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Rapid responses of root traits and productivity to phosphorus and cation additions in a tropical lowland forest in Amazonia

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Summary

- Soil nutrient availability can strongly affect root traits. In tropical forests, phosphorus (P) is often considered the main limiting nutrient for plants. However, support for the P paradigm is limited, and N and cations might also control tropical forests functioning.
- We used a large-scale experiment to determine how the factorial addition of nitrogen (N), P and cations affected root productivity and traits related to nutrient acquisition strategies (morphological traits, phosphatase activity, arbuscular mycorrhizal colonisation and nutrient contents) in a primary rainforest growing on low-fertility soils in Central Amazonia after one year of fertilisation.
- Multiple root traits and productivity were affected. Phosphorus additions increased annual root productivity and root diameter, but decreased root phosphatase activity. Cation additions increased root productivity at certain times of year, also increasing root diameter and mycorrhizal colonisation. P and cation additions increased their element concentrations in root tissues. No responses were detected with N addition.
- Here we show that rock-derived nutrients determine root functioning in low-fertility Amazonian soils, demonstrating not only the hypothesised importance of P, but also highlighting the role of cations. The changes in fine root traits and productivity indicate that even slow-growing tropical rainforests can respond rapidly to changes in resource availability.

Key words: Amazon rainforest; arbuscular mycorrhiza; fine root productivity; large-scale nutrient fertilisation experiment; multiple nutrient limitation; phosphatase enzyme; root morphology.

Introduction

Tropical rainforests are the most diverse and productive terrestrial ecosystem on Earth (Beer *et al.*, 2010) representing a terrestrial carbon (C) sink of 2.89 ± 0.6 Pg C per year (Pan *et al.*, 2011), with the Amazon forest alone storing about one quarter of global terrestrial C sinks (Le Quéré *et al.*, 2018). Moreover, tropical net primary production (NPP) may be further stimulated under atmospheric CO_2 enrichment (Kimball & Idso, 1983; Ainsworth & Long, 2004; Norby *et al.*, 2005). Future CO_2 uptake could, however, ultimately be controlled by the amount of available nutrients in the soil to support new growth (Hungate *et al.*, 2006; Fleischer *et al.*, 2019) as well as by how efficiently plants can acquire and use nutrients. In temperate forests, nitrogen (N) is

usually considered to limit plant growth, whereas phosphorus (P), or other rock-derived elements are considered more likely to be the limiting nutrient in tropical lowland forests (Walker & Syers, 1976; Vitousek & Sanford, 1986; Wardle, 2004). Phosphorus and cations, are supplied to soil predominantly by weathering of the parent material (Walker & Syers, 1976), and are essential in several metabolic process of plants, such as ATP production, stability of cells and enzyme activation (Aerts & Chapin, 1999; Lambers *et al.*, 2006; Hawkesford *et al.*, 2012). Approximately 60% of the Amazonian forests grow in highly-weathered soils, characterised by very low concentrations of rock-derived P and cations, with evidence for P affecting plant growth (Aragão *et al.*, 2009; Quesada *et al.*, 2010, 2012). However, even in tropical forests, N availability may be important in controlling key aspects of forest function (Wright *et al.*, 2011; Wright, 2019), and/or greater N availability could help alleviate limitation by other elements (Chen *et al.*, 2020). Therefore, there remain major gaps in our understanding of the role different elements play in controlling tropical forest function, especially in Amazonia.

Plants can adapt their root morphological, physiological, biochemical and molecular properties to optimise nutrient acquisition (Chapin, 1980; Bloom *et al.*, 1985; Aerts, 1999; Raghothama, 1999; Addo-Danso *et al.*, 2020). Because of the low mobility of P in soils, roots usually move towards P, getting thinner and longer to facilitate the exploration of greater soil volume in P patches (Hodge, 2004; Lambers *et al.*, 2008; Metcalfe *et al.*, 2008; McCormack & Iversen, 2019). Alternatively, roots displaying more conservative morphological features (*i.e.* lower specific root length - SRL, greater diameter) may invest more in mycorrhizal associations to meet nutrient demands (Hodge, 2004; Comas *et al.*, 2014; Eissenstat *et al.*, 2015; Liu *et al.*, 2015; Kong *et al.*, 2016; Ma *et al.*, 2018). The very fine hyphal network typical of arbuscular mycorrhizas (AM) allows the fungi to forage for P away from P-depleted zones around roots, resulting in high inorganic P uptake in exchange for photosynthetically fixed C from the host plant (Hodge, 2004; Smith & Read, 2010; Eissenstat *et al.*, 2015). There is also evidence for the role of AM in acquiring other elements, such as Ca, Mg, K and sulphur (Siqueira *et al.*, 1998; Zangaro *et al.*, 2003) and micronutrients such as zinc and copper (Smith & Read, 2010). The main source of P in low-fertility tropical soils is, however, bound in organic compounds or occluded in secondary minerals (Walker & Syers, 1976; Cross & Schlesinger, 1995; Quesada *et al.*, 2010) and, consequently, they need to be degraded before being assimilated by roots (Lambers *et al.*, 2006). The hydrolysis of organic P happens mainly through the activity of phosphatase enzymes released by microbes and plant roots (Hinsinger, 2001; Treseder & Vitousek, 2001; Vance *et al.*, 2003;

Olander & Vitousek, 2004). Therefore, strong investment in the production of phosphatase enzymes that can become bound to root surfaces or released into the soil matrix may be necessary to mine organic P in these forests (Liu *et al.*, 2015; Kong *et al.*, 2016; Lugli *et al.*, 2020).

Plant trait-based approaches are especially useful tools to increase understanding of plant function in species-rich environments, such as tropical forests. Although tropical trees may use a range of complementary adaptations to optimise P-uptake (Zemunik *et al.*, 2015; Lugli *et al.*, 2020), it remains uncertain how plastic these strategies are in response to short-term changes in the availability of different nutrients. Root functional traits are considered to represent a balance between maximising the acquisition of limiting resources and minimising the costs of root tissue construction and maintenance (Bloom *et al.*, 1985; Aerts & Chapin, 1999; Wurzburger & Wright, 2015; McCormack & Iversen, 2019). For example, about 20% of plant C could be transferred to AM fungi associates, whilst root exudates (*i.e.* organic acids, enzymes) can represent up to half of belowground C allocation (Bago *et al.*, 2003; Lynch *et al.*, 2005; Parniske, 2008). Therefore, trade-offs between uptake strategies are likely, with plant investment in root biomass and nutrient uptake strategies usually increasing with decreasing supply of the limiting nutrient (Bloom *et al.*, 1985). In naturally P-poor soils in Central Amazon, Lugli *et al.* (2020) demonstrate that due to the different levels of soil P availability in different pools (*i.e.* organic and inorganic P), plants need to invest in multiple P-uptake mechanisms.

