δt), e.g., stand-level aboveground biomass growth between the year 2018 and 2019 = (aboveground biomass_2019 – aboveground biomass_2018)/ Δt.

4.2.7 Statistical analysis

First, we used mixed-effects models to test the effects of nutrient addition on the nutrient concentration (carbon, nitrogen, and phosphorus) and composition (ratios of carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorus) in all tissues, and total nutrient allocated to tissues, and the ratio of total nutrient allocated to different tissues across all forests. The mixed-effects models included nitrogen addition, phosphorus addition, forest age, and their interactions as fixed effects, with block as a random effect. After that, we used post-hoc tests, using the 'emmeans' function from the 'emmeans' package in R, to test the effect of nutrient addition on changes in nutrient concentration (carbon, nitrogen, and phosphorus), composition, and allocation in tissues among forest ages.

Second, we used mixed-effects models to test the effects of nutrient concentration, nutrient treatment (interaction between nitrogen and phosphorus), and forest age on change in forest growth. The mixed-effect models included nutrient concentrations (nitrogen or phosphorus), interaction between nitrogen and phosphorus treatment, and forest ages as fixed effects, with block as a random effect.

For all models, natural or log-transformed response variables were conducted to meet the model's assumptions of normality, and residual and Q-Q plots were applied to evaluate the model's quality. All these analyses were conducted in RStudio (4.0.2).

4.3 Results

4.3.1 Flexible nutrient composition in tropical forests

We found support for the hypothesis that tropical forests utilize stoichiometric flexibility over forest succession and across gradients in nutrient availability. The nutrient composition of tissues, expressed as the ratio of carbon to nitrogen, carbon to phosphorus, and nitrogen to phosphorus, changes over the course of forest succession (**Figure 4.2**). As the recovering forest gains biomass, the ratio of carbon to nitrogen decreased in all tissues ($p<0.05$ in all tissues, **Table A4.2**), the ratio of carbon to phosphorus increased in wood and fine roots ($p<0.1$ for wood and $p<0.05$ for fine roots, **Table A4.2**), and the ratio of nitrogen to phosphorus increased in wood ($p<0.05$) and fine roots ($p<0.0001$) but not in leaves (**Table A4.2**). These changes in nutrient compositions are mainly caused by an increase in nitrogen concentration (%N; $p<0.05$

for all tissues) and a decline in phosphorus concentration in both wood (%P; *p*<0.05) and fine roots (*p*<0.05) along forest succession (**Figure A4.1** and **Table A4.3**). They reflect the shift in nutrient limitation that we observed on forest net growth (Chapter 2) from nitrogen limitation in the 0- and 10-year old forests to phosphorus limitation in the 30-year forest.

Nutrient composition in all tissues also changed in response to nutrient additions (**Figure 4.2**). Phosphorus addition had a substantial effect on the ratio of carbon to phosphorus, decreasing the ratio of carbon to phosphorus in all tissues for all forest ages (*p*<0.0001, **Table A4.2**). Phosphorus addition was also associated with a decline in the ratio of carbon to nitrogen in leaves of the 0-year-old forest (*p*<0.05, **Figure 4.2**). Nitrogen addition decreased the ratio of carbon to nitrogen in all tissues of the 0 and 10-year-old forest (*p*<0.05 for all tissues), but did not change the ratio of carbon to nitrogen in tissues of the 30 and 300-year-old forests (**Figure 4.2**). This was consistent with the pattern of nitrogen limitation on growth in the younger forests but no nitrogen limitation in the older forests (Chapter 2). Nitrogen addition also increased the ratio of carbon to phosphorus in fine roots of the 300-year-old forest (*p*<0.05, **Figure 4.2**). These changes in ratios of carbon to nitrogen and carbon to phosphorus are mainly caused by higher nutrient concentrations (especially nitrogen and phosphorus) following specific nutrient addition rather than a change in carbon concentration (*p*<0.05 for all tissues, **Figure A4.1** and **Table A4.3**). The ratio of nitrogen to phosphorus decreased substantially in all tissues in the phosphorus treatments in all forest succession stages (*p*<0.0001, **Figure 4.2** and **Table A4.2**). After nitrogen addition, the ratio of nitrogen to phosphorus rose in wood (*p*<0.1) and fine roots (*p*<0.05) but not in leaves (**Figure 4.2** and **Table A4.2**).

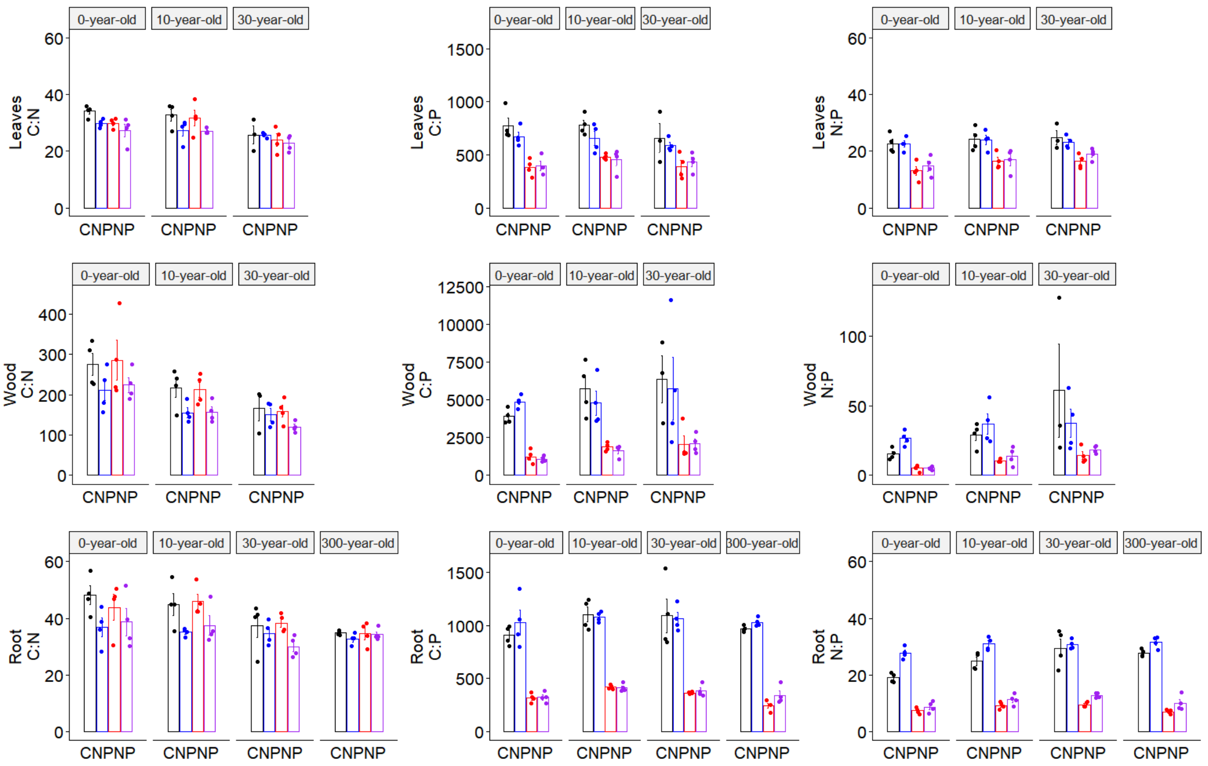

Figure 4.2 | The responses of nutrient composition in tissues to nutrient addition over forest succession.

In the x-axis of each sub-figure, C, N, P, and NP stand for control, nitrogen addition, phosphorus addition, and nitrogen plus phosphorus addition, respectively. In the y-axis, C:N, C:P, and N:P stand for the ratio of carbon to nitrogen, the ratio of carbon to phosphorus, and the ratio of nitrogen to phosphorus, respectively. Find the statistical analysis results in **Table A4.2**.

4.3.2 Flexible nutrient allocation in tropical forests

The total amount of nutrient allocated to each tissue can also change in response to nutrient addition over forest succession, consistent with the hypothesis that tropical forests use a strategy of flexible nutrient allocation to address nutrient constraints (**Figure 4.3**). In the 0 and 10-year-old forests, nitrogen addition increased the ratios of aboveground (leaves and wood) to belowground (fine roots) nutrient pools for nitrogen (p<0.05) and phosphorus (p<0.05) (**Figure 4.3**), with the leaf and woody nitrogen (p<0.05 for 0-year-old forest) and phosphorus (p<0.05 for both 0 and 10-year-old forest) pools both increasing, while fine root phosphorus pools decreased (p<0.05 in both forests) (**Figure A4.2**). In the 30-year old forest, nitrogen addition had no effect on the nitrogen and phosphorus allocations among tissues (**Figure 4.3**). Following phosphorus addition, the ratio of root to wood nitrogen pools in the 30-year old forest decreased (p<0.05) (**Figure 4.3**), with the fine root nitrogen pools decreasing in turn (p<0.05, **Figure A4.2**). Phosphorus addition did not change the ratio of leaf to fine root phosphorus pools in all 0, 10, and 30-year-old forests, but only decreased the fine root to wood phosphorus pool ratios in 0-year-old forest (**Figure 4.3**) and increased the phosphorus pools in all tissues (p<0.05) (**Figure A4.2**).

The ratio of leaf to wood nitrogen and phosphorus pools also changed following nutrient addition over forest succession (**Figure 4.3**, and **Table A4.4**). In 0 and 10-year-old forests, both the ratio of leaf to wood total nitrogen (p<0.05) and the ratio of leaf to wood total phosphorus (p<0.05) decreased in plots with nitrogen addition (**Figure 4.3**), reflective of an accumulation of wood biomass over time (Chapter 2). Further, phosphorus addition also lowered the ratio of total leaf to wood phosphorus in all 0, 10, and 30-year old forests (p<0.05, **Figure 4.3** and **Table A4.4**).

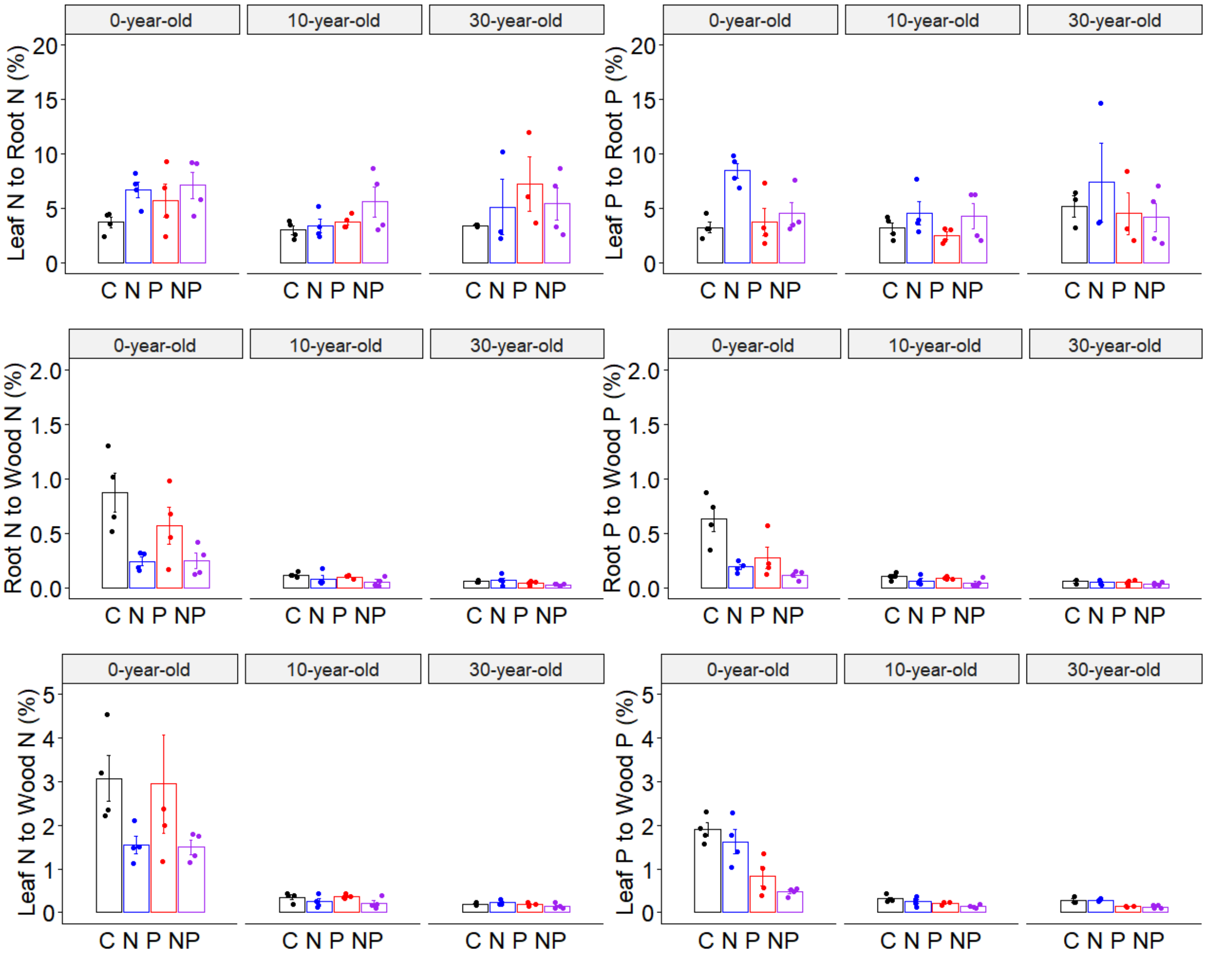

Figure 4.3 | Ratios of total nutrient allocated to tissues change in response to nutrient addition over forest succession.

In the x-axis of each sub-figure, C, N, P, and NP stand for control, nitrogen addition, phosphorus addition, and nitrogen plus phosphorus addition, respectively. Find the statistical analysis results in **Table A4.4**.

4.3.3 Relationships between nutrient concentrations and growth

Tissue nitrogen and phosphorus concentrations had no relationships with forest growth rate (growth rate between 2018 and 2019 for Agua Salud forests and between 2013 and 2018 in Gigante forest, Mg/ha.yr^{-1}), when controlling for nutrient treatment, forest age, and site (p>0.1, **Figure 4.4**).

