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Effects of Amazonian Dark Earths on growth and leaf nutrient balance of tropical tree seedlings

Estela Quintero-Vallejo · Marielos Peña-Claros · Frans Bongers · Marisol Toledo · Lourens Poorter

Abstract

Background and aims Amazonian Dark Earths (ADE) are ancient anthropogenic soils distributed in the Amazon basin. They are characterized by high nutrients such as phosphorus, calcium, potassium and nitrogen. We studied the effect of ADE on growth, morphology and physiology of 17 tree species from a Bolivian tropical moist forest.
Methods We conducted a greenhouse experiment where seedlings were grown for 2–4 months on ADE and non-ADE. We evaluated soil nutrient concentrations, seedling growth, leaf and root functional traits, and leaf nutrient concentrations.
Results Soil type affected 10 out of 24 evaluated seedling traits. Seedlings did not invest more in roots in non-ADE, but they invested in leaves and leaf area in ADE, although this did not lead to faster growth rate. Species responded differently to soil Ca increment; some species seemed to suffer from Ca toxicity as indicated by low survival, others from nutrient imbalance, whereas other species increased their leaf calcium, phosphorus and nitrogen concentration in ADE. Only for this latter group of nutrient accumulators, there was a positive interspecific relationship between leaf Ca and seedling growth rates.
Conclusions ADE did not lead to increased seedling growth. The ability of plants to colonize patches of ADE might depend on plant responses to increased soil Ca and their capacity to regulate internal tissue calcium to balance nutrition.

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Introduction

Nutrient availability in tropical soils is the result of complex biogeochemical processes that generate high spatial heterogeneity (Townsend et al. 2008). This heterogeneity is important, since it can determine tree species distribution at different spatial scales (John et al. 2007). However, the exact mechanisms that drive the underlying patterns of plant distribution are still far from being fully understood. This lack of explanation is partly because plants respond differently to a multitude of soil-

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related factors, such as water availability (Brenes-Arguedas et al. 2013) and nutrients (Ordoñez et al. 2009). Among nutrients, nitrogen (N) and phosphorus (P) are the major limiting elements for primary productivity of forests (Vitousek et al. 2010). Nevertheless, recent fertilization studies have found that potassium (K) and calcium (Ca) influence plant growth in tropical forest, in similar ways as N and P (Baribault et al. 2012; Wright et al. 2011).

Plants respond to soil nutrient availability through a suite of traits, by adjusting their allocation patterns, morphology, tissue chemistry and physiology. With an increase in soil nutrient availability, plants shift biomass allocation to aerial structures, such as leaves, instead of underground structures such as roots (Poorter et al. 2012). This is usually accompanied by changes in other plant traits such as an increase in specific leaf area (SLA), N and P concentration in leaves, and growth (Ordoñez et al. 2010; Ordoñez et al. 2009). On the other hand, in soils with low-nutrient availability, plants grow slowly, have a smaller leaf area and allocate more biomass to roots, thus enhancing nutrient uptake (Baraloto et al. 2006). Plant tissue chemistry changes with soil fertility, which is usually reflected in the nutrient concentration of leaves, particularly leaf N and P and their ratio (Ågren 2004; Townsend et al. 2007). Apart from soil fertility, foliar nutrient concentrations are shaped by a number of other factors, such as soil pH (Lambers et al. 2008; Viani et al. 2014), plant demands for elements such as calcium (Ca) to increase tissue toughness (Baribault et al. 2012), negative interactions between tissue P and aluminum (Al) when plants tend to accumulate Al (Metali et al. 2015), species phylogeny (Watanabe et al. 2007), and symbiotic relationships between plants and microorganisms (Nasto et al. 2014). As a result, plant nutrient concentrations and their stoichiometry can be uncoupled from those in the soil.

Soil pH is an important factor in plant nutrition; it may limit the availability of a range of elements that are important for plant growth (e.g., iron [Fe] and zinc [Zn]), and may raise the availability of other elements, such as Al, that could cause plant mortality (Lambers et al. 2008). For example, in soils with a low pH (<5.8), increases in Al and Fe availability lead to decreases in P availability by reaction of Al and Fe with inorganic P to form plant unavailable insoluble compounds (McDowell et al. 2003). Furthermore, an increase in soil Al affects root elongation or plant uptake of other

elements such as Ca and magnesium (Mg), causing reduced development of roots, depression of growth and potentially plant death (George et al. 2012). On the other hand, in soils with high pH (~7.4) such as calcareous soils, increased concentrations of available Ca can react with inorganic P and form calcium phosphate, which makes P unavailable for most plants (Tyler 1996). Besides, in soils with high pH, some elements such as Fe, Mn, Zn and Cu form oxide-bound complexes and become unavailable, thus limiting plant growth (Lee 1998).

In general, soils in the tropics have low pH (Von Uexküll and Mutert 1995) and a low availability of a range of nutrients. However, Neotropical soils can also be diverse in their chemical properties as a result of different geological processes. In particular, the Amazon basin has experienced a long and dynamic geological history (Hoorn et al 2010). In combination with different biogeochemical processes (Quesada et al. 2010) this has led to a wide variation in soil characteristics and different availability of nutrients such P, Ca, Mg, and K. Despite the fact that most of the Amazon basin has a low soil fertility, there are patches of Amazonian Dark Earths (ADE) with high soil fertility that are the product of past human inhabitation of the region since pre-Columbian times (Glaser and Birk 2012).

Amazonian Dark Earths are characterized by a thick dark or gray top layer (up to 1.5 m deep) with presence of ceramics that indicates past indigenous settlements (Sombroek 1966; Woods and Glaser 2004). ADE have a black color due to high concentrations of organic matter and black carbon, they have high P and Ca, and higher pH than the natural soils commonly found in the Amazon, such as Ultisols and Oxisols (Falcão et al. 2009; Glaser and Birk 2012; Sombroek 1966). ADE seem to be the product of additions of charcoal and organic waste which change soil physic-chemical characteristics (Schmidt et al. 2014). For example, charcoal addition leads to a more stable organic matter content, and a high soil nutrient retention capacity (Glaser et al. 2002), while the addition of organic waste leads to high concentrations of P, N, and Ca (Glaser 2007). Compared with natural Central Amazonian soils, ADE are more fertile because of the combination of high pH and Ca- and P- concentrations. For this reason, ADE are often used by local farmers to grow crops faster for longer periods of time compared with slash and burn agriculture practiced on regular soils in the Amazon (German

2003). Similarly, a forest growing on ADE has increased gross primary productivity, net primary productivity, and higher rates of fine root production compared with a forest growing on non-ADE (Doughty et al. 2014).

By stimulating productivity, the high fertility of ADE could promote changes in plant traits associated with acquisition strategies such as higher relative growth rate, higher leaf biomass and higher nutrient concentrations in the leaves. Evaluating changes in plant traits associated with ADE provides a unique opportunity to understand tree responses to soil fertility as a consequence of past human inhabitation and soil modification. It could also help to understand how soil fertility influences species composition in habitats that do not vary in climatic conditions and water availability.

