Molybdenum and Phosphorus Interact to Constrain Asymbiotic Nitrogen Fixation in Tropical Forests

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Abstract

Biological di-nitrogen fixation ($N_2$) is the major natural source of new nitrogen to land ecosystems. Phosphorus ($P$) is thought to limit $N_2$ fixation in many tropical soils, yet both molybdenum ($Mo$) and $P$ are crucial for the nitrogenase reaction (which catalyzes $N_2$ conversion to ammonia) and cell growth. We have limited understanding of how and when fixation is constrained by these nutrients in nature. Here we show in tropical forests of lowland Panama that the limiting element on asymbiotic $N_2$ fixation shifts along a broad landscape gradient in soil $P$, where $Mo$ limits fixation in P-rich soils while $Mo$ and $P$ co-limit in P-poor soils. In no circumstance did $P$ alone limit fixation. We provide and experimentally test a mechanism that explains how $Mo$ and $P$ can interact to constrain asymbiotic $N_2$ fixation. Fixation is uniformly favored in surface organic soil horizons - a niche characterized by exceedingly low levels of available $Mo$ relative to $P$. We show that soil organic matter acts to reduce molybdate over phosphate bioavailability, which, in turn, promotes $Mo$ limitation in sites where $P$ is sufficient. Our findings show that asymbiotic $N_2$ fixation is constrained by the relative availability and dynamics of Mo and P in soils. This conceptual framework can explain shifts in limitation status across broad landscape gradients in soil fertility and implies that fixation depends on $Mo$ and $P$ in ways that are more complex than previously thought.

Introduction

Biological di-nitrogen fixation ($N_2$) fixation is the major natural source of new N to tropical forests [1] and is thought to influence whether N or phosphorus ($P$) limits the ability of these forests to respond to CO$_2$ fertilization [2,3]. The macronutrient $P$ has for a long time been thought to limit $N_2$ fixation in tropical forests, but some observations question how generalizable this might be. From previous work in Hawaii, Vitousek [4] noted that trace metals might together with $P$ influence asymbiotic $N_2$ fixation in soils of a montane tropical forest. Our own studies have demonstrated that molybdenum ($Mo$) but not $P$ limits asymbiotic fixation in a Panamanian lowland forest [5]. $Mo$ functions as a co-factor in the nitrogenase enzyme (which converts $N_2$ to ammonia) while $P$ is needed for ATP and cell growth [6].

Here we examine the influence of both $Mo$ and $P$ on asymbiotic $N_2$ fixation in mature lowland tropical forests of Panama. Asymbiotic fixers (bacteria that live freely in soils) are ubiquitous and may contribute substantial atmospheric $N_2$ in conditions where symbiotic $N_2$-fixers (bacteria that live in association with plant roots) are absent, rare or not fixing [as observed in some mature forests] [7–9]. Both asymbiotic and symbiotic fixation contribute to the common pattern of abundance of $N$ relative to $P$ or other plant resources in many tropical forests [7,10].

Limitation by $Mo$ alone has also been reported for asymbiotic fixers in a highly leached temperate forest soil [11] as well as for symbiotic fixers in some managed pastures and agricultural systems [12,13]. Responses to trace-metal cocktails combined with $P$ fertilizer in Hawaiian soils [4,14,15] also raise the possibility of a $Mo$ influence. Positive responses to $P$ fertilizer [4,5,14–16] are, however, complicated by the observation that superphosphate fertilizer often contains trace amounts of $Mo$ [5]. In our previous study of a Panamanian forest we found that asymbiotic fixation responded positively to $Mo$ alone, to superphosphate fertilizer in which $Mo$ was a “hidden” contaminant, but not to additions of trace-metal free $P$ [5].

These findings raise fundamental questions about the role of $Mo$ and $P$ in constraining $N_2$ fixation: a) Are fixers sensitive to variations in these soil nutrients across landscapes; b) Do fixers respond to both $Mo$ and $P$ in the same location; and c) What conditions and what mechanism(s) favor limitation by one element over the other? These questions identify the need to understand how $Mo$, $P$, or both elements together act to constrain $N_2$ fixation in nature.

Molybdenum and $P$ are both rock-derived and oxyanions in their bioavailable forms (molybdate, MoO$_4^{2−}$ and phosphate, PO$_4^{3−}$ respectively). It is plausible that $Mo$ limitation emerges because molybdate is susceptible to leaching, complexation by...
organic matter and adsorption to iron (Fe) oxides in highly weathered soils [13,17]. However, weathered tropical forest soils can also be substantially poor in P [18] and phosphate availability is reduced by sorption and immobilization processes [19]. The remarkable heterogeneity that exists in weathering conditions and distributions of soil nutrients across tropical forests [20] offers further motivation for resolving the dependence of N₂ fixation on Mo and P across tropical landscapes.

We chose six forest sites distributed along a steep soil P:Mo gradient in Panamanian lowland forests, across which total soil P spans the range reported from tropical forests world-wide while Mo remains relatively constant. The gradient derives from local variations in geology where andesitic and basaltic lithologies give rise to P-rich soils while marine sediments and rhyolitic tuff produce P-poor soils [21,22]. The forests are within 8 km of each other, share a similar pool of tree species, and experience a similar climate.