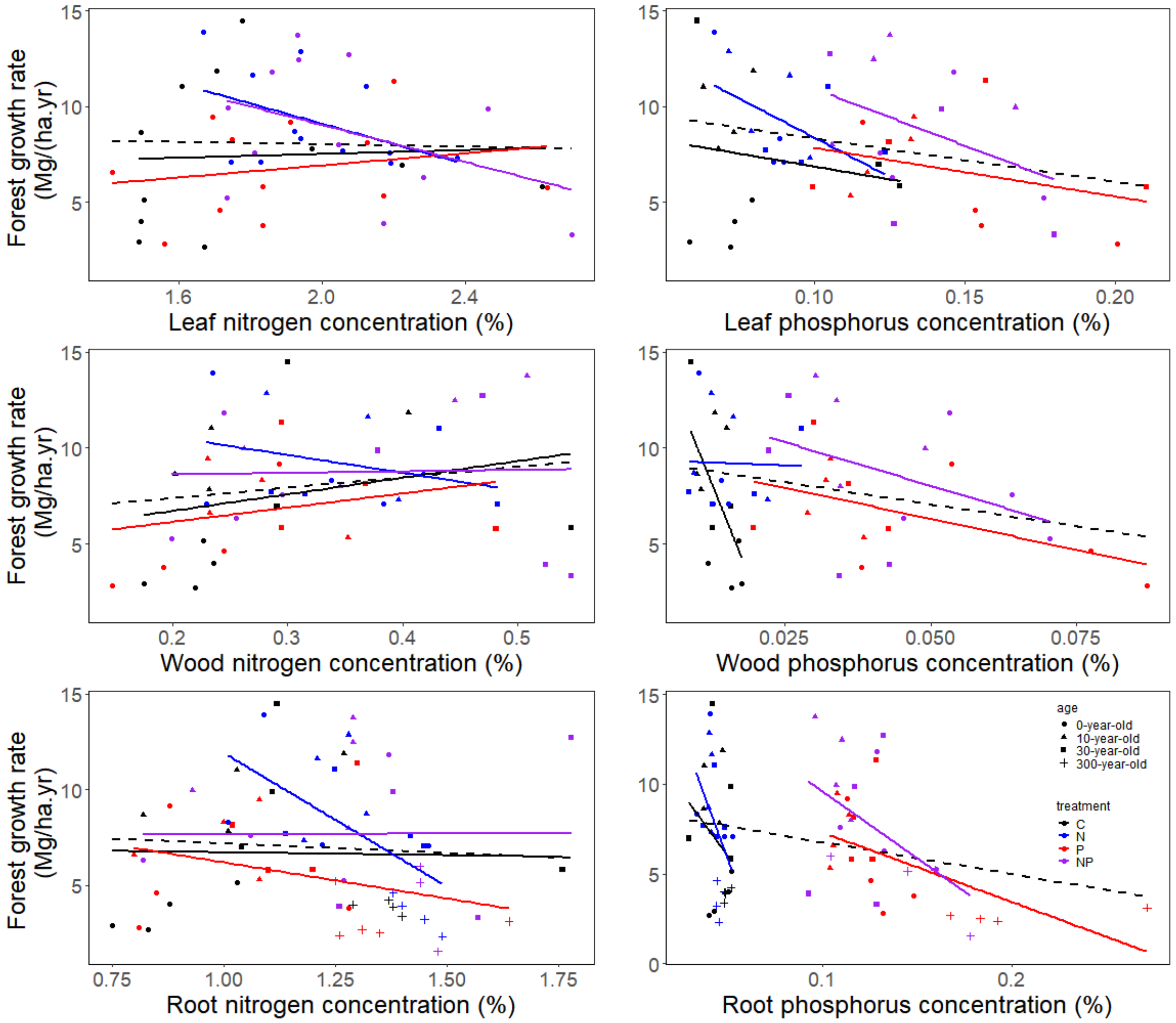

Figure 4.4 | The relationships between forest growth in the latest census year and nutrient concentration in tissues change in response to nutrient addition over forest succession.

In the figure, C, N, P, and NP stand for control (black solid lines), nitrogen addition (blue lines), phosphorus addition (red lines), and nitrogen plus phosphorus addition (purple lines), respectively. In each sub-figure, the black dash lines mean the relationship between forest growth and nutrient concentration in all treatment plots. Different shapes represent different forest ages: circle is for 0-year-old forest; triangle is for 10-year-old forest; square is for 30-year-old forest; cross is for 300-year-old forest.

4.4 Discussion

This work reports on the first nutrient manipulation experiment across a tropical forest successional gradient that attempted to assess changes in both nutrient compositions and allocations for all major plant tissues in response to nutrient addition. Our results support the first and third hypotheses (**Figure 4.1**), that tropical trees are flexible in their nutrient composition within tissues and allocation across tissues to address nutrient limitation. When forests are limited by soil nutrients, trees decrease nutrient concentrations, increase the ratios of carbon to nitrogen and/or carbon to phosphorus, and/or allocate more nutrients to leaves and fine roots. When forests are not limited by soil nutrients, trees increase (phosphorus) or do not change (nitrogen) nutrient concentrations, decrease the ratios of carbon to nitrogen and carbon to phosphorus, and allocate more nutrients to wood. Our results of nutrient composition changes are broadly consistent with findings from other tropical forest work conducted in other nutrient addition experiments (Wright et al., 2019), natural observations (Townsend et al., 2008; Cleveland et al., 2011), and some carbon dioxide enrichment experiments (Sardans et al., 2017). These results suggest tropical ecosystem nutrient use in plant tissues have the capacity to be flexible with environmental change.

The flexibility in adjusting nutrient compositions to address nutrient limitation results in changes of nutrient concentrations (nitrogen and phosphorus) over the course of forest succession. In our control plots, nitrogen concentration in tissues increases over the course of forest succession while phosphorus concentration decreases (**Figure A4.1** and **Table A4.3**), and these trends are consistent with how nutrient limitation changes over forest succession. We have found strong nitrogen limitation on forest growth in young secondary forests, phosphorous limitation on forest growth in the middle stage forest, and no evidence of nutrient limitation on forest growth in the mature forest (Tang et al., Chapter 2). Similar trends in nitrogen and/or phosphorus concentrations with forest succession were also observed in other tropical secondary forest successional gradients (Davidson et al., 2007; Liu et al., 2021; Poorter et al., 2021). For example, in a tropical forest successional gradient of the eastern Amazon, foliar nitrogen concentration increased with succession (Davidson et al., 2007), and in a sub-tropical forest successional gradient of southern China, the phosphorus concentration in tissues decreased with succession (Liu et al., 2021). These findings indicate that the shift of nutrient limitation patterns with succession that we detected may be widespread. However, the pattern of nitrogen concentration along the successional gradient may be affected by the abundance of nitrogen-fixing trees in ecosystems. Nitrogen-fixing trees have the capacity to support ecosystem-wide increases in nitrogen availability in tropical ecosystems and hence help to facilitate forest succession (Batterman et al, 2013; Levy-Varon et al. 2019).

Few studies report how trees adjust how their nutrient allocation to different tissues with environmental changes. Here, our findings indicate that when forests are nutrient-limited, more nutrients were allocated to leaves and fine roots than wood, and *vice versa* when forests are not nutrient-limited. This presumably allows plants to maximize capture of carbon (in leaves) and nutrients (by roots) when nutrients are limiting. Whilst changes in the total nutrient allocation to tissues in response to nutrient addition are associated with biomass change (Tang et al., Chapter 3), we still can conclude that forests will adjust nutrient allocation to address nutrient limitation (Chapin III et al., 1990). Flexible nutrient allocation allows tropical trees to have more capability to adjust nutrient stoichiometry in order to address nutrient limitation, as the flexibilities of nutrient concentrations in tissues exist in a certain range (**Figure 4.2** and **Figure A4.1**).

Beside changing nutrient composition and allocation to address nutrient shortage, trees also adjust nutrient composition and allocation to adapt to nutrient-rich environments. Our results demonstrated that phosphorus concentrations and pools in tissues significantly increase after phosphorous addition regardless of the pattern of nutrient limitation on forest growth. This substantial increase of phosphorus concentration in tissues was also found in other nutrient addition experiments (Wright, 2019) and natural observations where foliar phosphorus concentration increased by 50% across a four-fold gradient in total soil phosphorus in tropical forests (Cleveland et al., 2011). Yet, it is still unclear why trees increase phosphorus concentration and content in tissues beyond their growth requirement (**Figure 4.4**). There are two potential reasons for this phenomenon. First, when phosphorus is rich in soil, trees may take up more than they immediately need and store phosphorus for future use. This strategy may help trees survive when an extreme event happens, such as drought (Gessler et al., 2017), and also save trees' carbon cost to absorb phosphorus (Laliberte et al., 2015). Unlike nitrogen, phosphorus binds tightly to sand in soil, and it is therefore more easily taken up by trees' mycorrhizal partners to which carbon must be paid in exchange for phosphorus (Plassard and Dell, 2010). Second, trees may increase phosphorus absorption and store it for reproduction (Fortier and Wright, 2021), as phosphorus plays an important role in controlling the quantity and quality of reproductive tissues (Lasso and Ackerman, 2013; Fujita et al., 2014). Similar to phosphorus, nitrogen concentration changes in tissues are also not always associated with forest growth (**Figure 4.4**), indicating that trees can continue absorbing nitrogen beyond their requirement of growth. This may be because that trees absorb more nitrogen to increase defense with herbivory increase.

These nutrient use strategies of adjusting nutrient compositions and allocations in tissues may help forests acclimate to climate change and capture more carbon with increasing concentrations of CO_2 in the atmosphere. Biogeochemical theory and modeling suggests that the tropical forest carbon sink will be limited by soil nutrients (Cox et al., 2013; Huntingford et al., 2013) as carbon dioxide increases, because nutrients (especially phosphorus) in tropical soils are generally scarce. Some global terrestrial models predicted that, due to nutrient limitation, terrestrial net primary productivity will not be as great as predicted with rising atmospheric CO_2 alone in the coming decades (Fisher et al., 2012; Wieder et al., 2015; Fleischer et al, 2019). However, the projected slowing of the increase in net primary production caused by nutrient limitation may be overestimated, because global models generally do not incorporate the ability of trees to gradually adjust nutrient stoichiometry to address nutrient limitation. Our experimental results suggest that, despite increasing nutrient limitation under rising atmospheric CO_2 and nitrogen availability decreasing (Mason et al., 2022), forests may continue to contribute net carbon sequestration as carbon dioxide concentrations increase by adjusting nutrient use among tissues to increase photosynthesis and increasing total plant carbon per unit nutrient. Therefore, to improve our ability to predict the future of the tropical forest carbon sink, it is necessary to incorporate tissue, individual tree, and forest flexibility in nutrient stoichiometry in global ecosystem and Earth System models.

Whilst tropical forests use nutrient stoichiometry to address nutrient limitation, the extent to which the adjustment of nutrient stoichiometry addresses nutrient limitation may itself shift with forest succession. In the case of tropical mature forests, these nutrient use strategies may help to successfully address nutrient limitation, so that some forests may show no obvious growth response to nutrient fertilization (Wright et al., 2018; Tang et al., Chapter 2). Therefore, nutrients may less clearly control the growth of trees in mature forests than previously expected. Our results suggest that, in contrast, these nutrient use strategies may have limited ability to address nutrient limitation in young tropical forests, because in successional forests both stoichiometry and growth clearly respond to nutrient addition, suggesting that, even if flexible nutrient use strategies address nutrient limitation, they are not sufficient to overcome limitation. Therefore, nutrients are likely to strongly affect biomass accumulation in successional systems.

Chapter 5

Discussion

5.1 Summary

This thesis investigates how nutrients affect the potential of the tropical forest carbon sink in aboveground biomass. The primary goals of the research were to test (1) whether soil nutrients limit tropical forest aboveground biomass, and (2) if tropical forests can apply strategies to address nutrient limitation. These strategies could include adjusting above- and belowground carbon allocation, nutrient composition and nutrient allocation in tissues. In particular, I asked three questions across my three research chapters. In chapter 2, I asked whether soil nutrients limit tropical forest biomass sequestration, and, if so, how these patterns of nutrient limitation (strength and type) shift along forest succession. In addition, I explored how limiting nutrients affect biomass accumulation and regulate forest dynamics (growth, recruitment, and mortality) over the course of forest succession. In chapter 3, I asked if forests adjust above- and belowground carbon allocation to address nutrient limitation, and, if so, how carbon allocation changes in response to nutrient addition along forest succession. Finally, in chapter 4, I asked whether forests adjust nutrient allocation and composition to address nutrient limitation, and, if so, how does nutrient allocation and composition change in response to nutrient addition throughout forest succession.

To answer these questions, I used a nutrient addition experiment that spans a tropical forest successional gradient established by my first supervisor, Sarah Batterman, and collaborators at the Smithsonian Tropical Research Institute in Panama. The experiment includes forests aged 0, 10, and 30 years old following deforestation and cattle ranching, and a mature forest of more than 300 years. After up to 21 years (4 years in secondary forests) of nutrient addition, I first analysed the responses of aboveground biomass net change and forest dynamics to nutrient addition along the forest successional gradient (Chapter 2). I next analysed the changes in fine root biomass and the ratio of fine root biomass to aboveground biomass in response to nutrient addition along forest succession (Chapter 3). Finally, I analysed the changes in nutrient composition and allocation in tissues in response to nutrient addition along forest succession

(Chapter 4). I now discuss my findings and conclusions, putting them in the context of wider research and highlighting their importance in understanding the role of the tropical terrestrial carbon sink in mitigating climate change.

In Chapter 2, I identified consistent responses in aboveground biomass and forest dynamics – growth, recruitment and mortality – to the addition of the limiting nutrients over succession. The 0-year-old forest exhibited strong nitrogen and some phosphorus limitation, as reflected in accelerated biomass accumulation and increased tree recruitment and growth in response to nitrogen and phosphorus addition. The 10 and 30-year-old forests showed evidence of nitrogen and some phosphorus limitations, respectively. In both cases, the addition of the limiting nutrient accelerated aboveground biomass accumulation by increasing forest growth. The mature forest had no evidence of nutrient limitation on growth, and neither did the aboveground biomass stock respond to nutrient addition. Therefore, overall, soil nutrients constrained tropical forest carbon sequestration rates, but the strength and type of nutrient limitation shifted over the course of forest succession from strong nitrogen limitation, to phosphorus limitation, to no evidence of nutrient limitation. However, in this analysis it remained unclear if and how forests adjust above- and belowground carbon allocation and alter fine root biomass that is responsible for nutrient absorption from soils to address nutrient limitations.

In Chapter 3, I found that fine root biomass and the ratio of fine root biomass to aboveground biomass changed in response to nutrient addition. In addition, the changes in fine root biomass and the ratio of fine root biomass to aboveground biomass in response to nutrient addition shifted along the forest successional gradient. In the 0 and 10 -year-old forests, fine root biomass and the ratio of fine root biomass to aboveground biomass decreased following nitrogen addition. In the 30-year-old forest, fine root biomass and the ratio of fine root biomass to aboveground biomass decreased following phosphorus addition, with no effect of nitrogen addition. In the mature forest, fine root biomass and the ratio of fine root biomass to aboveground biomass had no evident change following nutrient addition. These patterns were consistent with the patterns of nutrient limitation identified in Chapter 2. Therefore, I demonstrated that tropical forests adjust above- and belowground carbon allocation to address nutrient limitations. However, it remained unclear whether tropical forests apply stoichiometric strategies, adjusting nutrient composition and allocation in tissues, to address nutrient limitation.

In Chapter 4, I found that nutrient composition and allocation in plant tissues change in response to nutrient fertilization. In addition, the results demonstrated that changes in nutrient composition and allocation in response to nutrient additions shift along the forest succession gradient. In nitrogen-limited forests, nitrogen addition decreased the ratio of carbon to nitrogen in all tissues and increased nitrogen content allocation from leaves and fine roots to wood. In phosphorus-limited forests, phosphorous addition decreased the ratios of carbon to phosphorous in all tissues in all forests, regardless of limitation status. In addition, phosphorous addition shifted phosphorus from belowground to aboveground tissues in the phosphorus-limited forest. Therefore, I offer conclusive evidence that tropical forests can adjust stoichiometric strategies to address nutrient limitations.