Nutrient manipulation experiments greatly contribute to directly testing for nutrient limitation in terrestrial ecosystems (Cleveland *et al.*, 2011; Sullivan *et al.*, 2014; Wright *et al.*, 2018). Although the hypothesis of P-limitation in tropical forests is widely accepted, clear evidence from large-scale experiments is variable and limited (Yavitt *et al.*, 2011; Mirabello *et al.*, 2013; Wurzburger & Wright, 2015; Alvarez-Clare & Mack, 2015; Wright, 2019). In a recent meta-analysis, Wright (2019) compiled data from 48 nutrient manipulation experiments in tropical forests and concluded that N and P limitation are widespread, but no evidence was found for a greater role for P than N, and it is uncertain how other nutrients, including cations, affect these ecosystems. Furthermore, root responses are particularly poorly understood, with nutrient addition experiments in Central America tending to have not measured productivity responses and having observed contrasting changes in standing stocks and root traits. For example, after two years of nutrient addition, root biomass (<2 mm diameter) decreased with K addition but increased with P addition for thicker roots (2-5 mm diameter) in Panama (Yavitt *et al.*, 2011), and no root biomass responses were detected in Costa Rica (Alvarez-Clare & Mack, 2015). In these same experiments,

changes in fine root morphology following P addition were observed in Panama, with roots becoming less dense and with greater specific root length (Wurzburger & Wright, 2015), whilst increased root nutrient concentrations were detected in Costa Rica (Alvarez-Clare & Mack, 2015). However, current experiments in Neotropical forests are located on natural soils with total P concentrations ranging from 400-1,600 mg kg^{-1} (Wright *et al.*, 2011; Alvarez-Clare *et al.*, 2013). In contrast, in the dominant soil type across Amazonia, the world's largest tropical forest, total P ranges from 100-200 mg kg^{-1} (Quesada *et al.*, 2010). Given the range of responses observed in these Neotropical studies and the differing soil fertilities, we clearly cannot extrapolate to how fine root traits and productivity are controlled by soil nutrient status in Amazonian forests.

We used the first large-scale nutrient manipulation experiment installed in Central Amazonian forests (the Amazon Fertilisation Experiment; AFEX) to determine whether key nutrient uptake mechanisms adopted by fine roots were altered by the factorial addition of N, P and cations (Ca, Mg and K) in low-fertility soils. Our study quantified the short-term responses in the first year of manipulations, thus investigating how rapidly roots can respond to the addition of the different nutrients. We hypothesized that given the low availability of P in soils at our site, there would be a strong and immediate effect of P addition on root traits and productivity, but that N addition would have limited impacts. This is based on the high C-costs of production and maintenance of fine roots as well as allocation towards nutrient uptake strategies. Thus, we expected that fertilisation would decrease plant investment in such traits. Consequently, we predicted that with P addition alleviating belowground P limitation, there would be decreased root productivity, together with a reduction in root phosphatase activities and AM colonisation, with morphological changes reflecting shifts from acquisitive to more conservative traits, decreasing, for example, SRL and SRA and increasing tissue density and mean diameter. Furthermore, due to the very low concentrations of cations in Central Amazonian soils, we also expected that cations would trigger changes in root traits, shifting from acquisitive to more conservative morphological traits, but with no effect on root phosphatase activity.

Materials and Methods

Site description and experimental design

This study was carried out within the AFEX experiment in Central Amazonia, installed ca. 70 km north of Manaus/Amazonas, Brazil in the area of the Biological Dynamics of Forest Fragments Project (BDFFP) Reserve at ZF-3, a collaborative project between the National Institute for

Amazonian Research (INPA) and the Smithsonian Institute (STRI). Mean air temperature is 26 ºC and mean annual precipitation is 2,400 mm (Araújo *et al.*, 2002). The vegetation is an old growth, lowland *terra firme* forest, associated with clay-rich (75%) Ferralsols and very low total P content (~ 85 mg kg^{-1} for the 0-30 cm soil depth). AFEX is composed of thirty-two 50 m x 50 m plots separated at least 50 m from each other and distributed in four blocks. Each of the four blocks (installed at least 300 m apart) includes eight plots representing seven nutrient addition treatments and one control applied in a factorial design: control (with no addition of nutrients), N, P, cations (Ca, Mg, K), N+P, N+cations, P+cations, and N+P+cations. All plots (n=4 for each treatment and control) were established in areas with similar soil, vegetation, and terrain, being restricted to plateaus.

Nutrient additions are split into three equal applications over the course of each wet season, with nutrients added every year since 2017 at the following total rates: (1) N: 125 kg ha^{-1} yr^{-1} as Urea; (2) P: 50 kg ha^{-1} yr^{-1} as triple superphosphate, and (3) Cations: 160 kg ha^{-1} yr^{-1} as dolomitic limestone for Ca and Mg, plus 50 kg ha^{-1} yr^{-1} as potassium chloride for K. Aiming to make our data comparable to other nutrient fertilisation experiments, the amount and rates of nutrients added to our site follow rates proposed by Wright *et al.* (2011) in Panama. Dry fertilisers were applied to the soil surface by hand covering the whole plot area (50 m x 50 m), including the surface of the ingrowth cores. Our results represent the root responses to the first year of nutrient additions, and thus also investigate how rapidly trees can respond to changes in soil fertility.