We examined whether limitation on N₂ fixation depended on landscape-scale variations in the relative abundances of soil Mo and P. We predicted that Mo would limit fixation in P-rich soils while P would limit in P-poor soils. In each forest, we evaluated the response of N₂ fixation to four nutrient addition treatments: control (C), Mo only (+Mo), trace-metal-free P only (+P) and both elements in combination (+Mo+P). To further understand the mechanism by which nutrient limitation differed across sites we intensively sampled vertical distributions of fixation, Mo and P within each soil and conducted laboratory experiments on the ability of organic matter to interact with bioavailable Mo and P.

Materials and Methods

Study Sites

Our study sites are located in mature lowland tropical forests within and south of the Barro Colorado Nature Monument, Republic of Panama. We selected six forests along a steep and well-characterized [21–24] gradient in total soil P, from high (AVA and Fairchild) and medium (Gigante) levels on andesitic and basaltic lithologies to low (Barro Verde and Zetek) and very low (Rio Paja) levels on marine sediments and rhyolitic tuff. All forests share a diverse community of tree species, receive 2600 mm annual rainfall, and are subject to a Jan–April dry season. AVA, Fairchild, Barro Verde and Zetek are all located on Barro Colorado Island, while the remaining sites are located on the nearby mainland. All necessary permits were obtained for the described field studies from the National Environmental Authority of Panama (ANAM).

Soil Nutrient Analysis

We sampled soils down to 80 cm depth from the surface organic layer (O horizon with little O₂ and O₃ development) followed by 10 cm depth increments of mineral soils using an auger or pits at each site. Three replicate samples were homogenized, oven dried (60°C, until stable), and ground by hand in a ceramic mortar and pestle (with liquid N₂ for organic horizons) to prevent Mo contamination introduced by mechanical grinders made of stainless-steel. Samples were analyzed for total C and N by infrared gas analysis combustion and total Mo and P via microwave digestion (CEM, MARS-5) in trace-metal-free nitric acid and ICP-MS analysis (Thermo-Finnigan, Element 2 at medium resolution). Due to high Fe concentration in tropical mineral soils, digests were diluted and this resulted in a limit of quantification of 0.3 ppm for total Mo in soil. Available Mo and P were extracted from all soils using anion-exchange resin beads. For mineral soils, we conducted extractions (beads:soil:water ratio of 1:5:60) where beads (Dowex 1×4–200) were free in solution, shaking at 80 rpm for 16 hrs. Beads were separated from soil using a sucrose-density gradient [25], eluted with 10% HNO₃ [26] and analyzed via ICP-MS. We conducted extractions of O horizon soil samples (beads:soil:water ratio of 1:0.5:30), using similar methods but with resin beads (Dowex 1×4–50) enclosed in nylon bags. This resin extraction method can be applied to both organic and mineral soils without the confounding effects of altering soil pH or osmotic potential during the extraction procedure. The limit of detection for resin-extractable Mo was 0.25 ppb.

Nitrogen Fixation Experiments

We followed a method designed and tested within our Panamanian forests for evaluating the response of N₂ fixation to nutrient amendments [5]. We collected field moist O horizon soils along 100 m transects at each forest site in the late dry season to wet season of 2008 (May–July). Samples were well mixed before nutrient additions and incubations. To each ~40 g sample (wet weight) we delivered one of four treatments in 14 mL (n = 5–15): distilled water (control), 667 µg Mo/kg as Na₂MoO₄ (+Mo), +203 mg P/kg as NaH₂PO₄ (+P), and 667 µg Mo/kg+283 mg P/kg (+Mo+P). To test that P addition levels were adequate, we also added +2.83, +28.3, +283 mg P/kg as NaH₂PO₄ to soil samples from each site (n = 5). Soils were well-mixed after the treatment application, placed in 0.5 L glass jars, and equilibrated outdoors at ambient forest temperature for 10–15 h. To commence acetylene reduction assays (ARA), jars were sealed and 10% of the headspace was replaced with C₂H₂. Mixed headspace samples were collected at 5 and 10 h, stored in gas-tight sampling vials and measured within 48 h for C₂H₄ on a gas chromatograph (SRI) equipped with a flame-ionization-detector and a Porapak N column. We selected the incubation time based on the physiological response of Azotobacter to Mo addition in our own laboratory experiments and to soil communities in the field [27]. In addition, results from our short-term laboratory incubations in all cases matched results from long-term fertilizations in large-scale field plots [5]. Following incubations, we dried and stored samples for moisture and nutrient content. Soil samples from the ARAs were subsequently extracted for available Mo and P content following resin methods described above. Acetylene reduction activity was calculated from the slope of C₂H₄ production over two time points and accounting for background levels of C₂H₄. Nutrient limitation of N₂ fixation was tested at each site with a 2-factor ANOVA, with two levels (without addition, with addition) of Mo and P using a general linear model and Tukey post hoc means separation tests (all statistical tests evaluated with SAS software, SAS Institute Inc, Cary, NC).