5.2 Synthesis

Multiple lines of evidence from our findings support the hypothesis that tropical forest carbon sink in aboveground biomass is limited by soil nutrients, and the type and strength of nutrient limitation shifts as forest proceed over secondary succession to mature forests. First, both the net change of aboveground biomass and forest growth showed changes in response to nutrient addition, with different responses to nutrient addition along forest succession. Both the net change of biomass and forest growth significantly increased following nitrogen addition in young secondary forests (0 and 10-year-old forests), and had some increase after phosphorus addition in the middle stage forest (30-year-old forest), but had no evident change after nutrient addition in mature forest. Second, strategies used by trees to address nutrient limitation had similar changes following nutrient addition along forest succession. Biomass allocation, nutrient composition, and nutrient allocation had evident changes following nitrogen addition in young secondary forests (0 and 10-year-old forests), and they had some changes following phosphorous addition in the middle stage forest (30-year-old forest), but had no evident change following nutrient addition in the mature forest (300-year-old forest). Therefore, we conclude that there are interactions between nutrient limitation and forest age: nitrogen (with some phosphorus) limits the young forests, phosphorus has some limitation in the middle stage forest, and there is no evidence of nutrient limitation in mature forests. Efforts to resolve growth dynamics in tropical forests would benefit from considering forest age in their analysis.

Trees apply nutrient acquisition and usage strategies to address nutrient limitation, such as adjusting biomass allocation, nutrient composition, and nutrient allocation. These strategies show different sensitivity and flexibility among tissues in addressing nutrient limitation.

Nutrient stoichiometry is more sensitive than biomass allocation in address nutrient limitation. For example, in the mature forest which had no evidence of nutrient limitation, biomass allocation did not show any change, but nutrient concentration and composition changed following nutrient addition (especially phosphorus). In addition, these strategies show different extent of flexibility among tissues in response to nutrient addition. Fine root biomass has more flexibility than wood biomass in response to nutrient addition. For example, in the 30-year-old forest following phosphorus addition, fine root biomass decreased by 40% (**Figure, 3.2**), wood (aboveground) biomass only increased by 20% (**Figure 2.3**). This divergent response results in the ratio of fine root biomass to aboveground biomass decreasing following nutrient addition. In addition, the extent of these strategies in addressing nutrient limitation also shift along forest succession. In young secondary forests (0 and 10-year-old forests) which have strong nutrient limitation, these strategies did not successfully address nutrient limitation, as the aboveground biomass had significant increase following nutrient addition, even when they substantially adjusted their strategies. However, in the 30 and 300-year-old forests, aboveground (wood) biomass had no evident change following nutrient addition, which indicates that these strategies can successfully address nutrient limitation.

Our findings of shifts in nutrient limitation from nitrogen to phosphorous or no limitation in tropical forests of Panama likely can be extended to other tropical forests for four reasons. First, our findings are consistent with biogeochemical theory that nutrient (especially nitrogen) availabilities and nutrient requirements of forest growth shift along secondary forest succession (Walker and Sayer, 1976; Menge et al., 2012; Nagy et al., 2017). Second, nitrogen fixation rates are higher in young forests than old forests, which suggests that young forests are nitrogen limited compared to old forests (Batterman, et al., 2013; Sullivan et al., 2014; Levy-Varon et al., 2019). Third, field observations in Amazon forests indicate that the nitrogen cycle becomes more open over forest succession (Davidson et al., 2007). Fourth, although evidence from fertilization studies is scares and no other study has test how nutrient limitation shifts over secondary succession in lowland tropical wet forests, our results are consistent with a fertilization experiment in the Amazon that demonstrated a young secondary forest is limited by nitrogen (Davidson et al., 2004), and another experiment from the Amazon that showed no nitrogen limitation in mature forests (Cunha et al., 2022). The latter experiment, which had substantially lower total phosphorus in the soil (85 mg/kg) than that in our site (400mg/kg), did find phosphorus limitation on leaf and fine root productivity, but no indication of phosphorus limitation on wood growth which comprises the majority of aboveground carbon sink. While, over all, we believe our patterns will hold in other tropical forests, there may be some variations across sites. Any difference could emerge if research sites have different soil phosphorus level, climate, rainfall, and abundance of nitrogen fixers, which affect nutrient limitation patterns. We may expect some phosphorus limitation in mature forests in about more than half of the Amazon which have lower phosphorus levels than our site (Wright, 2022), although recent experimental findings suggest this may not extend to the aboveground carbon sink (Cunha et al., 2022). In addition, experimental results also suggested dry forests may show less response to nutrient addition in dry years (Waring., 2019). Finally, some tropical forests containing high abundance of nitrogen fixers may not be limited by nitrogen but phosphorus, as nitrogen fixers increase the availability of nitrogen in the ecosystems (Hedin et al., 2009; Brookshire et al., 2012).

5.3 Implication

My work demonstrates that soil nutrients limit the carbon sink in successional tropical forests and that the pattern of nutrient limitation shifts throughout forest succession following disturbance. Nutrient limitation constrains tropical forest aboveground biomass accumulation in secondary forests and affects forest dynamics (Chapter 2). Forests can adjust above- and belowground carbon allocation (Chapter 3) and nutrient stoichiometry (nutrient compositions and allocations in tissues, Chapter 4) to address nutrient limitation. These results help to improve the assessment of the tropical forest carbon sink and the prediction of future carbon sequestration with climate change.

First, my work helps to improve the assessment of the carbon sink in successional tropical forests. In the past, the carbon sink in tropical successional forest was assessed according to the change of aboveground biomass without considering changes to the substantial belowground carbon pool. My results, especially in my third chapter, found that allocation of carbon to fine roots changes across forest succession and in response to nutrient availability. Belowground carbon allocation contributes an important part (about one third) of net primary production (Malhi et al., 2011). Therefore, including assessment of belowground carbon allocational changes along forest succession and its change in response to nutrient limitation is essential to improve predictions of the tropical forest carbon sink into the future.

Second, my work helps to improve our understanding of nutrient limitation patterns in mature forests using a nutrient addition experiment. The existence of nutrient limitation in mature forests is difficult to determine, and often in experiments these forests show no clear response to nutrient addition (Wright et al., 2018; Wright, 2019), including in our analysis. This may be because mature forests experience little nutrient limitation, and trees adjust their carbon allocation and nutrient stoichiometry to successfully address any weak nutrient limitation. My results suggest that changes in above- and belowground carbon allocation, nutrient composition, and nutrient allocation in response to nutrient addition are consistent with the pattern of nutrient limitation throughout forest succession (Chapter 2 and 3). Therefore, analysis of responses of carbon allocation, nutrient composition, and nutrient allocation to nutrient addition can be used as a tool to assess nutrient limitation in mature forests where previous methods have failed. This is particularly important as mature forests contain the most carbon in tropical forest ecosystems and the carbon sink in mature forest is expected to increase with CO_2 fertilization (Cox et al., 2013; Huntingford et al., 2013).

Third, my work helps to improve the prediction of the future carbon sink in tropical mature forests. Recently studies suggested the carbon sink in Amazon mature forests has been decreasing, with the previous gains in forest productivity slowing down and mortality increasing over past decades (Brienen et al., 2015), and early ground evidence and statistical models analysing forest dynamic records suggest similar changes will continue in Amazonia and emerge in African tropical forests in coming decades (Hubau et al., 2020). However, these findings contrast with results of dynamic global vegetation models, which predict that the carbon sink would increase with CO_2 fertilization in the atmosphere (Cox et al., 2013; Huntingford et al., 2013). The difference between these may relate to several factors, including potentially because (1) belowground carbon change is not measured directly in permanent plots, and/or (2) nutrient limitation of the carbon sink and strategies applied by trees to address nutrient limitation (increase belowground carbon allocation and carbon capture per unit of nitrogen or phosphorous) were not included in models. Therefore, to improve the prediction of the future carbon sink in tropical mature forest ecosystems, we suggest models include not only the effect of nutrient limitation on forest carbon sink but also the strategies that trees apply to address nutrient limitation, which I have identified in this thesis.

Fourth, my work provides suggestions for policymakers to manage secondary forests re-growing after disturbance. Secondary forests have high carbon capture rates per unit area, and recovery of tropical forests by succession is a promising way to slow global warming if these forests stay as forests. Many global schemes, such as the Bonn Challenge, the Convention on Biological Diversity Aichi Targets, and the New York Declaration on Forest, have been launched to help address the potential role of forests in meeting global climate goals. The primary aim of these schemes is to help keep global warming below 1.5 ^{o}C with 200 Pg C to be absorbed by 2100 (Lewis et al., 2019). To help realize this climate goal, it is necessary to understand the factors altering forest carbon accumulation. While some analyses (Poorters et al., 2016) suggest tropical forest recovery is climate-controlled, results from my work and some other studies clearly show that nutrients can strongly constrain forest carbon sequestration rates (Chapter 2). Therefore, taking nutrient limitations properly into consideration, such as restoring forests in fertile soils, will help to support the effectiveness of these schemes.

5.4 Future research

My research, on the community scale, demonstrates that soil nutrients limit tropical forest carbon sink and nutrient limitation constrains tropical forest carbon sequestration by affecting forest dynamics. To address nutrient limitation, trees increase belowground carbon allocation, increase ratios of carbon to nitrogen (phosphorus), and adjust nutrient allocations across tissues. However, more work needs to be done in the future to test (1) if soil nutrient limitation contributes to the slowdown of the carbon sink in tropical mature forests by establishing a network of large-scale fertilization experiments across Neotropical, African and Asian tropical forests, (2) whether soil nutrient limitation causes higher forest mortality in other tropical forest sites and ages, and if so, what is the mechanism, (3) how soil carbon in tropical secondary and mature forests will change in the future with climate change, and (4) on the species scale, what is the effect of nutrient addition on tropical forest dynamics and the strategies that trees apply to address nutrient limitation.

First, the cause of carbon sink stalling in tropical mature forests. Whilst my results demonstrated that nutrient limitation can constrain carbon sequestration by limiting biomass productivity and enhancing mortality, it is still unclear if the nutrient limitation widely exists in tropical mature forests and if nutrient limitation is responsible for the decline in the carbon sink in the Amazon tropical mature forest (Brienen et al., 2015). Therefore, more work will be required to test if nutrient limitation exists across other sites in tropical mature forests.

Detecting nutrient limitation patterns in mature forest is very difficult, especially on continent scales. The best way to demonstrate nutrient imitation patterns in a forest is by applying a nutrient fertilization experiment, but it is impossible to manipulate nutrient availability on a continent-scale. An easy method should be applied to test the nutrient limitation pattern in tropical mature forests. Methods, such as measuring the ratio of nitrogen to phosphorous (Koerselman and Meuleman, 1996) and the ratio of nitrogen resorption efficiency to phosphorus resorption efficiency (Du et al., 2020), were used to assess the nutrient limitation pattern (mainly the type of limitation) in mature forests. These two methods are based on the assumption that the nutrient concentrations change is associated with only forest growth and stoichiometric homeostasis theories, respectively. However, these methods may need more consideration, because my results demonstrated that nutrient concentration in tissues can be flexible in response to nutrient limitation and trees can increase phosphorus concentration in tissues without enhancing forest growth (Chapter 3). Therefore, these two methods cannot be applied to assess the nutrient limitation in the specific forest, and more work, including method innovation, should be done to test if soil nutrients limit the carbon sink in tropical mature forests.

Second, the effect of nutrients on forest mortality. Tree mortality is critical for carbon storage and forest dynamics (McDowell, et al., 2018), so it is important to understand the factors regulating it. My results showed that forest-wide mortality had some decrease following the addition of limiting nutrients in both 10 and 30-year-old forests, which have high forest growth rates. In contrast, my results also demonstrated that mortality increased in both 0 and 30-year-old forests following limiting nutrients and nitrogen additions, respectively. Yet, it is still unclear why forest mortality changes following nutrient addition. The reason for mortality decreased following limiting nutrient addition may be because limiting nutrient addition will relieve trees' competition for limiting nutrients, so the mortality decreased with limiting nutrient addition in both 10 and 30-year-old forests. The reason for a mortality increase following limiting nutrient addition in the regenerated forest (0-year-old forest) may be because trees grow faster but die younger or because forests accelerated self-thinning. The reason for a mortality increase following nitrogen addition in both 0 and 30-year-old forests may be because that nitrogen addition increased herbivory. More work will be required to answer the effect of nutrients on forest mortality and the mechanisms.

Third, soil carbon retention and efflux. Soil contains a large amount of carbon, and minor changes in soil carbon may dramatically affect global carbon cycling and climate change, so it is necessary to understand how soil carbon will change in the future. My study indicated that the soil carbon in tropical mature forests may increase if the tropical forest carbon sink is limited by soil nutrients - because more carbon will be invested belowground to increase nutrient absorption. On the other hand, soil carbon may decrease if the soil carbon efflux increases with climate change and nutrient limitation. Yet, it is still unclear how soil carbon retention and emission will change in the future. Therefore, to improve the prediction of future carbon cycling and climate change, it is critical to study the change of soil carbon and its mechanisms.

Fourth, on the species scale, the effects of nutrient addition (or limitation) on forest dynamics, and strategies applied to address nutrient limitation. My research was conducted on a community scale. However, the response of forest dynamics to nutrient addition and strategies applied by trees to address nutrient limitation may differ across species, due to species-specific variation in functional traits. Investigating how plants adapt to nutrient limitation at the species scale is critical for us to understand forest dynamics and predict successional paths, and this analysis will help us to predict changes in species composition along forest succession and how much carbon can be captured in the restored lands. Furthermore, it will also provide suggestions for policymakers to assess if the climate schemes, such as Bonn Challenge, will successfully help us tackle global warming. Therefore, more work should be done to focus on species scale dynamics and strategies.

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Appendices

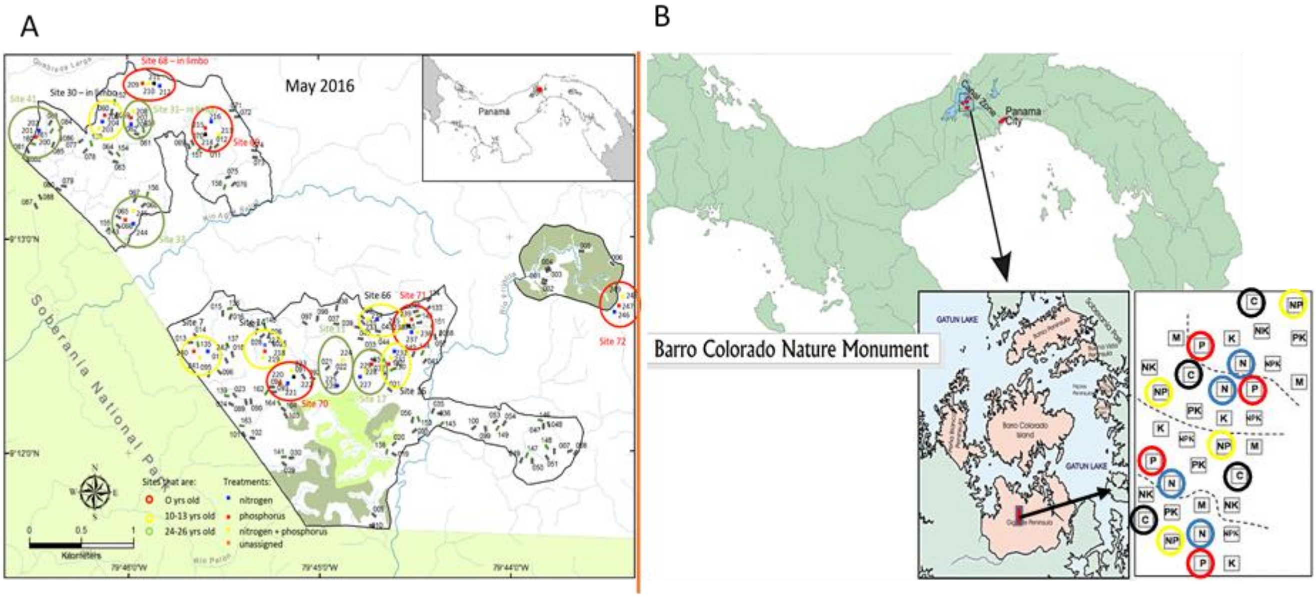

Figure A2.1 | The location of our experiments and the layout of the nutrient addition plots.