Fine root productivity

Key monitoring measurements were limited to the central 30 m x 30 m (900 m^2 area) of each plot. In each plot (n=32), five 12 cm-diameter, 30 cm-deep, root-free ingrowth cores (2 cm plastic mesh) were installed in August 2017 in the central 30 m x 30 m plot area. Ingrowth cores were collected every three months after installation and the five core replicates were homogenised in the field by plot and by soil depth (0-10 and 10-30 cm; N=64) in each collection. Fine roots (< 2mm in diameter living roots) produced in the first year of nutrient addition (four ingrowth core campaigns from August 2017-September 2018) were used to determine productivity. All fine roots from the two soil depths were manually extracted during a period of 60 minutes in four intervals of 15 minutes and root-free soil reinserted into the existing holes (Metcalfe *et al.*, 2007). After sampling, roots were washed and cleaned by gently brushing to remove soil particles. The cumulative root biomass sampled at each time point (one sample for every 15 minutes = four

samples) was used to estimate the amount of roots that would be sampled after the 60 minutes sampling collection (Metcalfe *et al.*, 2007). We tested four different types of curves (logarithmic curve, Michaelis-Menten asymptotic curve, power law curve and asymptotic exponential curve) to extrapolate to the amount of roots that would be sampled during 180 minutes, choosing the curve that resulted in the best model fit (Michaelis-Menten asymptotic curve; Equation 1).

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

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

Fine root productivity was calculated as dry mass of roots produced per day for the entire ingrowth core sample and by depth (0-10 and 10-30 cm). Root net primary productivity was calculated summing the biomass of fine roots produced in each ingrowth core census and was expressed in Mg ha^{-1} $year^{-1}$.

Root morphology

Subsamples of fine roots from the ingrowth core campaign held in February 2018 (newly produced roots < 3 months old) were used to determine morphological traits. Fine roots from both soil depths (0-10 and 10-30 cm) were cleaned and fresh root samples (<2 mm diameter) were spread homogeneously in a plastic tray with approximately one quarter of the root biomass picked randomly for the subsequent scanning (Holdaway *et al.*, 2011). Roots were scanned at 600 dpi and images analysed using WinRHIZO (WinRHIZO Regular 2015, Regent Instruments, Canada) to provide root mean diameter, total length, area and volume, then samples were dried at 60 ºC for 72 hours to determine dry root mass. These were used to determine specific root length (SRL), specific root area (SRA), root tissue density (RTD) and mean root diameter (Metcalfe *et al.*, 2008). SRL (cm g^{-1}) was calculated as root length per unit root dry mass, SRA (cm^2 g^{-1}) was calculated as root superficial area per unit dry mass and RTD (g cm^{-3}) was calculated as root dry mass per unit root volume.

Root phosphatase activity

Root subsamples collected in February 2018 were analysed for root-surface potential acid phosphomonoesterase activity (phosphatase). Phosphatase was measured within 3 days of root sampling using triplicate subsamples per plot and per soil depth (0-10 and 10-30 cm) using a fluorimetric microplate assay (Turner & Romero, 2010; German *et al.*, 2011) as described in Lugli *et al.* (2020). About 10 mg of the root sample (washed, fresh weight basis) were incubated with Methylumbelliferyl-phosphate (MUF), which was used as an analogue substrate for the enzyme acid phosphomonoesterase. In addition, sample, buffer and substrate blanks were prepared. Samples were incubated for 30 min at ~ 25 ºC while gently shaking, then 50 µL of 1 M NaOH were added to all samples and standard vials to terminate the reaction. Aliquots of the sample solution were pipetted into a black 96-well microplate and 20 min after termination, fluorescence was read on a fluorometer (Tecan Infinite® 200 PRO, Grödig, Austria), at 365 nm excitation and 450 nm emission. Roots were removed from vials, rinsed with Milli-Q water, scanned and subsequently dried at 60 ºC for 72 hours. Root phosphatase activity per plot and depth was expressed in µmol MUF g^{-1} root dry mass h^{-1}.

Mycorrhizal colonisation

To determine AM colonisation, roots collected in February 2018 were subsampled, cleaned and scanned, and segments were stored in 50% ethanol. Only root fragments from the 0-10 cm soil layer were used for AM analyses. The clearing and staining processes were adapted for tropical roots based on Brundrett *et al.* (1984) and Wurzburger and Wright (2015). Briefly, roots were cleared using a 2.5% KOH solution and autoclaved at ~ 120 °C for ± 10 minutes, then placed in alkaline H_2O_2 solution for further bleaching for ± 30 minutes. Before staining, roots were acidified in 2% HCl solution for 30 minutes and were then added to a beaker with Trypan Blue 0.05% until constantly blue. Roots were rinsed in tap water and ten uniformly stained 1 cm root fragments per plot were mounted on slides to quantify total root length colonised by AM fungi (40 x optical) (McGonigleE *et al.*, 1990). Mycorrhizal colonisation was assessed as the percentage of the total root points along the root length that had any mycorrhizal fungi structures.

Nutrient concentration in fine roots

To ensure there was enough material for nutrient analysis, root material <2 mm diameter from all four collections spanning the first year of fertilisation (August 2017-September 2018) was bulked.

Dried and ground roots from each collection were composited by plot and soil depth. Analyses were performed at the Soil and Plant laboratory (LTSP) at the National Institute of Amazonian Research (INPA) in Manaus, Brazil, and followed established methods that have also been used to characterise variability in the plant and soil variables across the Amazon basin (Quesada *et al.*, 2010). Carbon and N contents were determined using an automatic C and N analyser (VARIO MAX CHN Element Analyzer) (Nelson and Sommers, 1996). Concentrations of P and cations in roots were analysed by nitroperchloric digestion described by Malavolta *et al.*, (1989). Phosphorus concentrations were determined by colorimetry (Anderson and Ingram, 1993), and quantified by spectrophotometry (UV-120-01, Shimadzu, Kyoto, Japan). Ca, Mg and K were determined by atomic absorption spectrophotometry (AAS, 1100 B, Perkin-Elmer, Ueberlingen, Germany).

Statistical analyses

Linear mixed-effect models were used to test the effect of added nutrients and their interaction in the factorial design N*P*cations. The presence/absence of each of the main nutrients were used as a fixed factor and the four blocks as random factor. All models were run in the R packages 'lme4' and 'lmerTest' (Bates *et al.*, 2014; Kuznetsova *et al.*, 2017). Full factorial models were simplified using backward elimination performed by the *step* function in 'lmerTest' package. The significant model was then re-run and only the significant effects of nutrient additions are reported. Since no significant interaction effects were detected between the different nutrients added, results are shown for single nutrient additions only, following Wright et al., (2011). To graphically assess the effect of specific nutrients, all plots where a specific nutrient was not added (*i.e.* –P; n=16) are compared to all plots where that nutrient was added (*i.e.* +P; n=16) (Wright et al. 2011). Results are shown for the whole soil core and for both soil depths separately, but since our aim was to detect the effect of the addition of different nutrients, depth was not used as a factor in the statistical models and differences between depths themselves are therefore not discussed in detail (Supporting Information; Table S1 and S2). Data were checked for normality and variance homogeneity and the selection for the best model was made based on functions from 'LMERConvenienceFunctions' R package (Tremblay & Ransijn, 2015). All analyses were conducted in R version 3.4.4 (R Core Team, 2018).