Here we present results of a greenhouse experiment, in which seedlings of 17 tropical tree species were grown on ADE and non-ADE. We examined how soil fertility of ADE affected seedling growth, biomass allocation, and morphological and chemical traits of tropical tree species. We hypothesized that plants growing on ADE would have higher growth rates compared with plants growing on non-ADE. We also hypothesized that the higher growth rates of seedlings on ADE would be associated with increases in biomass allocation to stem and leaves, leaf N, Ca and P concentrations, specific leaf

area and leaf area ratio, which are traits that enhance light capture and carbon gain. We also predict that seedlings growing on less fertile non-ADE will have higher biomass allocation to (secondary) roots and produce roots with higher specific root length to enhance nutrient uptake.

Methods

Species and collection site Seventeen tree species were selected for the study based on seed availability. Hereafter species will be referred by the genus name (Table 1). Most of the seeds were collected from a semi-evergreen forest (La Chonta forest concession) located in the province of Guarayos, Bolivia (15° 47′ S, 62° 55′ W) in July and August of 2012. The site has a mean annual temperature of 24.3 ° C and mean annual rainfall of 1580 mm (data from 2000 to 2006 from La Chonta) with a dry season (<100 mm/month) from May through September (Peña-Claros et al. 2012). Soils at La Chonta are a mosaic of poor soils from the Brazilian Precambrian Shield (Navarro and Maldonado 2002) and more fertile soils that are the product of sedimentation and erosion originated from the uplift of central Andes (Latrubesse et al. 2010). A previous study

Table 1 Scientific names and families of tree species used for this study

Scientific Name	Family	Nodules present in roots	
		Reported	Observed
Albizia niopiodes (Spruce ex Benth.) Burkart	Fabaceae	+ [1]	+
Anadenanthera colubrina (Vell.) Brenan	Fabaceae	+ [3]	−
Enterolobium contortisiliquum (Vell.) Morong	Fabaceae	+ [2,3,4]	−
Erythrina crista-galli L.	Fabaceae	+ [4]	+
Hymenaea courbaril L.	Fabaceae	− [2,5]	−
Machaerium villosum Vogel	Fabaceae	+ [2]	−
Ormosia nobilis Tul	Fabaceae	+ [1]	−
Poeppigia procera C. Presl	Fabaceae		−
Samanea tubulosa (Benth.) Barneby & J.W. Grimes	Fabaceae		+
Schizolobium parahyba (Vell.)S.F. Blake	Fabaceae		
Terminalia oblonga (Ruiz & Pav.) Steud.	Combretaceae		
Cariniana ianeirensis R. Knuth	Lecythidaceae		
Chorisia speciosa A. St.-Hil.	Malvaceae		
Guazuma ulmifolia Lam.	Malvaceae		
Cedrela fissilis Vell.	Meliaceae		
Swietenia macrophylla King	Meliaceae		
Sapindus saponaria L.	Sapindaceae		

Information on presence of nodules in roots, as reported in the literature, is shown with the number corresponding to its reference. Information on observed nodules in roots of seedlings used in this experiment is shown. (+) represents nodules present, and (−) represents nodules absent

[1] (de Faria and de Lima 1998)

[2] (Barberi et al. 1998)

[3] (Sprent and Parsons 2000)

[4] (Frioni et al. 2001)

[5] (Lima et al. 2006)

showed that La Chonta also has Amazonian Dark Earth (ADE), which are anthropogenic soils indicating inhabitation of this forest in the past (Paz-Rivera and Putz 2009).

Study site The experiment was carried out in a shade house at the Agronomy Faculty of the Universidad Autónoma Gabriel Rene Moreno in Santa Cruz de la Sierra, Bolivia (16° 30′ S, 63° 10′ W). The climate in this city is warm, with an average temperature of 24.2± 0.8 ° C, and mean annual rainfall of 1308±391 mm (Data from National Institute of Meteorology, Bolivia; 1949–2013). The 6×3 m shade-house was built and covered with black mesh (2×1.5 mm) to give an irradiance of about 10 % of full sunlight (around 400 lux). This level of light is similar or even higher than the light encountered in the understory of a tropical rain forest (~1–2 %), where seedlings of most tropical tree species grow well (Poorter 1999).

Soils and shade-house experiment The soil used for testing germination and plant growth was collected at La Chonta, where we previously identified a site with dark earth and the presence of pottery shards, hereafter ADE, and a site at least 500 m away with more clear soils, hereafter non-ADE. Soils were mixed with river sand at a ratio of 4:1 (w/w), to allow adequate drainage and to facilitate harvesting of the whole root system, including fine roots. Although the use of sand could

dilute the effect of soil nutrients, we considered that this dilution might not affect our experiment because the differences between ADE and non-ADE were retained for most of the soil variables except total N (Table 2). Chemical and physical characteristics of the original soils and of the mixture with sand were assessed in the Centro de Investigación Agrícola Tropical (CIAT), in Santa Cruz, Bolivia (protocols for chemical and physical properties can be seen in Online Resource 2). The main differences in soil variables between ADE and non-ADE sand mixes were found for Ca concentration, P concentration, total exchangeable base and cation exchange capacity (ratios between ADE and non-ADE were>2 in all cases); soil pH was slightly alkaline (>7) in both soils (ADE: 7.8; non-ADE:7.6) (Online Resource 1).

From April to September of 2013 seeds were germinated in trays of 25×30 cm filled with either ADE or non-ADE sand mixes. When seedlings produced their first leaves, eight seedlings per species per soil type were harvested and 12 seedlings per species per soil type were transplanted to pots of 650 ml (9.2 cm diameter×15 cm height). The transplanted seedlings were placed in a completely randomized design in the shade-house and were re-randomized every 3 weeks to ensure that all seedlings were growing under similar light and temperature conditions. Plants were watered daily or every other day depending on the weather, since the shade house allowed rain water to pass through.

Table 2 Soil characteristic of Amazonian Dark Earths and non-Amazonian Dark Earth collected in La Chonta forest concession, before and after mixing them with river sand

	ADE	non-ADE	ADE/River sand	non-ADE/ River sand	Ratio variables on ADE:non-ADE
pH	7.4	7	7.8	7.6	1
Electric conductivity (μS/cm)	226	108	218	149	1.5
Ca (cmol/kg)	12.2	4.8	10	4.2	2.4
Mg (cmol/kg)	1.3	1	1.1	0.8	1.4
Na (cmol/kg)	0.11	0.04	0.09	0.05	1.8
K (cmol/kg)	0.38	0.26	0.28	0.2	1.4
Olsen-P (mg/kg)	36	12	35	11	3.2
Total N (%)	0.31	0.16	0.18	0.11	1.6
Total Exchangable bases	14	6.1	11.5	5.3	2.2
Cation Exchange Capacity	14	6.1	11.5	5.3	2.2
Base Saturation (%)	100	100	100	100	1
Organic Mater (%)	3.3	2.7	2.9	2.2	1.3
Sand (%)	49	57	68	73	0.9
Silt (%)	39	36	25	20	1.2
Clay (%)	12	7	7	7	1

Ratios between values on ADE and non-ADE after the mixing with river sand are presented. Phosphorus reported on this table was obtained using Olsen Method for soil P extraction (See, Online Resource 1)

Once seedlings were established, at around half of the growing period, the number of leaves on each seedling was counted and the newest fully developed leaves were tagged with colored threads. At the time of harvesting we counted the number of newly produced leaves.