The experiments were established in two nearby sites in central Panama, Agua Salud (**A**, left panel) and Gigante (**B**, right panel). The Agua Salud experiment (**A**) includes three forests: 0-year-old forest (red cycles), 10-year-old forest (yellow cycles), and 30-year-old forest (green cycles). Each forest had five nutrient fertilization blocks (replicates), and each block contained a control plot (black rectangle), a nitrogen added plot (blue square), a phosphorus added plot (red square), and a nitrogen plus phosphorus added plot (yellow square). The Gigante experiment had one forest, the mature forest. This forest had a 2×2×2factorial NPK fertilization and a micronutrient addition with four replicates experiment. We selected the control plots (black cycles), the nitrogen added plots (blue cycles), the phosphorus plots (red cycles), and nitrogen plus phosphorus added plots (yellow cycles) for our analysis.

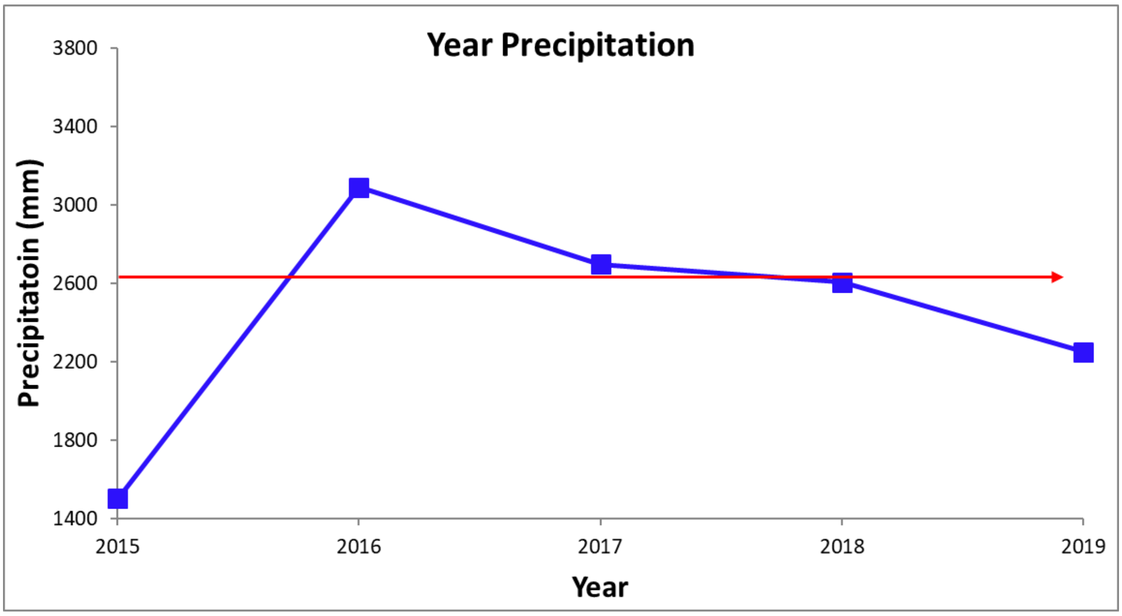

Figure A2.2 | Annual precipitation at our research site.

Precipitation was measured monthly during the experiment. The blue dots represent the annual precipitation values and the red line the mean annual precipitation from 2009 to 2019.

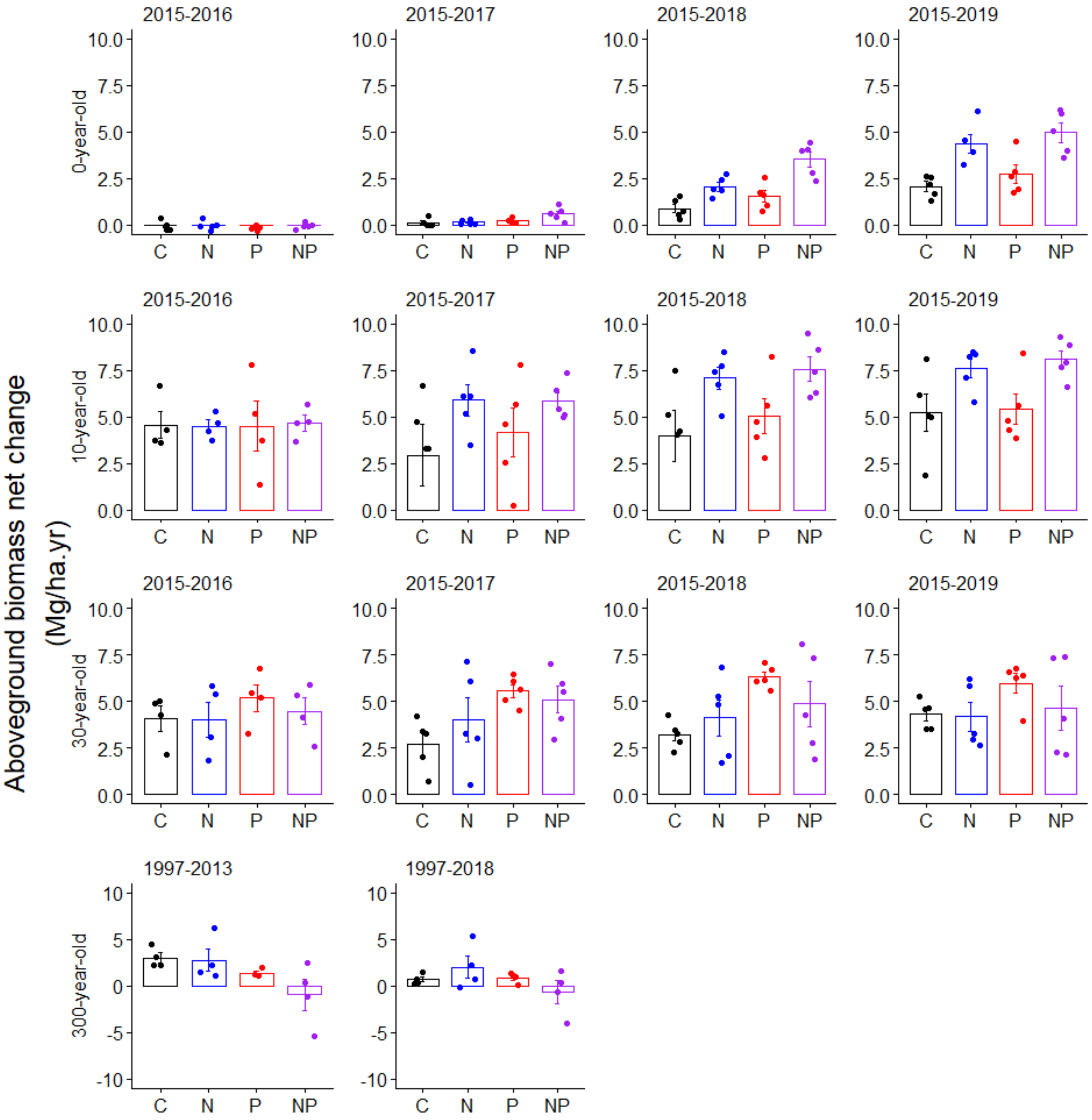

Figure A2.3 | Net change of aboveground biomass from the pre-fertilization census (2015) to fertilization censuses (2016, 2017, 2018, 2019) in each forest.

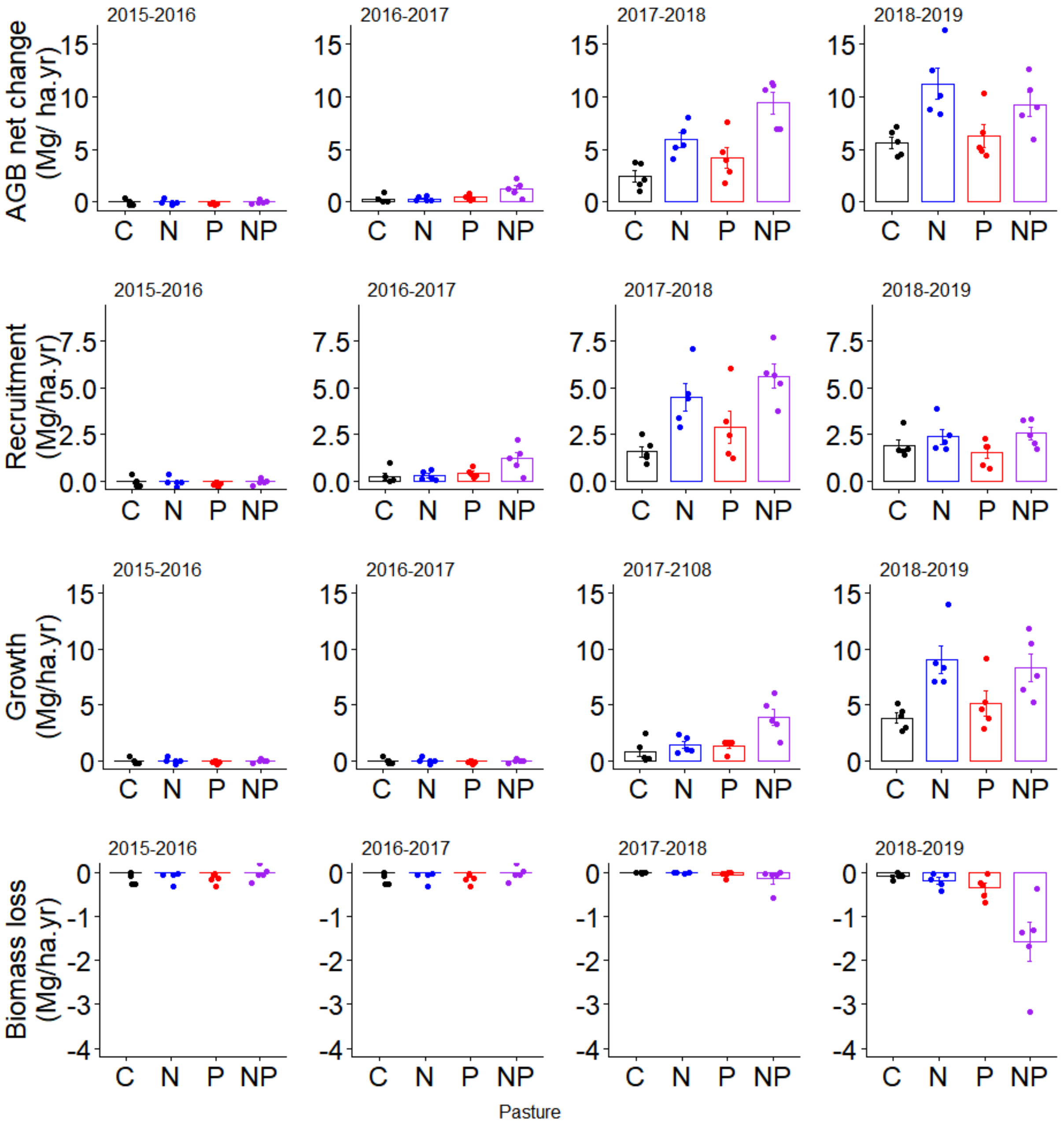

Figure A2.4 | The effect of nutrient addition on aboveground biomass (AGB) and its dynamics (recruitment, growth, and mortality) in 0-year-old forest for each annual census interval following onset of fertilization.

In the figure, different treatments were represented using different colors. Black columns and dots, control treatment (no nutrient addition); blue columns and dots, nitrogen addition treatment; red columns and dots, phosphorus addition treatment; purple columns and dots, nitrogen plus phosphorus treatment.

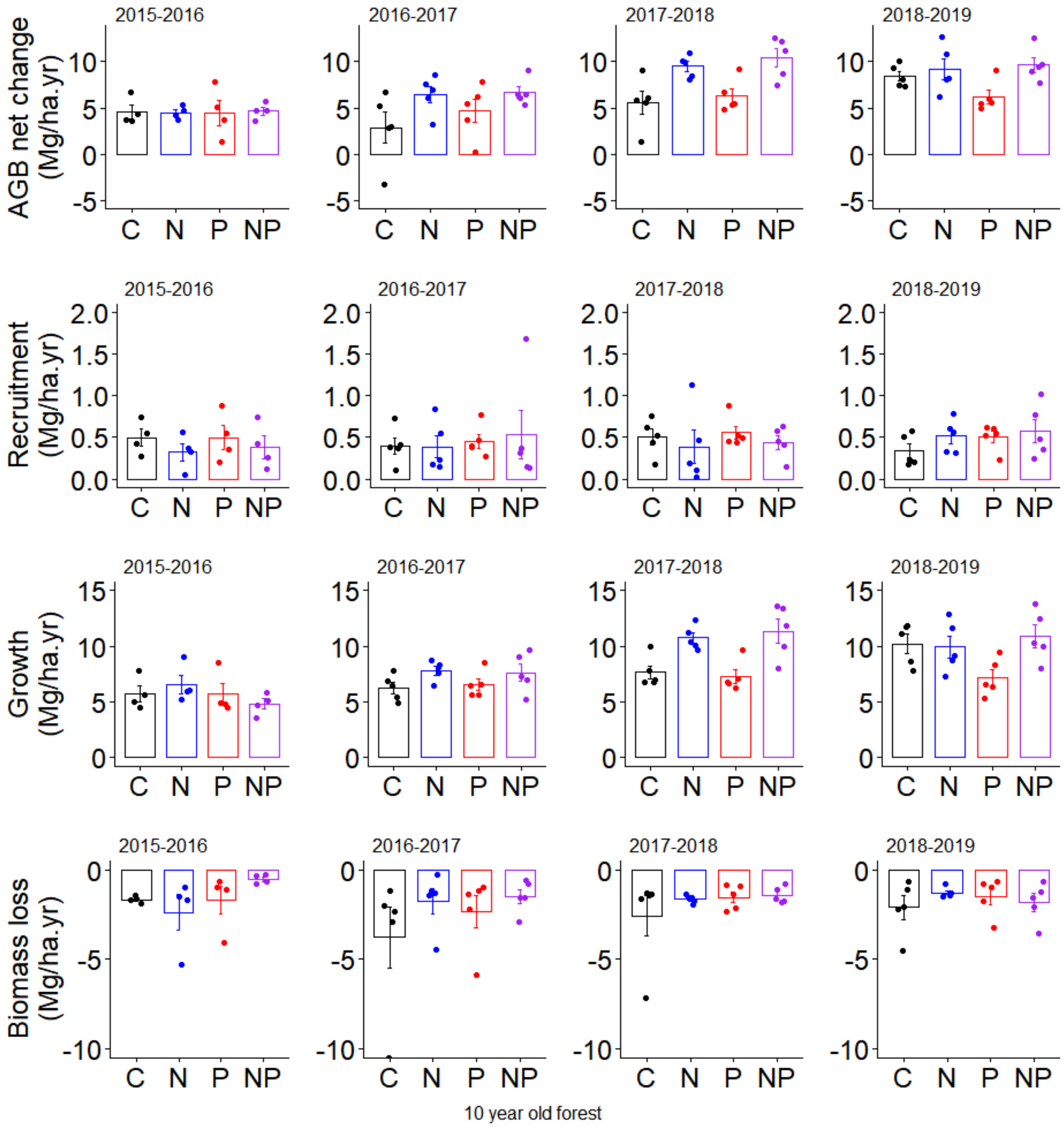

Figure A2.5 | The effect of nutrient addition on aboveground biomass (AGB) and its dynamics (recruitment, growth, and mortality) in 10-year-old forest for each annual census interval following onset of fertilization.