Results

Root productivity

After one year of nutrient addition, mean fine root productivity across all control plots (n=4) was 2.98 ± 0.33 Mg ha^{-1} year^{-1} (0-30 cm soil depth). Total root productivity for the 0-30 cm soil depth, significantly increased by 23% in P-addition plots compared to plots without added P (-P: 3.50 ± 0.30 *versus* +P: 4.31 ± 0.33 Mg ha^{-1} year^{-1}; $F_{1,24}$=4.67, p=0.04; Fig. 1). The significant increase in mean root productivity with P addition for the whole core was mainly driven by changes in the 0-10 cm soil layer (-P: 2.03 ± 0.15 *versus* +P: 2.64 ± 0.20 Mg ha^{-1} year^{-1}; $F_{1,24}$=6.62, p=0.017), with no significant effect in the 10-30 cm layer with the addition of any nutrient (Fig. 1). No significant effects were found for total root productivity with the addition of N or cations (Fig. 1). Although the addition of cations did not significantly affect annual root productivity, there were short-term effects of cations at certain times of the year. No interactions among nutrient treatments were found for root productivity in any sampling time. When analysing root productivity for the 3-month interval used for our root trait analyses (November 2017 – February 2018), the addition of cations increased fine root productivity by 52% for the whole 0-30 cm soil layer ($F_{1,26}$=8.28, p=0.008) and this increase was mainly driven by a significant effect detected for the 0-10 cm layer ($F_{1,26}$=12.32, p=0.002; Supporting Information Fig. S1).

Root morphological traits

Mean root diameter (0-30 cm) across control plots (n=4) was 0.99 ± 0.03 mm, SRL 1,310 ± 76 cm g^{-1}, SRA 311 ± 14 cm^2 g^{-1} and RTD 0.15 ± 0.007 g cm^{-3}. In plots where P was added, root diameter significantly increased in the 0-10 cm soil layer when compared to plots without P addition ($F_{1,26}$=4.78, p=0.038; Table 1), with no changes for the full 0-30 cm layer ($F_{1,25}$=3.61, p=0.07). The addition of cations increased mean root diameter from 1.03 to 1.12 mm for the whole 0-30 cm soil layer ($F_{1,25}$=8.55, p=0.007). The same trend was found for the 0-10 cm ($F_{1,26}$=3.78, p=0.06) and 10-30 cm ($F_{1,27}$=3.36, p=0.08) soil layer. For mean root diameter, the addition of N did not result in any changes for any soil layer. The addition of N, P and cations separately had no effect on SRL, SRA or RTD (Table 1).

Root phosphatase activity

Mean root phosphatase activity across control plots (n=4) was 40.80 ± 6.74 μmol g^{-1} h^{-1} for the 0-30 cm soil layer. Compared to plots without P, the addition of P significantly decreased root phosphatase activity only in the top 10 cm by 23% (-P: 41.84 ± 2.70 *versus* +P: 31.97 ± 2.95 μmol g^{-1} h^{-1}; $F_{1,27}$=7.30, p=0.01; Fig. 2). No significant changes in root phosphatase activity were detected with the addition of N, P or cations for the whole core (0-30 cm), although a decline of root phosphatase activity was captured with P addition (-P: 38.90 ± 2.52 *versus* +P: 33.21 ± 3.07 μmol g^{-1} h^{-1}; $F_{1,27}$=3.45, p=0.07; Fig. 2). When analysing soil layers separately, the addition of N or cations did not affect root phosphatase activity.

Mycorrhizal colonisation

Mean total root AM colonisation in control plots was 38.46 ± 4.75% for the 0-10 cm soil layer. The addition of cations increased total AM colonisation from 41.90% in plots where cations were not added to 50.40% with cation addition ($F_{1,27}$= 4.57, p=0.042; Fig. 3). Neither the addition of N nor the addition of P significantly affected root AM colonisation. No significant effects of nutrient addition were detected when analysing AM structures separately (Supporting Information; Table S3).

Nutrient concentration in fine roots

Mean C and N concentrations in roots growing in control plots were 43.82 ± 0.19 and 0.74 ± 0.13 %, and mean P, Ca, Mg and K concentrations were 0.46 ± 0.02, 0.92 ± 0.09, 0.84 ± 0.12, 2.80 ± 0.20 g kg^{-1} for the whole 0-30 cm soil layer. In plots where P was added, concentrations of P and Ca increased in roots growing in the 0-10, 10-30 and for the mean 0-30 cm soil layer (Fig. 4). Concentrations of P in roots more than doubled with P addition ($F_{1,27}$= 40.97, p<0.001), whilst Ca concentrations increased by at least 50% ($F_{1,26}$= 17.08, p=0.0003). The addition of cations significantly increased Ca, Mg and K concentrations in roots. Ca concentrations increased about 30% in plots where cations were added, being significantly higher only for the 0-10 cm ($F_{1,25}$= 4.29, p=0.048; Fig. 4) and mean 0-30 cm soil layer ($F_{1,26}$= 4.67, p=0.04; Fig. 4). Mg concentrations increased by more than 50% ($F_{1,26}$= 23.81, p<0.0001 for the 0-30 cm layer), with K concentrations increasing by 20-30% with cations addition ($F_{1,27}$= 7.02, p=0.013 for the 0-30 layer; Fig. 4). The addition of N did not significantly affect the concentrations of nutrients in roots one year after fertilisation commenced.

Discussion

Here we demonstrate experimental support for the hypothesis that rock-derived nutrients play a more important role than N in controlling fine root functional traits in highly weathered, ancient soils, such as those found in most Amazonian forests. Phosphorus addition had major impacts on root productivity and functional traits analysed here, but cation additions also affected root dynamics. The addition of N, as expected, did not affect root productivity or any root trait analysed here. Overall, the results demonstrate that trees in these slow-growing forests show high plasticity in response to shifts in P and cation availability.