A second harvest was done between 2 and 4 months after transplanting, depending on the species. Differences in length of the growing period were due to differences in germination time, and lack of seed availability of some species at the time we started the experiment. At each harvest, we determined the fresh mass of roots, stems, and leaves; leaves were sectioned into the leaf lamina and the petiole, or rachis for compound leaves, and sections were weighed separately. Laminae were scanned with a desktop scanner (Cannon LIDE 20, Canon, USA) and leaf area was determined using the software program ImageJ (Rasband 2008). Leaf resistance to mechanical damage was measured for nine of thirteen species using force to punch. Force to punch was determined on one or two of the newest expanded leaves using a field penetrometer, which consisted of a flat-end nail of 3.2 mm in diameter; the nail was attached to the inner part of a syringe and a water basin on top. A leaf was placed between two acrylic plates both having a 6 mm diameter hole. The holes were located in the same position so that the nail could cross the leaf between the two plates similar to Aranwela et al. (1999). Force to punch the leaf was determined by the weight (converted to Newton) of the water added to the basin that was necessary to penetrate the leaf, divided by the circumference of the punch nail.

Root length (RL) was determined following Newman (1966). Roots were placed in a tray filled with water. The tray was covered by a transparent sheet marked with 1×1 cm square grid and the number of intersections between roots and the gridlines were counted horizontally and vertically. Thereafter, total root length was determined as $R = \pi NA/2H$, where R is the total length of the root (cm), N is the number of intersections between the root and the gridlines, A is the area of the rectangle (cm^2) and H is the total length of the straight lines of the grid (cm) (Newman 1966). Root morphology was described by the presence/absence of tap roots and by the number of secondary roots. When tap roots were present both the diameter of the base (close to the transition to stem) and the diameter of the tip were measured. The diameter of three secondary roots was measured with a digital caliper.

We measured stem length from the base to the apical bud, and top and base stem diameter. Stem volume was calculated using the formula for a section of a cone. Lamina and petioles, roots and stems were stored in separate paper bags, oven-dried at 70 °C for 72 h, and weighted again to determine dry weight of plant sections. Using the dry weights we calculated root mass fraction (RMF, g g^{-1}), leaf mass fraction (LMF, g g^{-1}) and stem mass fraction (SMF, g g^{-1}) as the weight of each plant part over the total seedling dry mass. We also calculated stem, root and leaf dry matter content (SDMC, RDMC, LDMC, respectively, in g g^{-1}) as the dry mass over the fresh mass of that section. Specific leaf area (SLA, m^2 Kg^{-1}) was calculated as the ratio between leaf area and leaf dry mass, leaf area ratio (LAR, m^2 Kg^{-1}) as the total leaf area over total seedling dry mass, specific root length (SRL, m Kg^{-1}) as root length over dry root mass and root length per plant mass (RLPM m Kg^{-1}) as the root length over total seedling dry mass.

These traits determine important functional characteristics for the plants. Biomass fractions of seedling sections describe how plants allocate biomass to light intercepting tissue in the case of leaves, or nutrient capturing tissue in the case of roots. Stem, root and leaf dry matter content, and force to punch indicate tissue toughness, which are thought to be good proxies for tissue longevity. SLA and LAR indicate how efficiently plants invest in light interception. Measurements of SRL and RLPM indicate how the biomass that is allocated to roots can be efficient in nutrient capture through increase of absorption surface (Markesteijn and Poorter 2009).

Leaf nutrient N, P, and Ca concentration was determined for three randomly selected seedlings per species per soil type. For five species (*Albizia, Anadenanthera, Cedrela, Machaerium* and *Samanea*) we selected a seedling that had a leaf dry weight>50 mg, the minimum quantity required for leaf tissue analyses in the laboratory. Extraction of N, P and Ca were done using digestion with H_2SO_4, Se, and salicylic acid (Novozamsky et al. 1983). After digestion, N and P content was measured with a continuous-flow analyzer (SAN Plus Segmented Flow Analyser Skalar SA-4000, Skalar UK, York, UK), and Ca content was measured using an Atomic Absorption Spectrometer (AAS-Varian Spectra AA-600, Varian, Palo Alto, USA). Chemical tissue analyses were done at the facilities of Wageningen University, The Netherlands.

Data analyses We quantified survival percentage for each species by dividing the number of seedlings alive at the time of the second harvest by the number of transplanted seedlings. We compared survival percentage between soil types using a chi-square test. Relative height growth rate (RGRh) was calculated for each seedling as: $RGRh = \frac{\ln h_2 - \ln h_1}{t_2 - t_1}$, where h_n represents the seedling height at time n; t_1 is time at transplanting, t_2 time at harvesting, and ($t_2 - t_1$) is the number of days between transplanting and harvest. RGR based on plant biomass (RGRb) was calculated for each species using the information of both harvests as: $RGRb = \frac{\overline{\ln M_2} - \overline{\ln M_1}}{t_2 - t_1}$, where $\overline{\ln M_n}$ is the average of the natural log of the dry mass of each seedling at harvest n.

Continuous variables were log$_{10}$-transformed, whereas ratios were arcsine-transformed prior to statistical analyses. To test for the effect of ADE and non-ADE on total biomass, RGRh, and plant traits, we performed a two-way ANOVA, with soil type and species as factors. For this analysis we used 13 species instead of 17 because we had significant mortality in four species that left us with few replicates per treatment to make comparisons between the two soils. We used an unbalanced design because two species (*Cariniana* and *Hymenaea*) had different number of seedlings per soil type. The interaction between soil type and species was also evaluated. For all traits but RGRh, data of the second harvest were used. The amount of variance that was explained by each factor was determined by dividing the sum of squares associated with each factor (soil or species) and their interaction by the total sum of squares of the model. A separate analysis was done for each species using one-way ANOVA, with soil type as factor. The effect of soil type on leaf production rate was tested using a non-parametric Kruskal-Wallis test because the data were not normally distributed.

We detected that the percentage of leaf Ca was always higher on ADE, whereas the percentages of leaf P and leaf N were similar or lower on ADE than in non-ADE, despite the higher concentration of these elements in ADE (Table 1; see results; Fig. 3). Therefore, we explore the relationships between pairs of tissue nutrient concentration (Ca and P; Ca and N) from each soil. We averaged the Ca, N and P concentration in the leaves of seedlings growing in ADE and non-ADE, and calculated the difference between these amounts in each soil type per species, as follows: $Difference_{ADE-nonADE} = \left(\overline{Nutrient_{i,ADE}} - \overline{Nutrient_{i,non-ADE}}\right)$, where *Nutrient* stands for the concentration in the leaf, and i stands for Ca, N or P. Using the information of all species, we performed linear regressions to test the relationship between the difference in leaf Ca (Ca$_{ADE-nonADE}$) and the difference in leaf P (P$_{ADE-nonADE}$) as well as the relationship between the difference in leaf Ca (Ca$_{ADE-nonADE}$) and the difference in leaf N (N$_{ADE-nonADE}$).