In the figure, different treatments were represented using different colors. Black columns and dots, control treatment (no nutrient addition); blue columns and dots, nitrogen addition treatment; red columns and dots, phosphorus addition treatment; purple columns and dots, nitrogen plus phosphorus treatment.

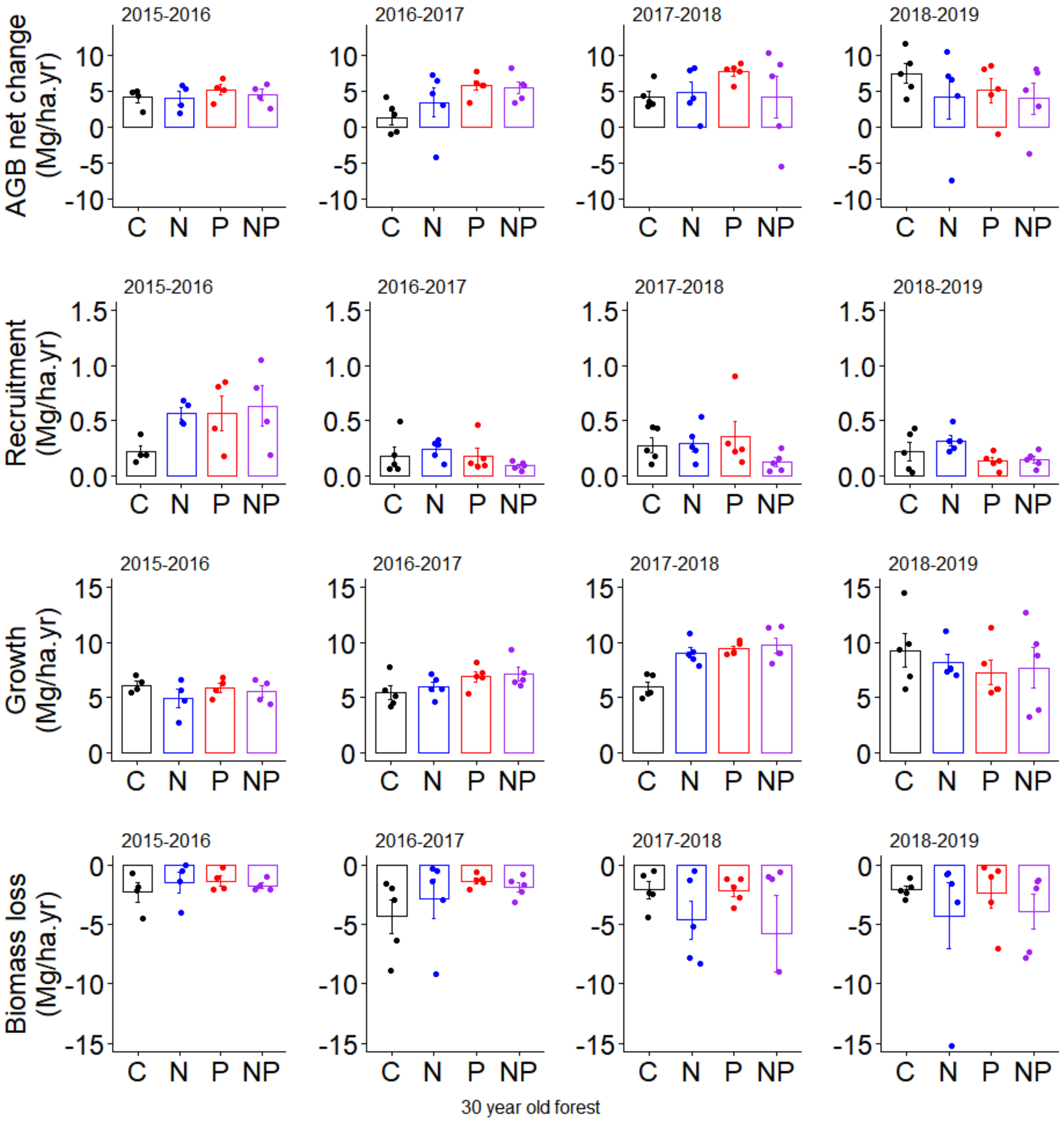

Figure A2.6 | The effect of nutrient addition on aboveground biomass (AGB) and its dynamics (recruitment, growth, and mortality) in 30-year-old forest in each annual interval following onset of fertilization.

In the figure, different treatments were represented using different colors. Black columns and dots, control treatment (no nutrient addition); blue columns and dots, nitrogen addition treatment; red columns and dots, phosphorus addition treatment; purple columns and dots, nitrogen plus phosphorus treatment.

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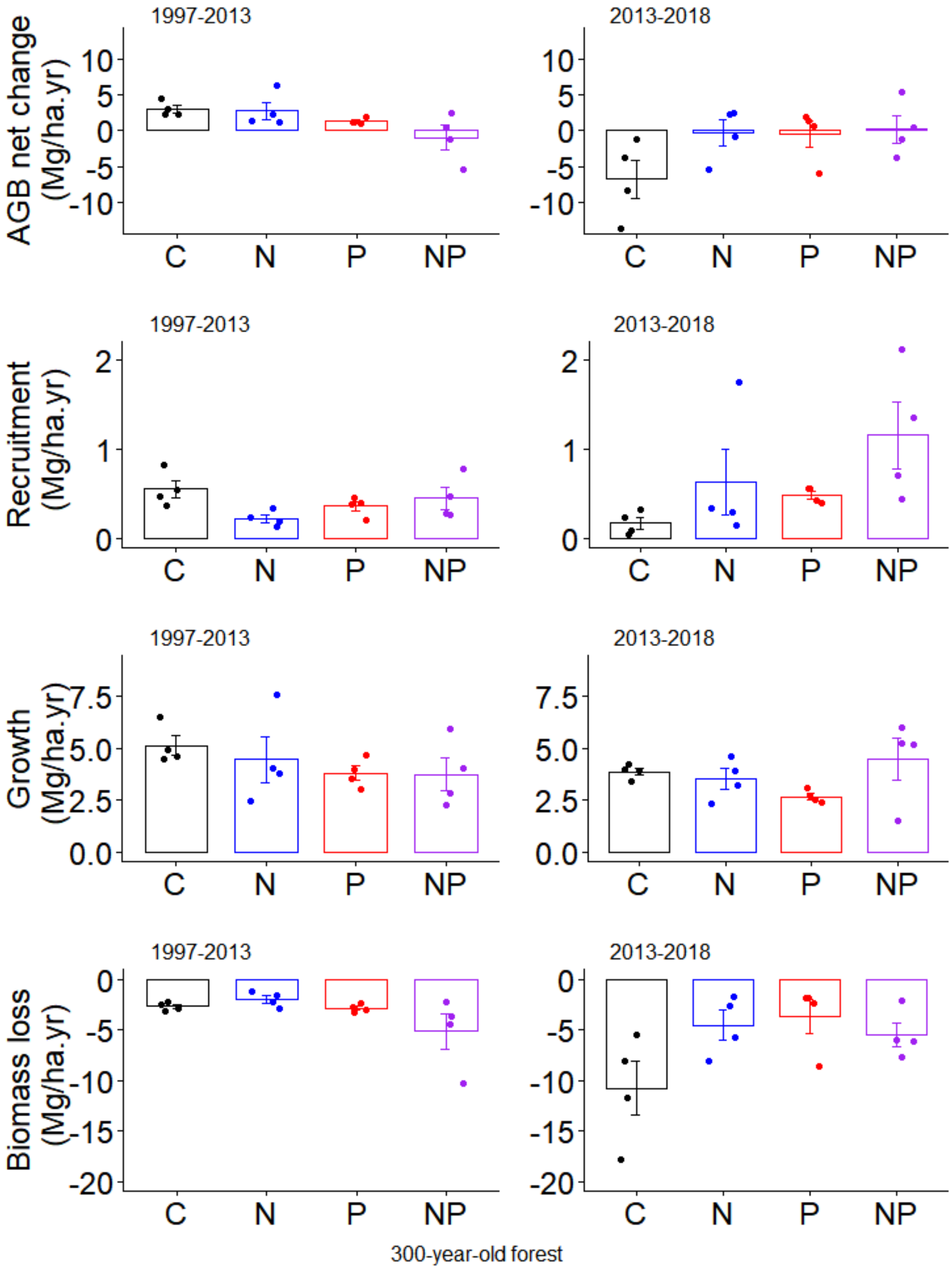

2538

2539 **Figure A2.7 |** The effect of nutrient addition on aboveground biomass (AGB) and its dynamics
2540 (recruitment, growth, and mortality) in 300-year-old forest in multi-annual periods with nutrient
2541 addition.

2542 In the figure, different treatments were represented using different colors. Black columns and dots,
2543 control treatment (no nutrient addition); blue columns and dots, nitrogen addition treatment; red
2544 columns and dots, phosphorus addition treatment; purple columns and dots, nitrogen plus phosphorus
2545 treatment.

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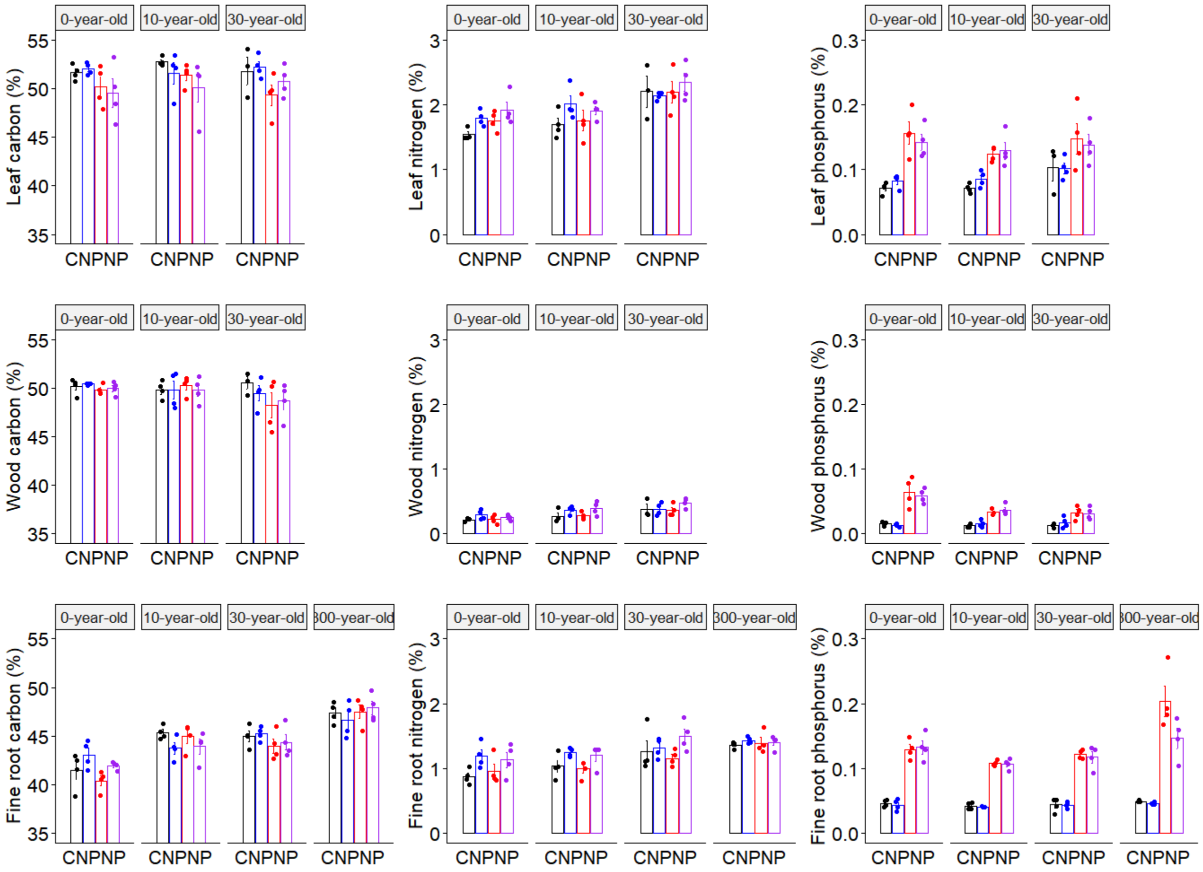

Figure A4.1 | The response of nutrient concentrations (carbon, nitrogen, and phosphorus) in tissues (leaves, wood, and fine roots) to nutrient addition over forest succession.

In the x-axis of each sub-figure, C, N, P, and NP stand for control, nitrogen addition, phosphorus addition, and nitrogen plus phosphorus addition, respectively. Find the statistical analysis results in **Table A4.3**.

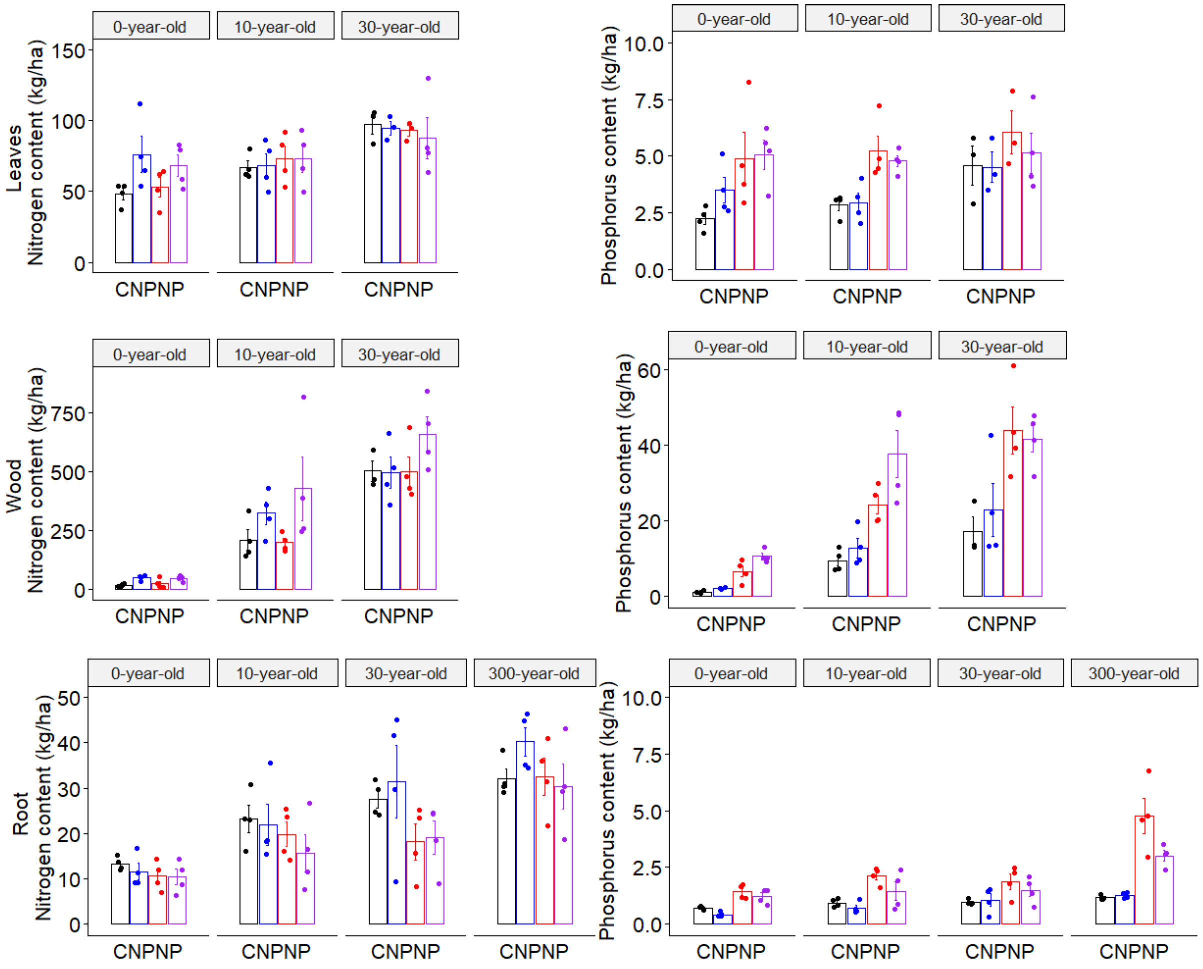

Figure A4.2 | The response of nutrient contents (nitrogen, and phosphorus) in tissues (leaves, wood, and fine roots) to nutrient addition over forest succession.