Fine root productivity was stimulated by short-term P addition

Due to the high costs of construction and maintenance of fine acquisitive roots (McCormack *et al.*, 2015), we expected that P addition would decrease fine root productivity as a sign of alleviation of P limitation. Our results demonstrate that in the short-term, P addition increased root productivity by 23%, suggesting that, contrary to what we expected, the construction costs of short-lived acquisitive roots might be less than the maintenance costs of long-lived fine roots. The increase in root productivity in our study is not consistent with the lack of responses (Alvarez-Clare & Mack, 2015) and declines in root biomass (Yavitt *et al.*, 2011) observed in previous fertilisation experiments in tropical forests, nor observed variation in root productivity between soil types with contrasting fertility in the Colombian Amazon (Jiménez *et al.*, 2009), but are in more agreement with the study of Waring *et al.*, (2019) in a tropical dry secondary forest. The response also contrasts with the reductions in root productivity and C allocation belowground following alleviation of N limitation in temperate and boreal forests (Janssens *et al.*, 2010; Peng *et al.*, 2017). However, our results are actually consistent with large-scale spatial patterns observed within Amazonia; higher fine root productivity has been observed in more fertile soils of the Western Amazon basin than in low-fertility soils in the Central portion of the basin (Aragão *et al.*, 2009). The agreement between our results and the broader spatial patterns in Amazonia (Aragão *et al.*, 2009) may suggest a common response to greater P and cation concentrations across natural gradients and in response to experimental manipulation. At this stage, however, it is not clear if the increase in root productivity in our study site after one year of P additions is transient and could change with chronic nutrient enrichment and how these responses, together with turnover rates, will affect partitioning of plant biomass allocation and stocks between above and

belowground compartments (Ostertag, 2001; Jiménez *et al.*, 2009; Wurzburger & Wright, 2015). Nonetheless, our results demonstrate a rapid change in productivity rates in response to P additions in Central Amazonia, pointing to an increased role of direct root nutrient uptake in a more P-fertile system.

A trend towards greater root productivity with cation addition was also observed, with the increase in productivity being greater in some of the sampling points but not overall. In Panama, four years of K additions elicited changes in fine root dynamics, decreasing root stocks while increasing root turnover (Yavitt *et al.*, 2011). Therefore, despite its potential importance, it remains less clear the extent to which the availability of specific cations controls root productivity in tropical forests and how such responses would change in the short and longer term.

Phosphorus and cations additions cause rapid increase in average root diameter

Together with other factors, soil fertility is expected to control the expression of fine root morphological traits (Valverde-Barrantes *et al.*, 2013, 2017; Freschet *et al.*, 2017; Addo-Danso *et al.*, 2020). Hence, we hypothesised that the addition of nutrients would alleviate limitation, resulting in a shift from acquisitive to more conservative root traits, decreasing, for example, SRL and SRA and increasing RTD and mean diameter. Root diameter increased ~10% with cation and P addition, but no responses were detected for SRL, SRA and RTD in our fertilisation experiment. The direct effect of P addition on root diameter is, however, not conclusively demonstrated in our study, since our P fertiliser (triple superphosphate) includes ~15% of Ca in its composition and root diameter also increased in plots where we added cations. Therefore, we cannot exclude the possibility that the responses in both treatments were driven by Ca. Contrary to our findings, Wurzburger & Wright, (2015) reported root morphological traits shifting toward more acquisitive roots with K, P and NPK additions, with lower tissue density and higher specific length after 14 years of nutrient manipulation in Panama. Such contrasting responses compared to our study could also be attributed to differences in root age, with our results representing < 3 month old fine roots, whilst Wurzburger & Wright, (2015) studied mixed-age roots sampled from standing stocks.

The addition of P and cations could have favoured the root production of some species with naturally thicker roots in our study site, but since our measurements refer to the community-level, we cannot determine the species-specific effect in our results. Also, roots can maximize nutrient uptake employing very contrasting root morphologies (Chen *et al.*, 2016) diluting the signal at the community level. Although small diameter roots are more efficient in exploring larger

soil volumes in terms of plant biomass investment per unit volume of soil (Bates & Lynch, 2001; Hodge, 2004; Liu *et al.*, 2015), the increase in root diameter detected here could also provide increased mechanical protection against pathogens and herbivores (Laliberté *et al.*, 2015; Valverde-Barrantes *et al.*, 2017) and increased number or size of root cortical cells which could consequently increase levels of mycorrhizal colonisation (Brundrett, 2002; Guo *et al.*, 2008; Comas *et al.*, 2014). Since nutrient concentrations in root tissues increased following fertilisation, thicker diameter roots could be related to increased nutrient uptake through AM networks, either as a result of greater nutrient delivery per unit root length colonised or due to greater AM colonisation (see below; Eissenstat & Yanai, 1997; Eissenstat *et al.*, 2000; McCormack & Iversen, 2019).

Reduction in fine root phosphatase activity with P addition

A strong line of evidence for the role of P in controlling nutrient uptake strategies used by plants in our study is the significant decrease in root phosphatase activity with short-term P addition. A previous study demonstrated that root-surface phosphatase potential activity was a prevalent mechanism adopted by fine roots in Central Amazonian forests (Lugli *et al.*, 2020). Our results, therefore, support the idea that the exudation of phosphatase by plants is an important avenue for P acquisition in soils with low P availability in Central Amazonia (Guilbeault-Mayers *et al.*, 2020; Lugli *et al.*, 2020), and its rapid reduction suggests that this is indeed a resource-costly strategy. Together with the increase in fine root productivity captured here, the decrease in phosphatase potential activity point to a possible shift in soil P sources in our system, from organic to inorganic P, benefiting root foraging (*i.e.* direct root nutrient uptake or AM colonisation) over mining strategies. In soils with low P concentrations, plants tend to be efficient in acquiring P, which is usually accompanied with higher root phosphatase activity (Raghothama & Karthikeyan, 2005; Kitayama, 2013). However, the negative relationship between root-surface phosphatase potential activity and P availability captured in previous soil gradient studies (*e.g.* Kitayama, 2013; Nasto *et al.*, 2014; Ushio *et al.*, 2015) could also be a result of differences in plant species composition and soil physical properties. By controlling such factors in our large-scale experiment, we demonstrate that plants can rapidly detect increased P availability, changing their investment in key root traits. The addition of N and cations, on the other hand, did not affect root-surface phosphatase potential activity rates, suggesting there had been no increase in P limitation following the addition of other nutrients.