Based on species leaf P and N responses to soils type (see results y axes; Fig. 4a and b), we separated the species into the following groups: species whose differences in leaf P (P$_{ADE-nonADE}$) were positive, implying higher leaf P on ADE (hereafter refered as *positive for* P); species whose differences in P (P$_{ADE-nonADE}$) were negative, implying lower leaf P on ADE (*negative for* P); species whose differences in leaf N (N$_{ADE-nonADE}$) were positive, implying higher leaf N on ADE (*positive for* N); species whose differences in leaf N (N$_{ADE-nonADE}$) were negative, implying lower leaf N on ADE (*negative for* N). Using these four response groups, we tested how Ca uptake was related with RGRb. We evaluated Ca because it was the nutrient in the leaves that consistently increased in ADE for all species (Fig. 3a). Therefore, we performed a linear regression for each response group between leaf Ca concentration in leaves as the independent variable and the average RGRb as the dependent variable. Finally, we also tested how Ca uptake was related with leaf P uptake or leaf N uptake for each response group using linear regressions. All statistical analyses were done using Genstat 16th ed. (VSN International Ltd).

Results

Eight out of 17 species showed some mortality during the experiment. *Terminalia* and *Poeppigia* had a significantly higher mortality in ADE than in non-ADE soil (χ^2:10.83, $p<0.05$; χ^2:12.08, $p<0.05$, respectively), whereas *Schizolobium* had a significantly higher mortality in non-ADE (χ^2:15.41, $p<0.05$) (Fig. 1).

Plant responses to ADE

We found significant differences between soil types for ten out of 24 traits, but the proportion of the variance explained by soils for each of these variables was very low (less than 2.8 %, Table 3). Species had a significant effect on all variables, explaining 35 to 96 % of the variance (Table 3).

Fig. 1 Seedling survival of 17 species of tropical trees in Amazonian Dark Earths (ADE) and non-Amazonian Dark Earth (non-ADE) soils after the growing period in the greenhouse
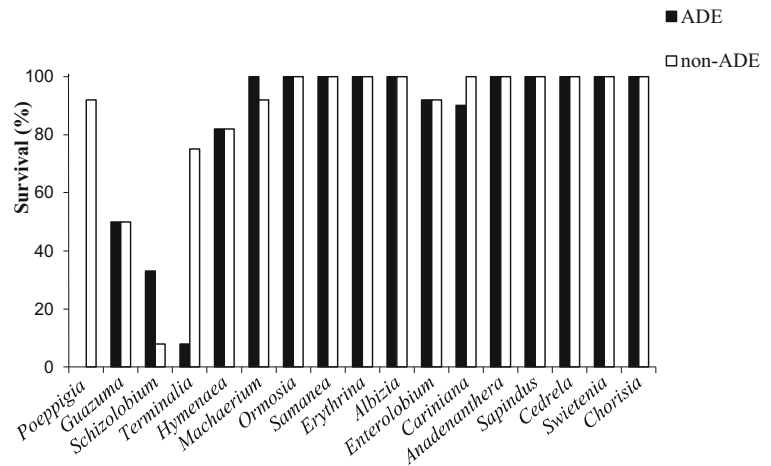
We expected soil fertility from ADE to have a positive effect on plant growth, but most of the species had a similar growth rate on both soils. Total biomass (Fig. 2a), height growth (RGRh, Fig. 2b) and biomass growth (RGRb) did not differ significantly between soil types (Table 3). However, the interaction between soil type and species was significant for total biomass and RGRh (Table 3), indicating that some species grew faster in ADE, whereas other species grew faster in non-ADE. Seedlings of *Cedrela* and *Chorisia* had significantly more biomass in ADE (one-way ANOVA: *Cedrela* $F=7.29$, $p=0.013$; *Chorisia* $F=5.40$, $p=0.03$), whereas *Machaerium* had a significantly higher biomass in non-ADE (one-way ANOVA, $F=4.87$, $p=0.039$) (Fig. 2a). Furthermore, *Swietenia* had a higher RGRh in ADE (one-way ANOVA, $F=9.04$, $p=0.006$) (Fig. 2b), whereas *Chorisia* had a significantly higher RGRh in non-ADE (one-way ANOVA, $F=18.88$, $p<0.001$).

Overall, leaf production rate was similar for both soil types (Kruskal-Walis $H=2.97$; $p=0.084$), although some species such as *Cedrela*, *Chorisia*, and *Cariniana* had a higher leaf production rate in ADE than in non-ADE, whereas *Swietenia* had higher leaf production rate in non-ADE soil (Online Resource 2). We found that biomass allocation to leaves (LMF) was significantly higher in ADE than in non-ADE (Table 3). There were no significant differences in biomass allocation to roots (RMF) between soil types. There was a significant interaction between soils and species in allocation to stems (SMF) (Table 3). Seedlings of *Anadenanthera* had heavier stems in ADE ($F=14.71$, $p<0.001$) than in non-ADE, whereas *Erythrina* and

Enterolobium seedlings had heavier stems in non-ADE than in ADE (Online Resource 2). Specific leaf area (SLA, a measure of area investment per leaf mass) and leaf area investment per plant mass (LAR) were significantly higher for seedlings in ADE (Table 3). We found a significant interaction term for specific root length (SRL) (a measure of plant investment in root length) (Table 3); *Enterolobium* seedlings had a significantly higher SRL in ADE, whereas *Swietenia* seedlings had higher SRL in non-ADE (Online Resource 2). Leaf and root dry mass content were significantly higher in non-ADE (Table 3). Leaf Ca concentration was significantly higher in ADE (Table 3) this was particularly the case for *Ormosia*, *Erythrina*, *Albizia*, and *Cariniana* (Fig. 3a). Leaf N concentration did not differ significantly between soil types (Table 3, Fig. 3b). We found a significant interaction between soil and species for leaf P concentration (Table 3), caused by a significantly higher leaf P concentration in ADE for *Machaerium* (one-way ANOVA, $F=8.36$, $p=0.04$) and in non-ADE for *Erythrina* (one-way ANOVA, $F=128.2$, $p<0.001$) (Fig. 3c).

Nutrient stoichiometry in relation with growth rates

We explored the differences in leaf nutrient concentration between seedlings growing in ADE and non-ADE soils. We found that all species had a higher leaf Ca in ADE than non-ADE (positive values on x axis, Fig. 4a), whereas species had either a higher (positive values on y axis, Fig. 4a) or a lower leaf P in ADE than in non-ADE (negative values on y axis, Fig. 4a). This resulted in a significant negative

Table 3 Averages of morphological, physiological and chemical variables measured on seedlings of 13 species of tropical trees growing on ADE ($n=12$ seedlings per species) and non-ADE soils ($n=12$ seedlings per species)