In the x-axis of each sub-figure, C, N, P, and NP stand for control, nitrogen addition, phosphorus addition, and nitrogen plus phosphorus addition, respectively. Find the statistical analysis results in **Table A4.5**.

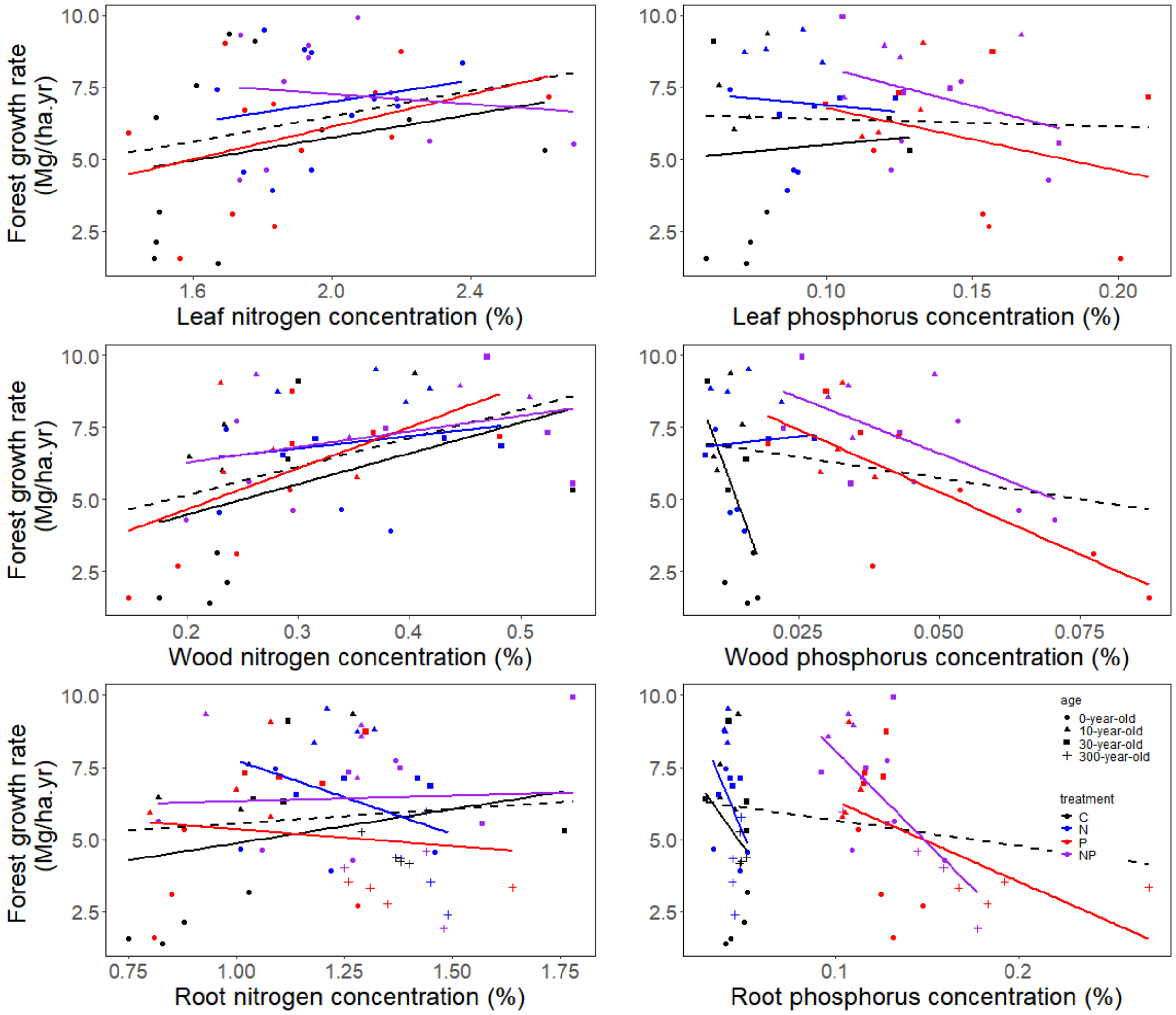

Figure A4.3 | The relationships between forest growth of all census period and nutrient concentration in tissues change in response to nutrient addition over forest succession.

In the figure, C, N, P, and NP stand for control (black solid lines), nitrogen addition (blue lines), phosphorus addition (red lines), and nitrogen plus phosphorus addition (purple lines), respectively. In each sub-figure, the black dash lines mean the relationship between forest growth and nutrient concentration in all treatment plots. Different shape of point stands for different forest age: circle is for 0-year-old forest; triangle is for 10-year-old forest; square is for 30-year-old forest; cross is for 300-year-old forest.

Table A2.1 | The statistical models of nutrient effect on AGB net change, Recruitment, Growth, and Mortality for all forest ages and censuses.

Variables	Models	Marginal R2	AIC
AGB Net change	factor(N)*factor(P)*factor(age)*factor(Interval) + (1|Block)	**0.6446339**	1233
Recruitment	factor(N)*factor(P)*factor(age)*factor(Interval) + (1|Block)	0.7138502	**560.8**
Growth	factor(N)*factor(P)*factor(age)*factor(Interval) + (1|Block)	0.7404324	**916.9**
Mortality	factor(N)*factor(P)*factor(age)*factor(Interval) + (1|Block)	0.7882741	598.6

Table A2.2 | The effects of nutrient addition on the aboveground biomass (AGB) net change and its dynamics (recruitment, growth, and mortality) across all census in all forest ages.

	Variables			
	AGB net change	Recruitment	Growth	Mortality
N	<0.05	<0.05	<0.0001	n.s.
P	<0.05	<0.05	n.s.	<0.05
age	<0.0001	<0.0001	<0.0001	<0.0001
Interval	<0.001	<0.0001	<0.0001	<0.0001
N:P	<0.1	n.s.	n.s.	<0.05
N:age	<0.0001	<0.0001	<0.05	<0.05
P:age	n.s.	<0.0001	<0.1	<0.0001
N:Interval	n.s.	<0.05	<0.05	n.s.
P:Interval	n.s.	n.s.	<0.05	n.s.
age:Interval	<0.0001	<0.0001	<0.0001	<0.0001

Table A3.1 | The statistical results of the effects of nutrient addition on fine root biomass, and the ratio of fine root biomass to aboveground biomass across forests.

	Variables	*p* values
Fine root biomass	factor(N)	<0.05
	factor(P)	<0.0001
	factor(age)	<0.05
	factor(N):factor(P)	n.s.
	factor(N):factor(age)	<0.05
	factor(P):factor(age)	n.s.
The ratio of fine root biomass to aboveground biomass	factor(N)	<0.0001
	factor(P)	<0.05
	factor(age)	<0.0001
	factor(N):factor(P)	n.s.
	factor(N):factor(age)	<0.0001
	factor(P):factor(age)	n.s.

Note: in this table, N means nitrogen addition treatment; P means phosphorus addition treatment; age stands for the forest ages; n.s. stands for no significant difference ($p>0.1$)

Table A4.1 | The ratio of selected species' aboveground to total aboveground biomass in the top canopy layer.

	0-year-old forest	10-year-old forest*	30-year-old forest*
C	82.44±1.11(n=4)	93.73±1.71(n=4)	81.48±4.96(n=3)
N	85.33±3.40(n=4)	90.87±3.18(n=4)	84.25±3.63(n=4)
P	85.35±1.09(n=4)	90.41±1.64(n=4)	87.84±2.85(n=3)
NP	87.31±1.02(n=4)	89.47±5.44(n=4)	89.72±1.43(n=4)

*In the 10 and 30-year-old forests, trees with > 5cm diameter at breast height were assumed as top canopy layer.

Table A4.2 | The statistical analysis results of the response of nutrient composition in different tissues to nutrient addition over forest succession.

	Leaf C:N	Wood C:N	Fine root C:N	Leaf C:P	Wood C:P	Fine root C:P	Leaf N:P	Wood N:P	Fine root N:P
factor(N)	<0.05	<0.05	<0.0001	<0.05	n.s.	<0.05	n.s.	<0.1	<0.0001
factor(P)	<0.05	n.s.	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
factor(plotage)	<0.05	<0.05	<0.0001	n.s.	<0.1	<0.05	n.s.	<0.05	<0.0001
factor(N):factor(P)	n.s.	n.s.	n.s.	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.
factor(N):factor(plotage)	=0.05	n.s.	n.s.	n.s.	n.s.	<0.1	n.s.	n.s.	n.s.
factor(P):factor(plotage)	n.s.	n.s.	n.s.	<0.05	n.s.	<0.05	<0.1	<0.1	<0.05

Table A4.3 | The statistical analysis results of the response of nutrient concentration in different tissues to nutrient addition over forest succession.

	Leaf C	Wood C	Fine root C	Leaf N	Wood N	Fine root N	Leaf P	Wood P	Fine root P
factor(N)	n.s.	n.s.	n.s.	<0.05	<0.0001	<0.0001	n.s.	n.s.	<0.05
factor(P)	<0.05	<0.05	n.s.	<0.1	n.s.	n.s.	<0.0001	<0.0001	<0.0001
factor(plotage)	n.s.	n.s.	<0.0001	<0.05	<0.05	<0.0001	n.s.	<0.05	<0.05
factor(N):factor(P)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
factor(N):factor(plotage)	n.s.	n.s.	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	<0.1
factor(P):factor(plotage)	n.s.	=0.05	<0.1	n.s.	n.s.	n.s.	<0.05	<0.05	<0.05

Table A4.4 | The statistical analysis results of the response of the ratio of nutrient content in among different tissues to nutrient addition over forest succession.

	Ratio of leaf N content to fine root N content	Ratio of fine root N content to wood N content	Ratio of leaf N content to wood N content	Ratio of leaf P content to fine root P content	Ratio of fine root P content to wood P content	Ratio of leaf P content to wood P content
factor(N)	<0.1	<0.0001	<0.0001	<0.05	<0.0001	<0.05
factor(P)	<0.05	<0.05	n.s.	<0.05	<0.05	<0.0001
factor(plotage)	<0.1	<0.0001	<0.0001	n.s.	<0.0001	<0.0001
factor(N):factor(P)	n.s.	n.s.	n.s.	<0.1	n.s.	n.s.
factor(N):factor(plotage)	n.s.	<0.05	<0.1	n.s.	<0.05	n.s.
factor(P):factor(plotage)	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05

Table A4.5 | The statistical analysis results of the response of nutrient content in different tissues to nutrient addition over forest succession.

	Leaf N	Wood N	Fine root N	Leaf P	Wood P	Fine root P
factor(N)	<0.1	<0.0001	n.s.	n.s.	<0.05	<0.05
factor(P)	n.s.	n.s.	<0.05	<0.0001	<0.0001	<0.0001
factor(plotage)	<0.05	<0.0001	<0.0001	<0.1	<0.0001	<0.0001
factor(N):factor(P)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
factor(N):factor(plotage)	<0.05	<0.05	n.s.	<0.1	<0.05	n.s.
factor(P):factor(plotage)	n.s.	n.s.	n.s.	<0.1	<0.05	<0.05

Supporting document

Summary of fertilization experiments in tropical forest

Site	Fertilizer	Forest age (years)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
San Carlos de Rio Negro, Venezuela	NPK	0, lowland	*Not studied*	*Not studied*	Increases with NPK addition	*Not studied*	Uhl 1987
Yucatan, Mexico	Factorial N and P	10, lowland	P addition increased foliar and litter P	Increased with NP addition	Increases with N, P and NP addition	*Not studied*	Campo & Dirzo 2003; Campo & Vazuez Yanes 2004; Campo et al., 2007
		60, lowland	NP increased foliar and litter P	Increased with NP addition	Increases with N, P and NP addition	*Not studied*	
Igarape Acu, Para, Brazil	Complete fertilizer combined with -1 treatments	0, lowland	P addition increased foliar P. Litter not studied	*Not studied*	Increased with P addition	*Not studied*	Gehring et al. 1999
Paragominas, Para, Brazil	Factorial N and P	6, lowland	P (N) addition increased foliar P (N). Litter not studied	*Not studied*	Increased with N addition	*Not studied*	Davidson et al.2004
Paragominas, Para, Brazil	P	24,lowland	*Not studied*	*Not studied*	Insignificant	*Not studied*	Markewitz et al., 2012
AFEX, Manaus, Brazil	Complete	Old growth, lowland	*Not studied*	P addition increased litterfall	Insignificant	P addition decreased fine root biomass	Cunha et al., 2022
DHSBR, Guangdong Province, Southern China	Factorial N and P	75 years pine forest, Lowland	*Not studied*	*Not studied*	*Not studied*	P addition increased fine roots, NP addition increased fine roots	Zhu et al., 2013
		75 years mixed pine and broadleaf forest, lowland	*Not studied*	*Not studied*	*Not studied*	P addition increased fine roots, NP addition increased fine roots	
		Old-growth (>400 years) forest, lowland	*Not studied*	*Not studied*	*Not studied*	N addition decreased fine roots biomass,	

Site	Fertilizer	Forest age (years)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
Hawaii*	Factorial N and P	Old growth in 300 years soil, montane	Litter and foliar concentration increased with N addition	Leaf litterfall increased with N and NP addition	Increased with N addition	Fine root biomass decreased after 1.5 yrs N addition	Vitousek et al., 1993; Vitousek and Farrington, 1997; Ostertag, 2001; Harrington et al., 2001
		Old growth in 20000 years soil, montane	*Not studied*	*Not studied*	*Not studied*	*Not studied*	
		Old forest in 4.1 × 10^6 year soil, montane	Litter and foliar concentration increased with P addition	N addition increased leaf litterfall; P addition increased other litterfall	Increased with P addition	BNPP increased after P addition, root turnover rate increased after nutrient addition	
Luquillo Experimental Forest, Puerto Rico	Control, N	Old forest, montane	*Not studied*	*Not studied*	Insignificant	Live fine roots declined with fertilization	Cusack et al., 2011
		Old forest, lowland	*Not studied*	*Not studied*	Insignificant	Live fine roots declined with fertilization	