Increase in AM colonisation with cations addition

We expected that the addition of nutrients would decrease root AM colonisation levels, under the assumption that with greater nutrient availability, plants would not invest as much in the fungal symbiosis to acquire nutrients. In contrast, we observed AM colonisation increasing with cation additions, suggesting that plants could be relying on the association with AM fungi to acquire cations or other nutrients. Long-term addition of P, but not K, increased AM colonisation in standing-stock roots growing in forests in Panama (Wurzburger & Wright, 2015). Although the major benefit of AM fungi symbiosis has been considered the translocation of P to the host plant (Smith & Read, 2010), AM fungi also have been shown to acquire other macro and micronutrients such as N, Ca, Mg, K and S in pioneer and early successional tree species (Siqueira *et al.*, 1998; Zangaro *et al.*, 2003). Moreover, the higher levels of AM colonisation found here could be related to thicker root diameter detected in plots where cations were added (Table 1; Supporting Information Fig. S2). Trees with thicker absorptive roots would benefit more from AM fungi increasing their nutrient foraging capacity (Eissenstat *et al.*, 2015; Liu *et al.*, 2015; Kong *et al.*, 2016; Chen *et al.*, 2016). In contrast, trees with with thinner roots may take up nutrients directly from the soil solution or rely on phosphatase activity, thus using complementary mechanisms to acquire nutrients (Lugli *et al.*, 2020). Nevertheless, the higher investment in AM fungi with cation addition detected in this Central Amazon forest, could also suggest AMs benefit plants by increasing the uptake of other macro and micronutrients. Alternatively, it has been suggested that greater investment in AM fungi can alter the microbial community in the rhizosphere and decrease plant susceptibility to pathogens (Koide, 1991; Herre *et al.*, 2007; Laliberté *et al.*, 2015).

Stimulation of fine root nutrient concentrations

The addition of P and cations increased the concentrations of most elements in fine roots. Due the chemical composition of the fertiliser used in our P treatment (triple superphosphate: about 45% of P_2O_5 and 15% of Ca), the addition of P not only increased P concentrations in fine roots but also of Ca (Wright, 2019). It is important to highlight that even with no changes in AM colonisation and lower levels of root-bound phosphatase activity, the addition of P increased both P and Ca concentrations in roots. This points to either i) a greater role of direct root nutrient uptake or ii) increased nutrient uptake efficiency per unit AM and/or per unit phosphatase exuded in our study site. Such trends are likely due to higher nutrient availability in the soil solution after fertilisation

and a change of P source from primarily organic to inorganic P. The higher concentrations of Ca, Mg and K in fine roots after cations addition, demonstrates that we successfully increased cation availability and could also be a result of the higher levels of AM colonisation detected in our study site (Siqueira *et al.*, 1998; Zangaro *et al.*, 2003). The addition of N, however, did not affect the concentration of N or any other element in fine roots, suggesting that the extra N added to these already N-rich soils was not taken up by plants and/or that N concentrations in the root could be already at their optimal levels, with N being retranslocated to other plant tissues (Wurzburger & Wright, 2015). Therefore, plants growing in this Central Amazon forest strongly respond to the alleviation of rock-derived nutrient limitation and increase nutrient uptake, with a potential role for AM fungi driving some of these responses.

Implications for root functioning in Amazonian forests

By analysing a range of key root traits and root productivity, our study supports the hypothesis that P availability controls root functioning in Central Amazon forests, but the responses to cations also suggests that the role of rock-derived elements other than P has previously been underestimated. We found partial support for our hypothesis that nutrient addition would shift root traits from an acquisitive to a more conservative strategy. With P addition, we did find evidence for reduced investment in P acquisition, with reduced investment in mining P via phosphatases, and there was equivocal evidence for increases in root diameter. On the other hand, in contrast to our hypothesis, total root productivity increased suggesting direct root foraging for available P had become a more important strategy. With cations, we did observe a shift towards more conservative root traits with greater average root diameter, but AM colonisation increased suggesting a change in nutrient acquisition strategy rather than an overall shift to less acquisitive root traits. Direct comparisons with other studies in tropical forests are complicated because there is limited information on root productivity responses to nutrient manipulation, and traits have tended not been measured on roots of a known age. However, previous tropical nutrient manipulation experiments, installed in relatively more fertile soils in Central America, did not find strong support for P controlling root traits and fine root biomass, and only one studied the effect of cations (K, only) (Yavitt *et al.*, 2011; Wurzburger & Wright, 2015; Alvarez-Clare & Mack, 2015). Based on the different responses among fertilisation experiments, we suggest that soil nutrient availability may be even more important in determining fine root dynamics in Amazonian forests than Central American forests. Nevertheless, we stress the importance of continuous monitoring in

long-term manipulation experiments, to determine whether responses persist with chronic nutrient addition. Overall, our findings increase understanding of the plasticity of belowground plant traits and the factors controlling these responses, demonstrating that multiple nutrients shape belowground processes in Central Amazonian forests and that even slow-growing tropical rainforest in low fertility soils can respond very rapidly to nutrient additions. Phosphorus and cation availability, and changes in resource allocation to nutrient acquisition by Amazonian trees, thus, will likely play a key role in determining responses to future environmental change in these forests.

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Author contributions

LFL, KMA, LMM and IPH designed the research; LFL, JSR, RVA, MP, RLA, KMA, ALC, HFVC, RDP, LF, JLC, NPM, ACMM, STS and KJS performed the research, assisting with field sampling, logistics and/or laboratory analyses; IPH, LMM, CAQ, LEOCA, LFL and PM and wrote the grants that funded this research; LFL, KMA and JSR analysed the data; LFL, IPH, KMA, LMM, RLA, ALC, HFVC, LF, JLC, LEOCA, PM, OJVB, and CAQ commented on the manuscript. All authors approved the manuscript.

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Tables

Table 1 Mean root diameter, specific root length (SRL), specific root area (SRA) and root tissue density (RTD) ± standard errors with and without the addition of N, P and cations in two soil depths (0-10 cm and 10-30 cm) and for the mean 0-30 cm depth.