Trait	ADE	N-ADE	R^2	Soil			Species				Soil x Species		
				F	p	SSs/SSt	F	p	SSspp/SSt		F	p	SSsxspp/SSt
Biomass (g)	0.932	0.94	0.89	0.39	0.531	0.0002	184.11	<.001	0.88		3	<.001	0.014
RGRh (mm.mm.d^{-1})	0.043	0.042	0.87	0.09	0.767	0.0000	147.47	<.001	0.84		6.53	<.001	0.037
RGRb (g.g.d^{-1})	0.016	0.016	–	0.005	0.944	0.0000	–	–	–		–	–	–
Root Mass Fraction (RMF) (g g^{-1})	0.232	0.244	0.68	3.36	0.068	0.0040	46.54	<.001	0.67		0.58	0.859	0.008
Stem Mass Fraction (SMF) (g g^{-1})	0.255	0.261	0.67	1.8	0.181	0.0022	43.4	<.001	0.64		2.15	0.015	0.032
Leaf Mass Fraction (LMF) (g g^{-1})	0.513	0.494	0.61	8.06	0.005	0.0117	33.19	<.001	0.58		1.14	0.331	0.020
Diameter of secondary roots (mm)	0.481	0.49	0.61	0.08	0.776	0.0001	31.74	<.001	0.59		1.24	0.253	0.023
Secondary to primary root mass (g g^{-1})	0.897	0.755	0.38	2.98	0.086	0.0069	12.57	<.001	0.35		1.05	0.401	0.029
Number of secondary soots	29.17	30.66	0.53	1.43	0.233	0.0026	22.77	<.001	0.49		1.66	0.076	0.036
Specific Root Length (m Kg^{-1})	17,860	15,310	0.57	0.36	0.549	0.0006	26.88	<.001	0.52		2.69	0.002	0.052
Root Length Ratio (m Kg^{-1})	98,740	74,760	0.78	0.11	0.736	0.0001	79.19	<.001	0.77		1.74	0.059	0.017
Root Dry Matter Content (RDMC) (g g^{-1})	0.173	0.187	0.78	15.91	<.001	0.0128	78.82	<.001	0.76		0.78	0.672	0.008
Stem Dry Matter Content (SDMC) (g g^{-1})	0.282	0.293	0.91	7.81	0.006	0.0027	218.1	<.001	0.90		2.12	0.016	0.009
Leaf Dry Matter Content (LDMC) (g g^{-1})	0.27	0.279	0.96	14.98	<.001	0.0022	551.38	<.001	0.96		0.98	0.464	0.002
Stem Density (g cm^{-3})	0.31	0.316	0.90	1.6	0.207	0.0006	188.95	<.001	0.89		1.93	0.031	0.009
Specific Leaf Area (SLA) (m^2 Kg^{-1})	41.66	39.46	0.74	4.8	0.029	0.0046	63.23	<.001	0.73		0.83	0.624	0.010
Leaf Area Ratio (LAR) (m^2 Kg^{-1})	18.83	17.27	0.61	9.54	0.002	0.0140	32.52	<.001	0.57		1.17	0.306	0.021
Force to Punch (N mm^{-1})	0.229	0.244	0.69	4.31	0.039	0.0065	49.62	<.001	0.68		0.88	0.543	0.012
Leaf Thickness (mm)	0.123	0.124	0.90	1.01	0.315	0.0005	202.28	<.001	0.89		1.87	0.058	0.008
Leaf Ca concentration (mg g^{-1})	19.2	16.8	0.95	26.52	<.001	0.0278	71.3	<.001	0.90		1.67	0.102	0.021
Leaf N concentration (mg g^{-1})	34.7	35.4	0.95	1.35	0.251	0.0012	88.88	<.001	0.94		0.87	0.579	0.009
Leaf P concentration (mg g^{-1})	1.9	2.1	0.90	6.78	0.012	0.0134	32.42	<.001	0.77		5.02	<.001	0.119
Ca:P	11.06	9.95	0.83	3.74	0.059	0.0124	18.73	<.001	0.74		1.79	0.075	0.071
Ca:N	0.59	0.51	0.94	22.58	<.001	0.0258	65.31	<.001	0.90		1.41	0.191	0.019
N:P	20.51	19.84	0.83	0.41	0.522	0.0013	19.86	<.001	0.77		1.52	0.146	0.059

The overall r^2 of the model and the ANOVA results are provided. The effect of soil, species or the interaction is presented with their F values and their significance level. The percentage of variation explained by each factor (SS$_{factor}$/SS$_{total}$) or the interaction was calculated as the ratio between the factor sum of squares or interaction sum of squares and total sum of squares; thus, variation explained by soils (SS$_s$/SS$_t$), variation explained by species (SS$_{spp}$/SS$_t$), variation explained by the interaction between soil and species (SS$_{sxspp}$/SS$_t$)

relationship between the difference in leaf Ca concentration (Ca$_{ADE-nonADE}$) and the concomitant difference in leaf P concentration (P$_{ADE-nonADE}$) (Fig. 4a). A similar trend was found for leaf Ca

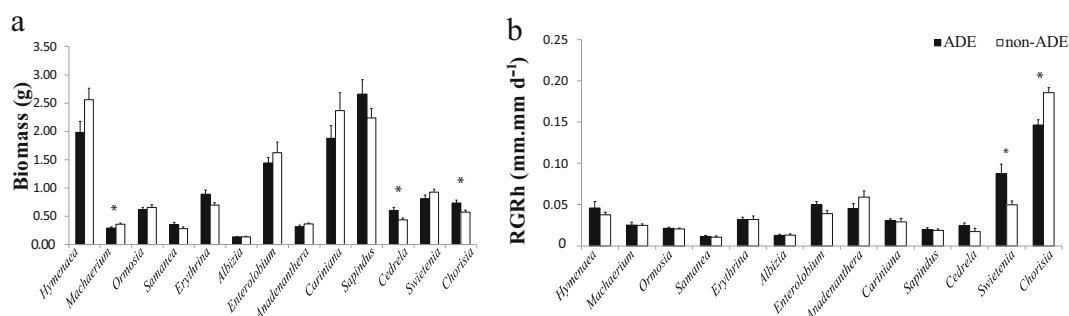**Fig. 2** Biomass at final harvest (**a**) and relative height growth rate (RGRh) (**b**) of seedlings of 13 species growing in Amazonian Dark Earth (ADE) (*black bars*) and non-Amazonian Dark Earths (*open bars*). Means and standard errors are shown. *Asterisks* indicate significant differences between soils at the species level ($p \leq 0.05$)
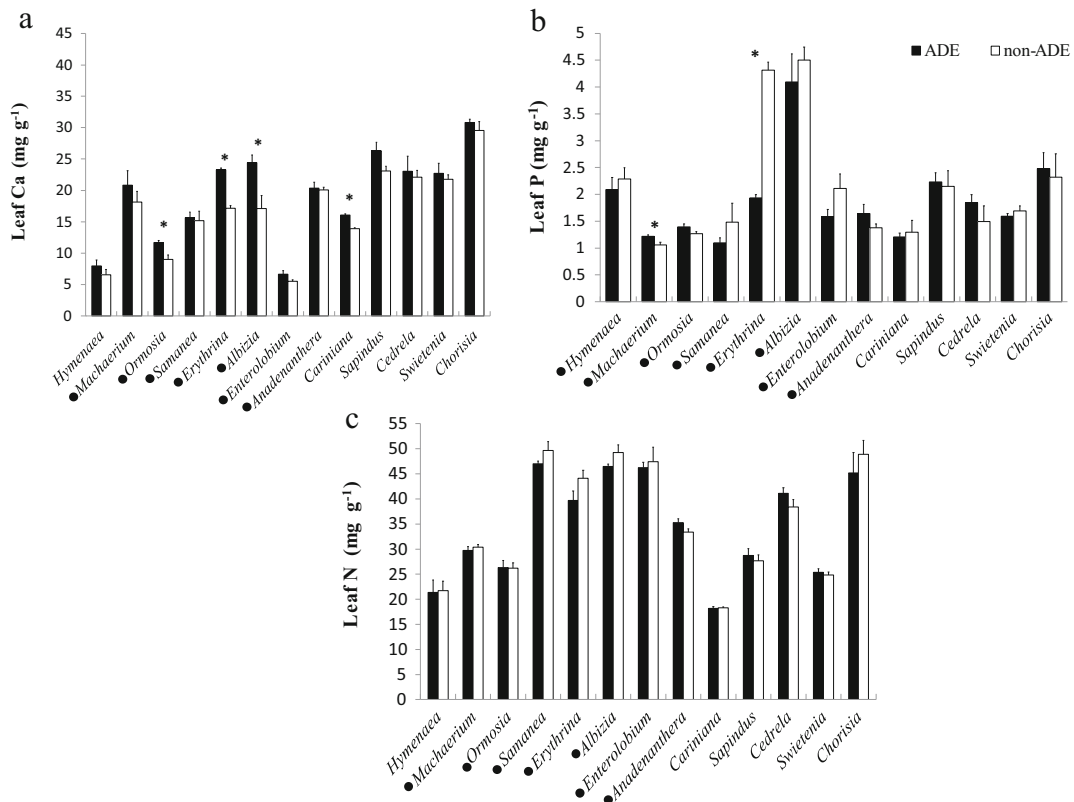
Fig. 3 Leaf calcium (a), phosphorus (b) and nitrogen (c) concentrations of seedlings of 13 tropical tree species growing in Amazonian Dark Earths soils (black bars) and non-Amazonian Darks Earth soils (open bars). Six seedlings per species were used per soil type. Species that are marked with a black point (•) are N-fixing legumes reported on Table 1. Bars represent the average per soil type, with standard errors. Asterisks indicate significant differences between the two soils (t-test, $p \leq 0.05$)