Site	Fertilizer	Forest age (years)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
Bombuscaro, Ecuador	Factorial N and P	Old growth, montane	*Not studied*	fine litter decreased after the first year of P addition at all elevations, whereas N and N + P addition had positive effects on litter production	N and NP increased growth	Nutrient addition, especially P, decreased fine root biomass	Homeier et al., 2012, 2013
San Francisco Reserve, Ecuador			Foliar N and P concentrations increased after N and P additions		N+P addition had some positive effect on growth	Nutrient addition, especially P, decreased fine root biomass	
Cajanuma, Ecuador						No change	
Parque Nacional Sierra Nevada,Venezuela	Factorial N and P	Old growth, montane	P concentrations significantly increased by P fertilization	Literfall significantly increased in NP plots in the 4th year, but not in the first 3 years	In N and P fertilized plots, trunk growth doubled	*Not studied*	Tanner et al., 1992
Jianfengling, Hainan Island, China	Factorial N and P	Old growth, montane	*Not studied*	*Not studied*	P addition increases small tree growth	*Not studied*	Jiang et al., 2018
		>60 year old secondary forest, montane	*Not studied*	*Not studied*	N addition increase the ANPP mainly growth (DBH>3cm)	*Not studied*	Ma et al., 2022
Xiaoliang, Guangdong,	Factorial N and P	75 years old mixed pine and broadleaf forest, lowland	Nutrient concentration increased following nutrient addition	*Not studied*	*Not studied*	*Not studied*	Mo et al., 2019
El Verde, Puerto Rico	complete	Old growth, lowland	*Not studied*	Increases with complete fertilizer	Insignificant	*Not studied*	Walker et al., 1996; Li et al., 2006

Site	Fertilizer	Forest age (years)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
La Selva, Costa Rica	complete	Old growth, lowland	*Not studied*	*Not studied*	Increases in high light with complete fertilizer	*Not studied*	Chou et al., 2017
Osa Peninsula, Costa Rica	Factorial N and P	Old growth, lowland	*Not studied*	*Not studied*	*Not studied*	N and NP addition increased fine root biomass	Cleveland and Townsend, 2006
Guanacaste, Costa Rica	Factorial N and P	~30 year old secondary forest, lowland	*Not studied*	No evident change	N and NP addition increase growth in wet year	P and NP addition increased fine root biomass	Waring et al., 2019
Limon, Costa Rica	Factorial N and P	Old growth, lowland	N (P) increased foliar N (P) in selected species	Insignificant	Increases with P for small trees. Trees>100 mm DBH unaffected	*Not studied*	Alvarez-Clare et al., 2013, 2015
Iguazu, Argentina	N and N and P together	Old growth, lowland	*Not studied*	*Not studied*	Increases in high light with NP addition	*Not studied*	Villagra et al. 2013
Korup, Cameroon	P	Old growth, lowland	P addition increased foliar and litter P	Insignificant	Insignificant	*Not studied*	Newbery et al., 2002
Tombopata, Peru	Factorial N and P	Old growth, lowland in Tombooata and montane in others	No evident change, Leaf P had some increase in some years	*Not studied*	N+P treatment increase growth for 10-20cm size	*Not studied*	Fisher et al., 2013
Tono, Peru					N+P treatment had some increase in growth		
San Pedro, Peru					N addition increase growth		
Wayqecha, Peru					N addition increase growth		
Barito Ulu, Central Kalimantan, Indonesia	Factorial N and P	Old growth, lowland	N, P and NP addition increased litter P and N	Increased with N, P and NP addition	No evident change	*Not studied*	Mirmanto et al., 1999

Site	Fertilizer	Forest age (yrs)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
Bukit, Singapore	Complete	Old growth, lowland	*Not studied*	*Not studied*	Nutrient addition increase seedling growth	*Not studied*	Burslem et al., 1995
Sabah, Malaysia	Complete	Old growth, lowland	NPK addition increased foliar nutrient concentrations	*Not studied*	Nutrient addition increase seedling growth	*Not studied*	Brearley., 2005
Gigante, Panama	Factorial N,P and K addition	Old growth, lowland	P addition increased foliar and litter P	Increased with P addition	Insignificant	K, NP addition reduced total fine-root biomass, but N addition did not change fine root biomass, nutrient addition increased fine root turnover	Kaspari et al., 2008; Wright et al., 2011, 2018; Mayer et al., 2014, Wurzburger and Wright, 2015; Yavitt et al., 2011
Agua Salud, Panama	Factorial N and P	0, lowland	P addition increased P in tissues; N addition increased N in tissues	*Not studied*	N and P addition increase growth	N addition decreased fine root biomass	This Study
		10, lowland		*Not studied*	N addition increases growth		
		30, lowland	P addition increased P in tissues	*Not studied*	P addition had some increase on growth	P addition decreased fine root biomass	

Site	Fertilizer	Forest age (yrs)	Nutrient Effects				Reference
			Tissue nutrient concentrations	Litter production	Tree/biomass growth	Fine roots	
Chiriqui, Panama	N	Old growth, montane	N addition increased foliar N	Annual fine litterfall increased only in the first year of N addition, leaf litterfall increased in both years	Overall stem growth and above-ground woody biomass production were not affected. But small dbh tree increase	Fine root biomass and production did not change. In organic layer, fine-root biomass decreased under N addition. At 10-20 cm in the mineral soil, fine-root biomass increase	Andersen et al., 2010; Adamek et al., 2009, 2011
Mor Ridge, Blue Montains, Jamaica	Factorial N and P	Old Growth, montane	N addition did not increase leaf N; P addition increase two species tree leaf P increase,	N addition increase leaf production	N addition increased wood growth; P addition increased one species wood growth	Nutrient addition increased fine root biomass	Tanner et al., 1990; Stewart, 2000
Mull Ridge, Blue Montains, Jamaica			N addition did not increase leaf N; P addition increase two species tree leaf P increase,	*Not studied*	*Not studied*		
Serrania de Macuira, Colombia	Factorial N and P	Old growth, montane	*Not studied*	*Not studied*	N, NP addition increase trunk growth	*Not studied*	Cavelier et al., et al., 2000

Note: * in Hawaii, 7 forests were fertilized (Wright, 2019), but three of them were mainly discussed.

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Soil physical and chemical properties in Agua Salud fertilization plots.

Year	Forest age	Treatment	pH (H2O)	NH4 (mg/kg)	NO3 (mg/kg)	Resin P (mg/kg)	Total C (%)	Total N (%)	Total P (mg/kg)
Pre-treatment	0	C	5.53 ± 0.14	9.32 ± 3.01	0.62 ± 0.3	0.84 ± 0.11	3.8 ± 0.16	0.3 ± 0.01	278 ± 30.56
Pre-treatment	0	N	5.52 ± 0.11	10.35 ± 3.06	0.58 ± 0.18	0.85 ± 0.2	3.9 ± 0.16	0.31 ± 0.02	294.4 ± 29.01
Pre-treatment	0	P	5.45 ± 0.13	10.31 ± 4.03	1.68 ± 0.95	1.16 ± 0.24	3.54 ± 0.18	0.28 ± 0.02	312.2 ± 25.77
Pre-treatment	0	NP	5.3 ± 0.24	16.51 ± 10.2	0.31 ± 0.09	0.59 ± 0.14	3.79 ± 0.11	0.3 ± 0.02	290.8 ± 33.6
Pre-treatment	10	C	5.31 ± 0.2	5.35 ± 1.26	0.2 ± 0.11	0.54 ± 0.08	3.79 ± 0.32	0.28 ± 0.02	277.2 ± 28.6
Pre-treatment	10	N	5.07 ± 0.12	5.64 ± 1.24	0.1 ± 0.1	0.57 ± 0.18	3.72 ± 0.23	0.27 ± 0.02	245 ± 17.75
Pre-treatment	10	P	5.29 ± 0.1	4.44 ± 1.13	0.07 ± 0.06	0.59 ± 0.09	3.97 ± 0.14	0.29 ± 0.01	260.6 ± 25.33
Pre-treatment	10	NP	5.31 ± 0.06	4.66 ± 0.55	0.09 ± 0.06	0.48 ± 0.11	4.07 ± 0.11	0.3 ± 0	238.8 ± 9.67
Pre-treatment	30	C	5.21 ± 0.21	7.61 ± 1.4	0.2 ± 0.13	0.77 ± 0.24	4.37 ± 0.11	0.32 ± 0.01	328.6 ± 33.41
Pre-treatment	30	N	5.18 ± 0.18	6.44 ± 1.22	0.25 ± 0.15	0.47 ± 0.16	4.03 ± 0.18	0.3 ± 0.02	272.6 ± 7.73
Pre-treatment	30	P	5.35 ± 0.17	6.59 ± 1.46	0.13 ± 0.05	1.03 ± 0.57	4.25 ± 0.32	0.33 ± 0.03	355 ± 46.58
Pre-treatment	30	NP	5.47 ± 0.17	6.89 ± 1.34	0.36 ± 0.29	0.78 ± 0.13	4.01 ± 0.18	0.32 ± 0.01	322 ± 20.83
2016	0	C	5.59 ± 0.11	6.28 ± 4.4	0.7 ± 0.22	0.53 ± 0.2	3.73 ± 0.26	0.32 ± 0.02	-
2016	0	N	5.65 ± 0.1	2.18 ± 0.28	0.95 ± 0.32	0.39 ± 0.17	3.51 ± 0.19	0.3 ± 0.02	-
2016	0	P	5.7 ± 0.08	2.32 ± 0.76	0.65 ± 0.08	1.71 ± 0.35	3.72 ± 0.25	0.3 ± 0.02	-
2016	0	NP	5.51 ± 0.14	3.06 ± 0.29	0.87 ± 0.22	3.19 ± 1.21	3.99 ± 0.18	0.33 ± 0.02	-
2016	10	C	5.46 ± 0.12	2.65 ± 0.48	0.67 ± 0.13	0.47 ± 0.12	3.82 ± 0.26	0.31 ± 0.02	-
2016	10	N	5.35 ± 0.15	4.51 ± 0.71	1.61 ± 0.74	0.43 ± 0.16	3.86 ± 0.22	0.3 ± 0.02	-
2016	10	P	5.4 ± 0.1	2.3 ± 0.53	0.58 ± 0.13	4.61 ± 3.07	4.32 ± 0.28	0.34 ± 0.01	-
2016	10	NP	5.55 ± 0.19	3.66 ± 0.55	0.56 ± 0.09	2.69 ± 0.65	4.39 ± 0.28	0.36 ± 0.03	-
2016	30	C	5.45 ± 0.13	3.81 ± 0.46	1.77 ± 1.09	0.25 ± 0.14	5.13 ± 0.4	0.38 ± 0.03	-
2016	30	N	5.38 ± 0.11	32.94 ± 27.24	2.93 ± 1.33	0.32 ± 0.18	4.69 ± 0.34	0.37 ± 0.02	-
2016	30	P	5.5 ± 0.14	3.05 ± 0.69	1.42 ± 0.45	4.42 ± 1.57	3.98 ± 0.17	0.36 ± 0.01	-
2016	30	NP	5.61 ± 0.15	4.75 ± 0.49	1.99 ± 0.4	3.4 ± 1.38	4.84 ± 0.18	0.41 ± 0.02	-

2017	0	C	5.64 ± 0.12	6.46 ± 3.88	0.88 ± 0.73	0.35 ± 0.12	4.12 ± 0.36	0.34 ± 0.03	308 ± 54.32
2017	0	N	5.71 ± 0.1	3.6 ± 0.93	0.7 ± 0.33	0.31 ± 0.1	4.24 ± 0.51	0.34 ± 0.03	243 ± 20.67
2017	0	P	5.7 ± 0.12	1.94 ± 0.26	0.1 ± 0.06	11.52 ± 7.41	3.77 ± 0.17	0.32 ± 0.02	342.75 ± 74.76
2017	0	NP	5.67 ± 0.09	3.3 ± 0.52	0.44 ± 0.15	14.86 ± 8.62	4.23 ± 0.24	0.35 ± 0.03	379 ± 49.2
2017	10	C	5.46 ± 0.15	4.62 ± 0.63	0.76 ± 0.49	0.48 ± 0.09	3.72 ± 0.17	0.29 ± 0.01	229 ± 15.14
2017	10	N	5.36 ± 0.11	4.88 ± 0.78	0.82 ± 0.34	0.17 ± 0.05	3.72 ± 0.2	0.28 ± 0.02	184.2 ± 16.04
2017	10	P	5.55 ± 0.05	5.5 ± 2.22	0.34 ± 0.17	10.45 ± 2.51	4.44 ± 0.23	0.34 ± 0.02	300.6 ± 49.98
2017	10	NP	5.49 ± 0.08	4.42 ± 0.18	0.54 ± 0.16	8.19 ± 1.38	4.38 ± 0.2	0.34 ± 0.02	243 ± 17.4
2017	30	C	5.32 ± 0.18	4.96 ± 0.85	0.86 ± 0.63	0.25 ± 0.04	3.96 ± 0.19	0.32 ± 0.01	253.2 ± 48.91
2017	30	N	5.35 ± 0.15	13.02 ± 7.26	1.3 ± 0.61	0.32 ± 0.09	4.29 ± 0.22	0.35 ± 0.02	217.4 ± 9.37
2017	30	P	5.68 ± 0.23	12.6 ± 7.69	2.1 ± 1.13	16.49 ± 9.22	4.15 ± 0.35	0.36 ± 0.03	362 ± 39.6
2017	30	NP	5.55 ± 0.15	5.78 ± 0.54	2.38 ± 0.75	31.47 ± 13.72	4.63 ± 0.21	0.39 ± 0.02	392.2 ± 25.45
2019	0	C	5.48 ± 0.1	0.55 ± 0.18	0.06 ± 0.02	0.31 ± 0.08	-	-	-
2019	0	N	5.47 ± 0.07	1.86 ± 0.21	0.89 ± 0.11	0.29 ± 0.11	-	-	-
2019	0	P	5.64 ± 0.11	0.67 ± 0.11	0.1 ± 0.05	7.35 ± 2.72	-	-	-
2019	0	NP	5.41 ± 0.08	1.53 ± 0.45	0.27 ± 0.07	4.27 ± 1.31	-	-	-
2019	10	C	5.39 ± 0.14	1.41 ± 0.5	0.24 ± 0.17	0.21 ± 0.03	-	-	-
2019	10	N	5.22 ± 0.17	3.34 ± 0.68	1.67 ± 1.13	0.13 ± 0.03	-	-	-
2019	10	P	5.28 ± 0.09	1 ± 0.25	0.17 ± 0.08	5.62 ± 3.09	-	-	-
2019	10	NP	5.31 ± 0.14	1.96 ± 0.19	0.47 ± 0.17	2.09 ± 0.23	-	-	-
2019	30	C	5.33 ± 0.14	2.08 ± 0.58	0.63 ± 0.26	0.31 ± 0.06	-	-	-
2019	30	N	5.36 ± 0.13	2.76 ± 0.55	1.02 ± 0.5	0.23 ± 0.08	-	-	-
2019	30	P	5.46 ± 0.13	2.05 ± 0.36	1.02 ± 0.46	4.71 ± 1.97	-	-	-
2019	30	NP	5.47 ± 0.13	1.99 ± 0.37	1.27 ± 0.24	11.23 ± 5.1	-	-	-

Note: in the table, N means nitrogen treatment; P stands for phosphorus treatment; ‘-’ means no data.

The allometric function selection

The allometric function is a good and commonly applied method to calculate the aboveground biomass. To improve the estimation of aboveground biomass, lots of works have been done to establish and delicate the allometric functions. Three kinds of methods, by doing a summary, were generated and widely used for the biomass estimation in different scales. First, global scale allometric functions, which were formed to estimate the aboveground biomass in the continent or global scale (Chave et al, 2004; Chave et al., 2014). Second, parameters modified global functions, which were formed according to the global functions and the climatic condition in the specific site, but these functions can be only applied to estimate the aboveground biomass in a specific location (Rutishauser et al., 2020). Third, the local allometric functions, which were established in some well-studied research sites and can be used to estimate the aboveground biomass of trees in the specific research site.