Nutrient	Depth	Diameter (mm)	SRL (cm g^{-1})	SRA (cm^2 g^{-1})	RTD (g cm^{-3})
	0-10 cm	1.05 ± 0.03	1,150 ± 56	290 ± 11.14	0.16 ± 0.005
-N	10-30 cm	1.06 ± 0.03	1,260 ± 69	308 ± 10.01	0.15 ± 0.004
	0-30 cm	1.05 ± 0.02	1,200 ± 53	299 ± 9.30	0.15 ± 0.004
	0-10 cm	1.09 ± 0.03	1,310 ± 102	320 ± 18.68	0.15 ± 0.004
+N	10-30 cm	1.11 ± 0.03	1,220 ± 82	308 ± 16.19	0.15 ± 0.007
	0-30 cm	1.10 ± 0.02	1,267 ± 64	313 ± 11.01	0.15 ± 0.003
	0-10 cm	1.02 ± 0.04	1,250 ± 94	304 ± 15.89	0.16 ± 0.005
-P	10-30 cm	1.07 ± 0.03	1,220 ± 61	305 ± 10.66	0.15 ± 0.005
	0-30 cm	1.05 ± 0.02	1,240 ± 60	304 ± 9.71	0.15 ± 0.005
	0-10 cm	1.11 ± 0.02*	1,210 ± 75	306 ± 15.81	0.15 ± 0.005
+P	10-30 cm	1.09 ± 0.03	1,260 ± 88	310 ± 15.74	0.15 ± 0.006
	0-30 cm	1.10 ± 0.02	1,230 ± 58	308 ± 10.94	0.15 ± 0.004
	0-10 cm	1.03 ± 0.03	1,260 ± 83	308 ± 14.63	0.15 ± 0.005
-Cations	10-30 cm	1.04 ± 0.03	1,330 ± 68	322 ± 13.20	0.14 ± 0.005
	0-30 cm	1.03 ± 0.03	1,290 ± 57	315 ± 10.60	0.15 ± 0.004
	0-10 cm	1.11 ± 0.03	1,200 ± 87	302 ± 16.95	0.15 ± 0.006
+Cations	10-30 cm	1.13 ± 0.03	1,150 ± 76	293 ± 12.61	0.15 ± 0.006
	0-30 cm	1.12 ± 0.02**	1,180 ± 57	297 ± 9.57	0.15 ± 0.003

n= 16 per treatment per depth. Significant effects of the N, P and cations by depth (*e.g.* -P *versus* +P) are indicated by * and **, representing probability at the 5 and 1 % levels, respectively.

Figures legends

Fig. 1. Fine root productivity in Mg ha^{-1} $year^{-1}$ for the 0-10 and 10-30 cm soil depths and sum of the whole soil core (0-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in Central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of nutrients in each depth. Means ±1SE (n=16) are presented. Significant effects of the N, P and cations are indicated by ** representing probability at the 1% level.

Fig. 2. Mean root phosphatase activity in μmol g^{-1} root dry weight $hour^{-1}$ for the 0-10 and 10-30 cm soil depths and mean of the whole soil core (0-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in Central Amazon, Brazil. Each panel contrasts 16 plots with and without the addition of nutrients in each depth. Means ±1SE (n=16) are presented. Significant effects of the N, P and cations are indicated by ** representing probability at the 1% level.

Fig. 3. Total root arbuscular mycorrhizal (AM) colonisation in % root length for roots from the 0-10 cm soil layer with and without the addition of N, P and cations in a lowland tropical forest in Central Amazonia, Brazil. Each panel contrasts 16 plots with and without the addition of nutrient. Means ±1SE (n=16) are presented. Significant effects of the N, P and cations are indicated by * representing probability at the 5% level.

Fig. 4. Element concentrations in fine root tissues for the 0-10 and 10-30 cm soil depths and mean of the whole soil core (0-30 cm) with and without the addition of N, P and cations in a lowland tropical forest in central Amazon, Brazil. Concentrations of C and N are given as % and concentrations of P, Ca, Mg and K are given in g kg^{-1}. Each panel contrasts 16 plots per depth with and without the addition of nutrient. Means ±1SE (n=16) are presented. Significant effects of the N, P and cations are indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively.

Supporting Information

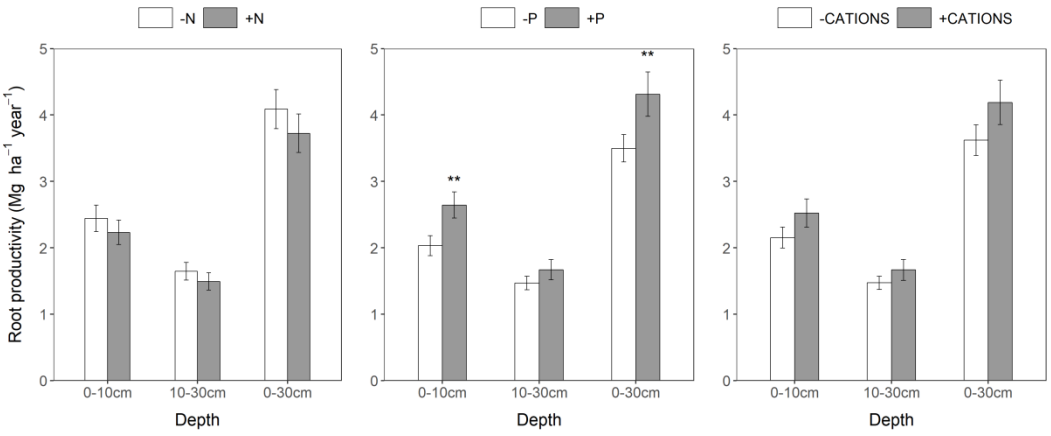
Fig. S1 Fine root productivity for a 3-month interval with the addition of N, P and cations in Central Amazonia.

Fig. S2 Relationship between mean root diameter and AM colonisation in Central Amazonia.

Table S1. Paired t-tests among soil depths for root productivity, morphological traits and root phosphatase activity.

Table S2. Paired t-tests among soil depths for nutrient concentration in fine roots.

Table S3. Root arbuscular mycorrhizal colonisation in different fungi structures.