concentrations (Ca$_{ADE-nonADE}$) and leaf N concentration (N$_{ADE-nonADE}$)(Fig. 4); species showed either a higher (positive values on y axis, Fig. 4b) or a lower leaf N concentration in ADE than in non-ADE soils (negative values on y axis, Fig. 4b). The latter relationship, however, was only a trend, but not statistically significant ($P=0.086$, Fig. 4b). It is worth to notice that most of the species located in the negative side of the y axis, where N-fixing legumes (Fig. 4a and b). We also found a positive relationship between the difference in leaf P and leaf N concentrations in ADE and non-ADE (Fig. 4c).

The relationship between leaf Ca concentration and RGRb showed that "positive for P" species increase in RGRb with increasing leaf Ca concentration (Adj-R^2:0.64; $p=0.034$) (Fig. 5a), but no relationship was found for "negative for P" species, (Adj-R^2:0.09; $p=0.26$) (Fig. 5a). Besides, we found that in "positive for P" species, leaf P concentration increased with leaf Ca concentration, although this was at the edge of

significance (Adj-R^2:0.56; $p=0.052$); but no relationship was found for "negative for P" species (Adj-R^2:-0.04; $p=0.42$) (Fig. 5b). Likewise, the relationship between leaf Ca concentration and RGRb showed that "positive for N" species increase in RGRb with increasing leaf Ca concentration (Adj-R^2: 0.86; $p=0.01$), but no relationship was found for "Negative for N" (Adj-R^2: 0.24; $p=0.12$) (Fig. 5c). Furthermore, we found that there was not significant relationship between leaf Ca concentration and leaf N concentration neither in "positive for N" species (Adj-R^2: 0.47; $p=0.12$), nor "Negative for N" species (Adj-R^2: -0.05; $p=0.46$) (Fig. 5d).

Discussion

The aim of this study was to evaluate whether tropical tree seedlings respond to the differences in nutrient concentrations between ADE and non-ADE. Seedlings indeed adjusted their morphology and tissue chemistry
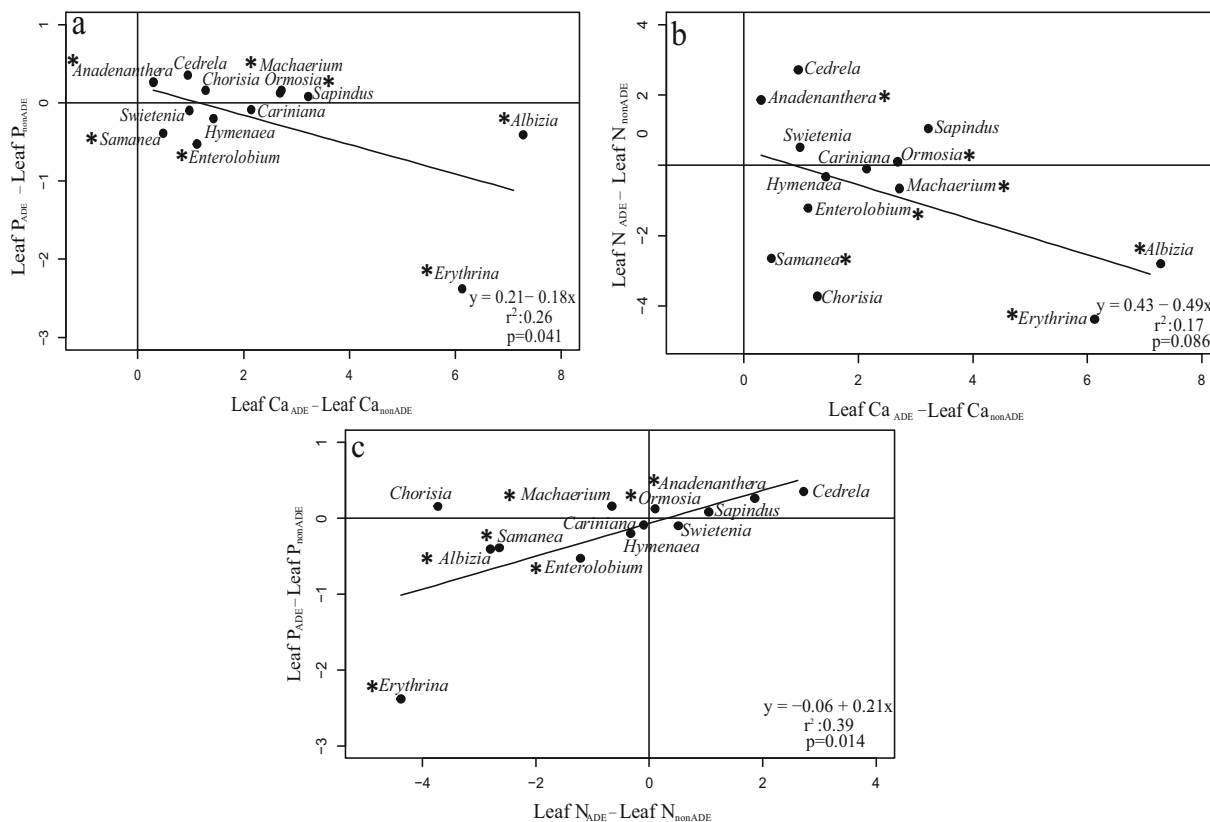
Fig. 4 Linear regressions of the differences in leaf nutrient concentrations between Amazonian Dark Earth and non-Amazonian Dark Earths. (a) Differences in leaf P against differences in leaf Ca. (b) Differences in leaf N concentration against differences in leaf Ca concentration; (c) Differences in leaf P concentration against differences in leaf N concentration. Species that are maked with an *asterisk* (*) are N-fixing legumes reported in Table 1. Results of linear regression are provided. Concentrations units are mg g^{-1}

in response to the increased fertility in ADE, but this did not lead to higher seedling growth in ADE. This lack of growth response seems to be determined by the interplay between the uptake of Ca and the uptake of other essential nutrients such as N and P.