All these three methods were applied to estimate the aboveground biomass of Panama forests. Four models, by searching the previous research, can be potentially applied to our project (**Table S1**). These four models include two global models which are function 4 (named 'Chave M4' below) and function 7 (named 'Chave M7' below) in the paper conducted by Chave et al (2014), the parameter revised global model (named 'Rutishauser' below) which was formed and used in the paper led by Rutishauser et al (2020), and one multispecies allometric function (named 'van M2' below) which was presented in the paper conducted by van Breugel et al (2011). However, these four functions were established and suitable for different tree sizes and forests (see **Table S1**), and no research discussed before that if any one of these allometric functions can be used to estimate the biomass of forests across a whole successional gradient with the trees' diameter at breast varying from 1cm to meters.

Biomass estimated by different allometric functions can have a big difference. If we applied different allometric functions to estimate the aboveground biomass of forests at different successional stages and to assess the aboveground biomass accumulation and its dynamics, some systematic errors will exist. Therefore, in our project, we need to choose a common allometric function that can be applied to calculate plot scale aboveground biomass across whole successional forests in Panama. We applied these four allometric functions to three different species, which have different wood densities, with the diameter at breast height of each species varying from 1cm to 150cm, and evaluated the models' performance (**Figure S1**).

We supposed that the model ‘Rutishauser’ had the best prediction for aboveground biomass of trees living in the Panama primary forest and having large diameters at breast height (>5cm), and the model ‘van M2’ had the best performance for the aboveground biomass of trees living in secondary forests in Panama and having small diameters at breast height (<30cm).

The aboveground biomass of large trees estimated by different allometric functions show a big difference (A, B, D, E, G, and H in **Figure S1**). The ‘Van M2’ model has a much lower estimation than other models’ estimations for large trees (diameter at breast height > 20cm). Therefore, this model is not suitable for the primary forest which has giant trees. In addition, looking at the small trees (C, F, and I in **Figure S1**), the aboveground biomass estimated by ‘Chave M7’ and ‘Chave M4’ is close to the aboveground biomass estimated by ‘Van M2’ and are better than the ‘Rutishauser’ model estimation result. Therefore, we conclude that the models ‘Chave M7’ and ‘Chave M4’ can replace the model ‘Van M2’ to estimate the aboveground biomass of trees having small diameters at breast height. Furthermore, whilst the aboveground biomass estimated by ‘Chave M7’ and ‘Chave M4’ is similar, the ‘Chave M4’ model includes the tree height parameter and our dataset does not include tree height. Therefore, to summarize the above discussion, we can use the ‘Chave M7’ function to estimate the aboveground biomass in our project.

Reference

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Table S1 | Four allometric functions which can be applied to calculate plot scale aboveground biomass

Model	Function	Fitness	Region	DBH(cm)	Reference
Chave M4	AGB=0.0673*((WD*(DBH^2)*H)^0.976)	PF	GS	5-212	Chave et al (2014)
Chave M7	AGB=exp(-1.803 - 0.976*E + 0.976ln(WD) + 2.673ln(DHB) - 0.0299*((ln(DBH))^2))	PF	GS	5-212	Chave et al (2014)
Rutishauser	AGB=exp(-2.024 - 0.896 * E + 0.920*ln(WD)+2.795*ln(DBH)-0.0461*ln(DBH)^2)	PF	Gigante	5-212	Rutishauser et al(2020)
van M2	AGB=exp(-1.130 + 2.267 * ln(DBH) + 1.186*ln(WD))	SF	AS	<30	van Breugel et al(2011)

Note: PF stands for primary forest, SF stands for secondary forest, GS represents global scale, AS represents Agua Salud, WD means species wood density, and DBH means diameter of breast height. In the model 'Chave M4', H means tree height and can be estimated using function: H=e^(0.83654015+0.76*ln(DBH)-0.034*[ln(DBH)]^2) (see the function 6a in Chave et al (2014)).

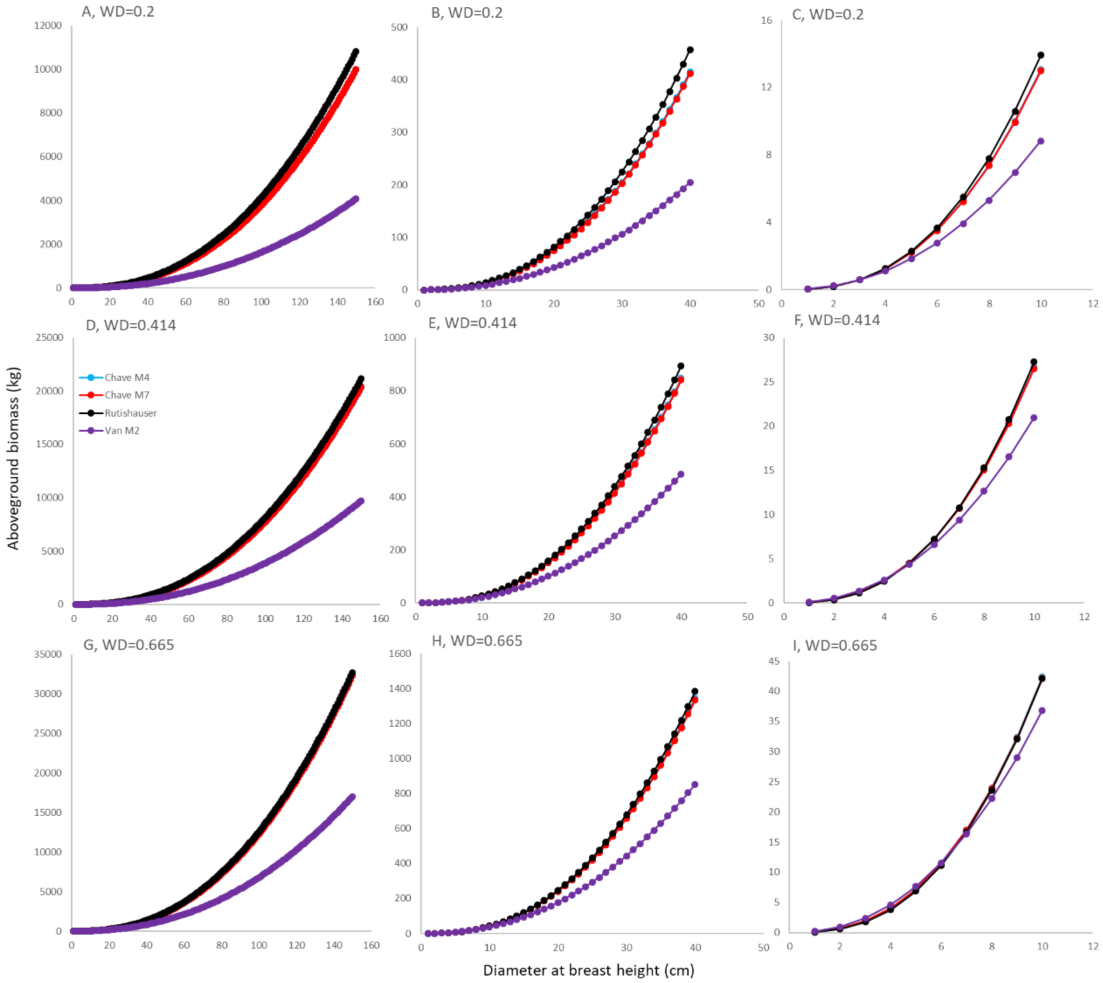

Figure S1 | The difference of biomass estimation using different allometric functions in different DBH range for three species.

In this figure, A, B, and C are for the species of which the wood density is 0.2. D, E, and F are for the species of which the wood density is 0.414. G, H, and I are for the species which has a wood density of 0.665. In each figure, the blue line is for the ‘Chave M4’ model, the red line is for the ‘Chave M7’ model, black line is for the ‘Rutishauser’ model, and purple line is for ‘Van M2’ model. A, D, and G are for DBH ranging between 1 and 150cm; B, E, and H are for DBH ranging between 1 and 40 cm; C, F, and I are for DBH ranging between 1 and 10cm.

Time efficiency in searching fine roots in tropical forests

Estimating fine root biomass is critical for precisely assessing the forest carbon sink and its biomass accumulation rate. However, fine root biomass is difficult to measure accurately. Picking fine roots from collected soil cores, at present, is a commonly applied method for fine root biomass estimation in forests, but this method is a time-consuming approach. There is a tradeoff between focusing efforts on collecting all the biomass from soil cores and collecting more soil samples that may represent the heterogeneity in the soil. To improve the time efficiency in fine roots picking, we applied a time interval method to search fine roots (Metcalfe et al., 2007).

To develop the time-interval approach for our site, we tested how much time it took to search through a whole soil sample and to pick out all fine roots. We collected two soil samples (5 soil cores for each sample, see **Methods** section in Chapter 3) from each of three plots in the 30-year-old forest where the fine root biomass was assumed to be similar to the fine root biomass of the mature forest, but be higher than fine root biomass of younger secondary forests. First, we flattened a soil sample from one plot on a large piece of white paper and spent six 20 minutes intervals (2 hours in total) picking fine roots. Second, we flattened a soil sample from the other plot on the white paper and accelerated the searching rate. We spent six 15 minutes (1.5 hours in total) picking the fine roots. Third, we flattened a soil sample from another plot and accelerated the searching rate again. We found that 10 minutes is enough for us to go through the soils and spent 65 minutes in total (15, 10, 10, 10, 10, and 10 minutes) to search fine roots in the soil. Therefore, we spent 65 minutes picking the rest three samples (from three plots). In each soil sample, the fine root searching rates were the same across intervals.

By comparing the fine root biomass collected using ≥ 65 minutes and using 65 minutes, we found fine root biomass has no difference between two time groups (p>0.1, paired t-test, **Figure S2**). Therefore, we applied 15, 10, 10, 10, 10, 10, and 10 minutes (65 minutes in total) interval approach to pick the fine roots in rest research plots (**Figure S3**). The fine roots biomass in our plots were estimated well because the curves are nearly flat after 45 minutes. The fine root biomass picked in 15 minutes, 25 minutes, 35 minutes and 45 minutes accounted for 82.3%, 89.0%, 93.1%, and 96% of the whole biomass collected within 65 minutes, respectively (**Figure S3**).

To minimize the systematic error of different people having different fine root picking rates, all these works were conducted by the first author alone.

Reference

Metcalfe, D., M. Williams, L. Aragão, A. Da Costa, S. De Almeida, A. Braga, P. Gonçalves, J. De Athaydes, S. Junior and Y. Malhi (2007). "A method for extracting plant roots from soil which facilitates rapid sample processing without compromising measurement accuracy." *New Phytologist* **174**(3): 697-703.

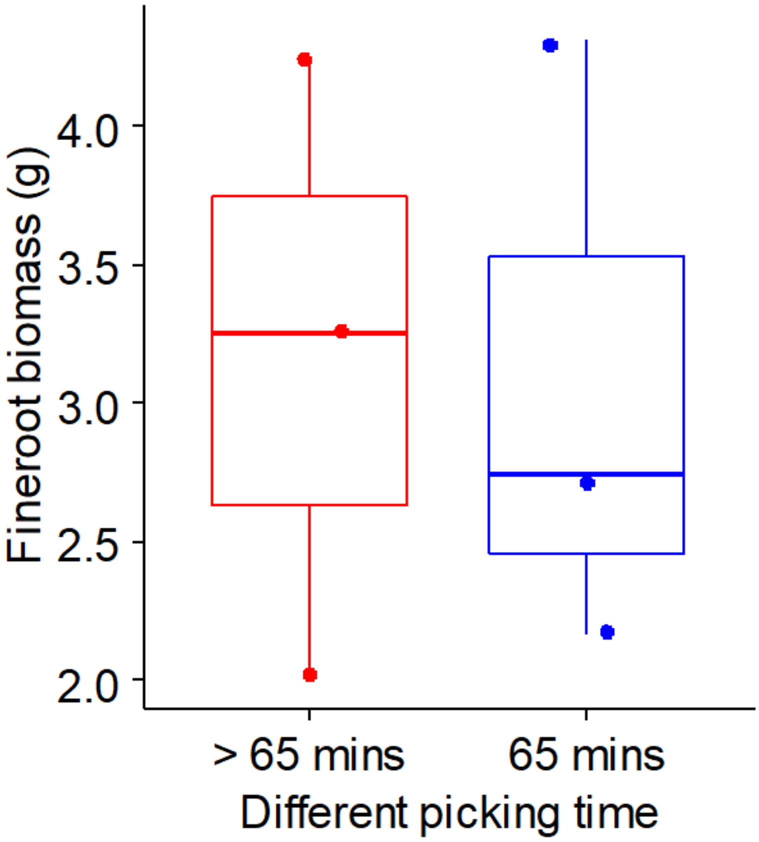

Figure S2 | The comparison of fine root picking using ≥ 65 minutes (≥ 65 mins in red colour) and using 65 minutes (65 mins in blue colour). ($p>0.1$, paired t-test)

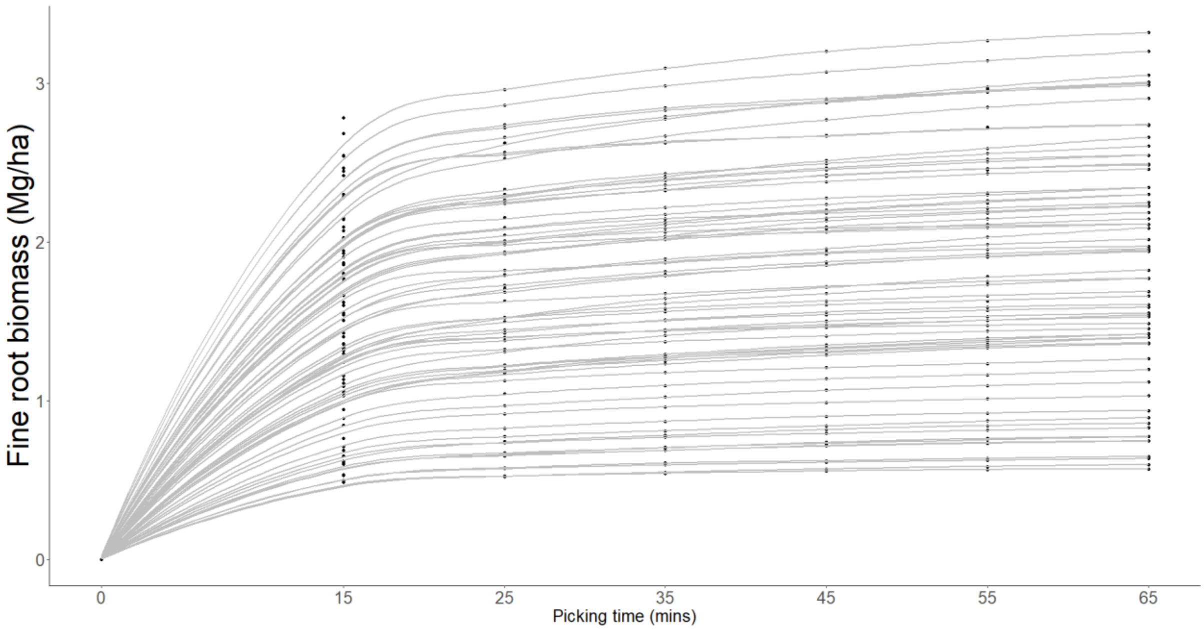

Figure S3 | The curves of fine root biomass accumulation in different picking times in each plot.