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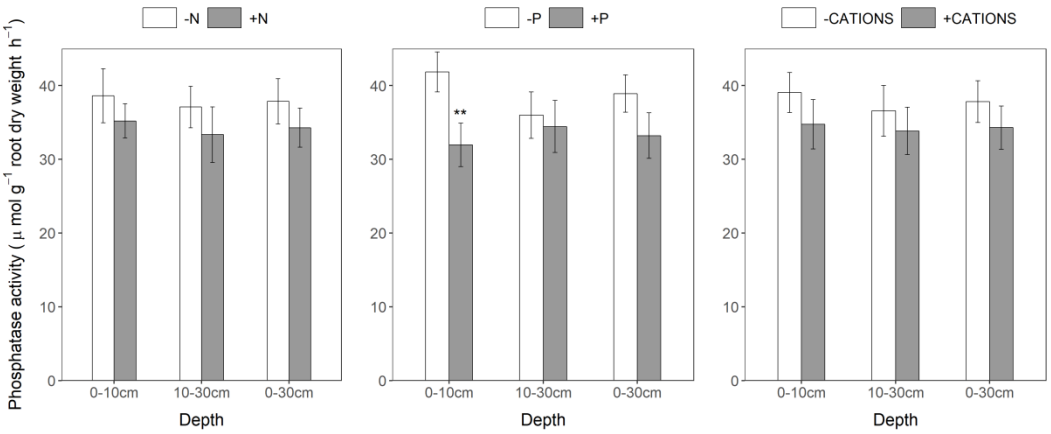

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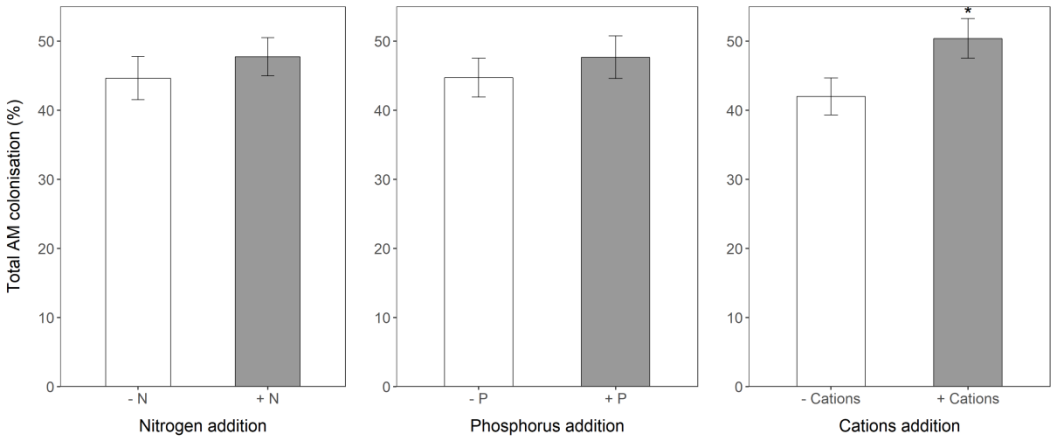

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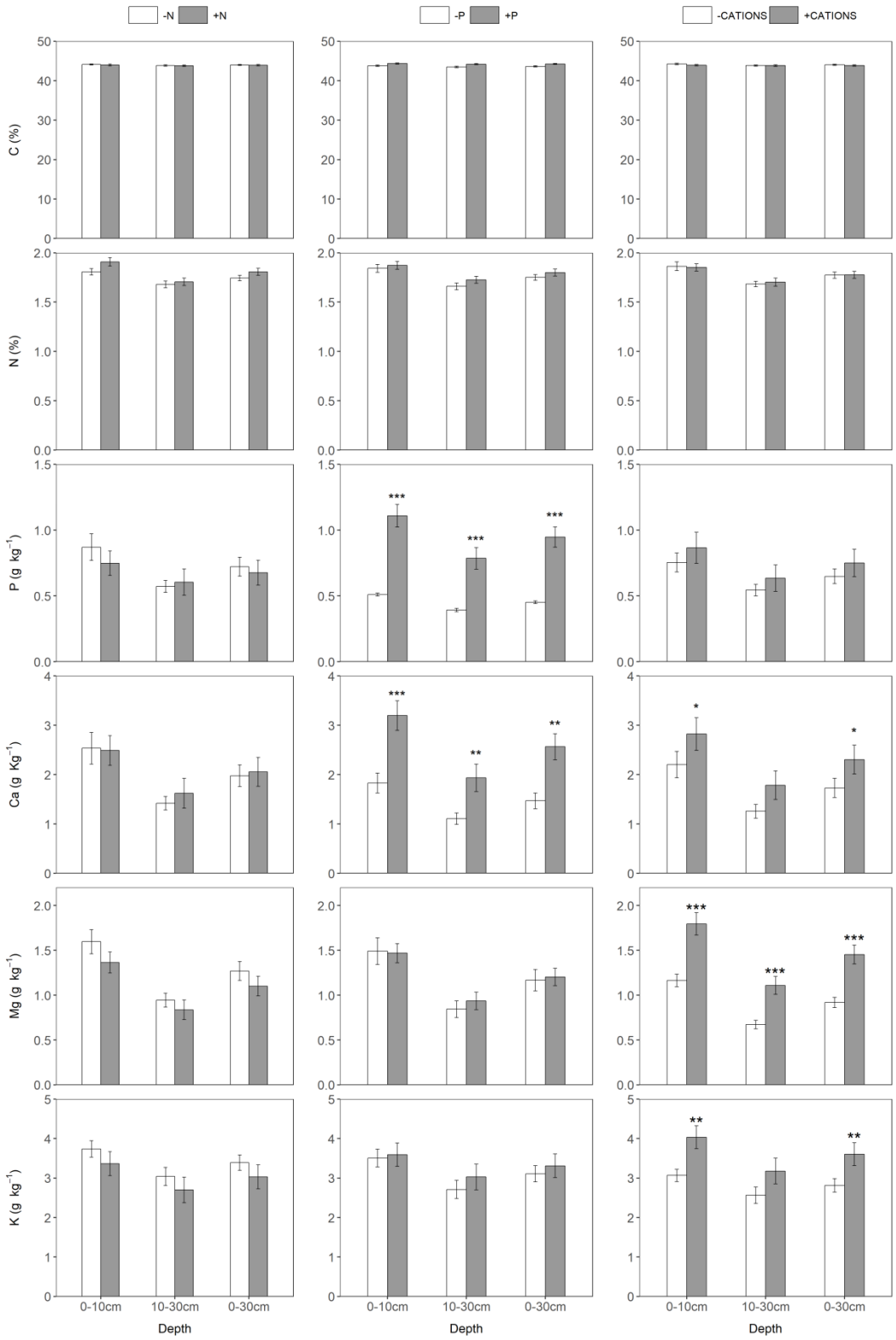

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