Plant responses to ADE

In our study we found lower survival in seedlings of *Poeppigia* and *Terminalia* that were growing in ADE, compared to those growing on non-ADE. Seedlings of *Poeppigia* were not able to develop a root system on ADE and the seedlings that could establish on non-ADE showed some chlorosis (E. Quintero, personal observation). These characteristics can be associated with inability of plants to regulate Ca uptake when they grow in soil with high Ca. Studies in calcifuges species have suggested that excess of Ca in the tissues translate to Ca toxicity, that inhibits growth, and causes mortality

(Jefferies and Willis 1964). This inhibition could be caused by metabolic disorders related to enzyme inactivation and to a decrease of P availability for metabolism due to the excess of cytosolic Ca (Grundon 1972; Jefferies and Willis 1964; Zohlen and Tyler 2004).

We predicted that in high-fertility ADE, plants would invest in aboveground light capture, whereas in the non-ADE, plants would invest in belowground nutrient capture. This is in line with Brower's hypothesis which states that plants invest in capturing the resource that is in most limiting supply (Brouwer 1962). We found that seedlings growing on ADE allocated more biomass to leaves (LMF) than seedlings on non-ADE, but we did not find support for higher investment in root biomass on non-ADE. Additionally, we found higher investment in leaf area per leaf mass (SLA) and per plant mass (LAR) on ADE. Greater biomass allocation to leaves in combination with higher SLA and LAR on ADE (Table 3) could increase seedlings capacity to capture
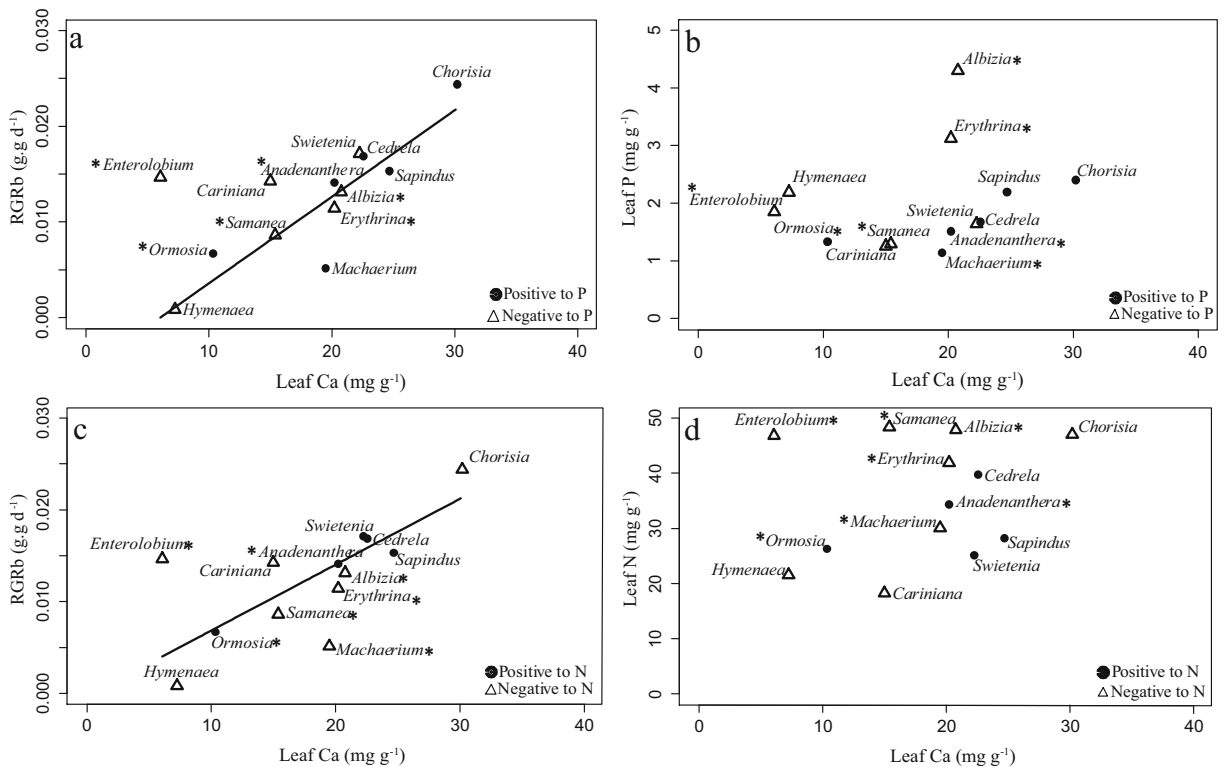
Fig. 5 Relation between (a) Relative biomass growth rate (RGRb) and leaf Ca concentration; (b) Leaf P concentration and leaf Ca concentration for species that responded positively or negatively to P. The regression line represent the significant relationship between RGRb and leaf Ca only for species that were "*positive* for P" (RGRb=-0.0054+0.009 (leaf Ca); R^2=0.64; *p*= 0.034). (c) Relation between relative biomass growth rate (RGRb) and leaf Ca concentration; (d) Leaf P concentration and leaf Ca

concentration for species that responded positively or negatively to N. The *regression line* represents the significant relationship between RGRb and leaf Ca only for species that were "*positive* for N" (RGRb=-0.0003+0.0071(Leaf Ca); R^2=0.86; *p*=0.014). Relationships used information of 13 tree seedling species. Species that are marked with *asterisk* (*) are N-fixing legumes reported in Table 1

light and should enhance biomass growth (Poorter 1999). However, in our experiment, seedling RGR was similar on both soil types, suggesting that an increase in leaf light capture capacity does not translate into faster growth. There are other factors, such as leaf tissue chemistry that could explain the observed limitation of seedling growth found in this study (Figs. 4 and 5).

High Ca in ADE soils affect leaf nutrient stoichiometry

One of the most interesting results of our study is the effect of ADE on leaf nutrient concentrations of some of the most important elements for plant growth. ADE were more fertile, and had 3.2 times more P, 2.4 times more Ca, and 1.6 times more N compared with the non-ADE (Table 2). All species had indeed higher leaf Ca on ADE than on non-ADE (Fig. 3a), but only 6 out of 13 species had higher leaf P and leaf N on ADE (Figs. 3b, c

and 4a, b). Seedlings of seven species had, on average, 1.3 times higher leaf P concentrations on non-ADE than on ADE, even though ADE had more P than non-ADE soils (Table 2). This result suggests that there is an imbalance between soil and tissue elements in some of the species that we studied. There are several possible explanations for this result that are related with soil chemistry and plants nutrient stoichiometry: first the effects of high Ca in soils and its consequences for P availability, second the effects of a high Ca supply on plant tissue and the interaction with inorganic P, and finally, a decrease in N-fixation leading to a decrease in P uptake by N-fixer species on ADE.

Firstly, soils that combine a neutral to alkaline pH with a high Ca concentration (as it is the case in this Bolivian forest) may have, in fact, a low effective availability of P. The relatively high Ca in ADE could lead to the formation of mineral insoluble calcium phosphate,

thus reducing P availability as it is reported in calcareous soils with pH around 7.8 (Lee 1998; Yang and Jacobsen 1990; Zohlen and Tyler 2004). In spite of higher plant available P extracted on ADE (Table 2), lower or not increase in seedlings leaf P could suggest some decrease in effective P availability due to P bounded to Ca (Yang and Jacobsen 1990). In such conditions, only species that can exude organic acids into the rhizosphere to increase the solubility of phosphate would be able to incorporate more P from the soil, as it has been shown in other studies (White and Broadley 2003). Further studies on the interactions between soil Ca and P on ADE could improve our understanding of P effective availability on those soils. Especially, because the major changes in soil chemistry on ADE is due to increases of Ca and P (Glaser and Birk 2012).

Secondly, high concentrations of Ca in the cytosol of plant cells can react with inorganic P and produce calcium phosphate, decreasing the concentration of P available for plant metabolic functions and consequently, the P concentration measured in leaves (Tyler 1996; Zohlen and Tyler 2004). Unfortunately, this mechanism has been mainly studied in temperate species that are not able to grow in calcareous soils with high soil Ca concentrations (i.e. calcifuge species). In the species used in this study this mechanism is speculative but, based in our results, we consider that it deserves further exploration. (Glaser and Birk 2012)

Third, some of the species belonging to Fabaceae (e.g., *Erythrina*, *Samanea*, and *Albizia*) might have increased N-fixation and higher P-uptake on non-ADE than on ADE. Presence of nodules have been reported for these species (Table 1). They had either a higher proportion of seedlings with nodules, or a higher number of nodules per root on seedlings growing on non-ADE than on ADE (data not shown). Thus, more nodules can increase N-fixation, that can increase P and N uptake on non-ADE (Fig. 3b and c). Recent studies suggests that N-fixers can increase the ability of P uptake by being able to invest more carbon for either association with mycorrhizal fungi or phosphatase release into the soil which can give them advantages to incorporate P (Nasto et al. 2014); nodulation process requires, in general, increase in soil pH, usually from acid to close to neutral (Epstein and Bloom 2005; Tisdale et al. 1985). Nevertheless, few studies have shown effects of soil pH≥7 on legume productivity. Three out of nine tropical herbaceous legumes from Australia showed lower productivity as a consequence

of liming that increased soil pH (> 6.0) and soil Ca (Munns and Fox 1977). Besides, studies on effects of liming on crop legumes such as pigeon peas (*Cajanus cajan*) showed a decrease in nodule weight and enzymatic activity with increase in soil pH from 4 to 6 (Ogata et al. 1988). Our results indicate that the nodulation process could be affected by increases in soil pH and Ca, on *Erythrina*, *Samanea*, and *Albizia* but we still need a better understanding of the environmental conditions that control the process of N-fixation in tropical trees in order to better understand this mechanism.

Leaf Ca predicts species growth, but only for those species that can cope with Ca excess

Growth of seedlings in this experiment did not appear to be nutrient limited. In both soil types, seedlings had higher leaf Ca and N concentration, (Table 3) than reference levels normally found (5 mg g^{-1} dry mass for Ca; 15 mg g^{-1} dry mass for N) to sustain plant growth (Epstein and Bloom 2005). On the other hand, leaf P concentrations were close to reference values (2 mg g^{-1} dry mass) (Epstein and Bloom 2005), with some exceptions such as *Erytrhina* (4.3 mg g^{-1} dry mass) and *Albizia* (4.5 mg g^{-1} dry mass) (Fig. 3b). But this "luxurious" nutrient consumption of Ca, N and P did not translate into higher RGR for these species (Fig. 2b). Lack of changes in plant growth with increased nutrient availability in ADE seems to be associated with species-specific abilities in tissue nutrient regulation: whereas some species increased RGR when leaf Ca, P and N increased simultaneously (Fig. 5a-d), other species increased leaf Ca with no consequences on leaf N or P and RGR (Fig. 5a-d). This implies that increases of Ca in the soils and in plant tissues are not beneficial for some species. In general, Ca enters the plants by diffusing among cells or through cell cytoplasm in the roots, then it goes to the xylem and accumulates in the leaves (White 2012). Plants that are not adapted to high Ca in the soils (such as calcifuge plants) cannot regulate the excess of Ca entering their tissues. This excess of Ca uptake is done at the expense of other ion uptake such as such as K, Fe and Mg, causing nutritional imbalance (Jefferies and Willis 1964). We cannot confirm if whether our plants had a nutritional imbalance, since we did not measure other nutrients in the leaves, although we did not notice any symptoms of nutrient deficiency. Additionally, excess of Ca can induce some metabolic problems. The excess of cytosolic

Ca can decrease cellular free P necessary for metabolic functions (Zohlen and Tyler 2004) or it can inhibit some enzymatic action by competing with enzyme cofactors such as Mg and manganese (Mn) (Jefferies and Willis 1964; Webb 1999). Unless plants are able to regulate the excess of Ca through storing it in the vacuole or cell wall (Kinzel 1989) or in epidermal cells or structures such as trichomes (Webb 1999), it is very likely that Ca can produce some metabolic dysfunctions. This has been suggested in studies that report poor plant development on soils with high concentrations of Ca (Jefferies and Willis 1964; Lee 1998).

Trees at La Chonta do not seem to be nutrient limited since natural soils are fertile in terms of N, P, Ca and pH (with average values>6.5). The improvement to the soil by increasing Ca and P levels, as it is the case in ADE, does not result in higher tree RGR, and it can be even detrimental for some tree species such as *Terminalia* and *Poeppigia* that seemed to suffered from Ca toxicity. Our results suggest "luxury" consumption of Ca by tree seedlings, but the advantages of this consumption depend on plant ability to maintain the balance among various nutrients needed for normal function. On one hand, it is likely that the species that did not improve RGR on ADE such as *Samanea, Erythrina* and *Albizia* are unable to keep levels of Ca that do not affect leaf P and N balance. On the other hand, species that had a higher RGR on ADE soils , such as *Cedrela*, and *Sapindus*, are able to keep levels of Ca that do not affect the balance with leaf P and N concentrations on ADE. Nevertheless, we did not elucidate the exact mechanism of Ca accumulation or detoxification (He et al. 2014). In general, our results suggest plant responses that could hamper species establishment on sites with high soil Ca and pH, contributing to understand soil factors that drive species distribution.

Concluding remarks

Past inhabitants of the La Chonta forest modified the soils by increasing mostly Ca and P on ADE. Contrary to our expectation, this soil enrichment did not lead to a general increases in tree growth, probably because the background soil fertility of non-ADE at La Chonta is already high. Our results suggest three possible ways in which increase in soil pH and Ca could affect seedling growth: first, the effects of high Ca in soils could decrease soil P availability for plants; second, high Ca concentrations in plant tissues could decrease the availability of cytosolic inorganic P for metabolic purposes; and third, an increment in soil pH could cause decrease in N-fixation leading to a decrease in P uptake by N-fixing species on ADE. Therefore, the ability of plants to colonize and grow in patches of ADE depends on their ability to cope with increases in soil nutrients, especially increases in Ca.

In the Amazon basin, sites where the natural soils are nutrient limited, ADE soils can improve conditions for plant growth whereas sites where nutrients in the soils are not limited, ADE potentially could cause nutrient imbalance that do not improve plant growth.

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