Experiments on molybdate and phosphate interactions with soil organic matter

We explored how organic matter controls the availability of phosphate and molybdate. First, we compared the fraction of available (i.e., resin-extractable) vs. total (i.e., digestible) P and Mo in O horizon samples across our sites. Second, we added and incubated available forms of Mo (as molybdate; 667 µg Mo/kg as Na₂MoO₄; 6-fold ambient) or P (as phosphate; 283 mg P/kg as NaH₂PO₄; 0.6-fold ambient) for 20–25 hours in fresh organic soil and then extracted available Mo and P. Third, to verify that soil polyphenols reduce Mo availability, we combined 0.1 mM solutions of molybdenum with increasing concentrations of tannic acid (0–1 mM) and extracted Mo with resin beads using the same methods as above. The quantity of Mo and P that could be recovered in available forms was compared with t-tests.
Results

N$_2$ fixation along a landscape gradient in soil nutrients

Our six forest sites span a five-fold range of total soil P (top 20 cm of mineral soil; Fig. 1a). Available P in the O horizon increased three-fold along this gradient in total soil P ($r^2 = 0.84$; log-log regression). In contrast, total Mo in mineral soils remained very low across all sites (0.3–0.9 ppm; see methods). In the O horizon, both total and available Mo were relatively stable across all sites (Table 1).

We observed a shift in the response of N$_2$ fixers to P and Mo additions across this soil P gradient. In Gigante and Fairchild at the P-rich end of our gradient, nitrogenase activity increased up to four-fold in response to additions of Mo alone and Mo and P combined, but did not respond to P alone (Fig. 1b); $F_{1,3} = 36$, $p < 0.0001$ for Gigante, $F_{1,3} = 16$, $p < 0.001$ for Fairchild, analysis of variance (ANOVA). In contrast, in Zetek and Rio Paja at the P-poor end of our gradient, nitrogenase activity increased 1.5 to 4 times only in response to the combined addition of Mo and P, but not to either element applied individually (Fig. 1b); Mo by P interaction, $F_{1,3} = 5.7$, $p = 0.023$ for Zetek and $F_{1,3} = 1.7$, $p = 0.039$ for Rio Paja, ANOVA). At AVA and Barro Verde we observed low to medium rates of ambient N$_2$ fixation but no clear response to nutrient amendments. To verify that we added sufficient available P, we dosed soils with phosphate at three additional levels (in total spanning four orders of magnitude) but observed no positive response in any forest (Table 2).

Table 1. Leaf litter chemistry from six forest sites in lowland Panama arranged from the high to low in total soil P.

| Site      | C (%) | N (%) | P (ppm) | Mo (ppb) | Resin Mo (ppb) |
|-----------|-------|-------|---------|----------|----------------|
| AVA       | 47.3  | 2.1   | 437     | 144      | 0.9            |
| Fairchild | 44.4  | 1.3   | 629     | 131      | 0.3*           |
| Gigante   | 47.9  | 1.6   | 384     | 70       | 2.0            |
| Barro Verde | 47.5 | 1.6   | 569     | 91       | 0.9            |
| Zetek     | 48.8  | 1.6   | 377     | 86       | 1.0            |
| Rio Paja  | 46.9  | 1.4   | 412     | 157      | 0.3*           |

Values are means and standard errors in parentheses from each sampling site. Chemical variables included total C and N (%), total digestible P (ppm) and Mo (ppb) and resin extractable Mo (ppb). Many values for resin Mo were at or below the limit of detection (0.25 ppb). doi:10.1371/journal.pone.0033710.t001

N$_2$ fixation and soil chemistry with depth

Individual soil profiles across all our study sites displayed similar and clearly delineated vertical gradients in N$_2$ fixation, mass ratios of total carbon (C) to N and of available Mo and P (Fig. 2). The highest rates of N$_2$ fixation always occurred in the upper soil horizon (O), which was rich in organic matter but poor in total N (C:N ratios ~30)(Fig. 2a). Deeper soil layers displayed negligible fixation, low C content, and abundant N relative to C (C:N ~10).

Figure 1. Soil P and the response of nitrogenase activity to nutrient additions from six forest sites arranged from high to low total P. A) Total P (0–20 cm depth; orange bars) and available P (O horizon; grey bars). Pale orange lines represent the range total P levels from montane tropical soils [31] and highly weathered soils in the Amazon basin [20]. B) Nitrogenase activity (acetylene reduction activity (ARA)) in response to nutrient additions: water only (–Mo – P), Mo only (+Mo + P), P only (–Mo + P) and both Mo and P (+Mo + P) in O horizon soil samples ($n = 5–15$). Mo was added as Na$_2$MoO$_4$ and P was added as NaH$_2$PO$_4$. Values represent means ± s.e.m. ARA data from Gigante (–Mo – P, +Mo – P and –Mo – P) have been previously reported [5]. Values reported on a mass basis in accordance with convention from global soil data; bulk densities of 0.07 and 0.7–1.2 g/cm$^3$ for organic and inorganic horizons respectively can be used to convert to an approximate soil volume basis. doi:10.1371/journal.pone.0033710.g001
Available Mo was exceptionally low in the organic soil layer, peaked in the upper mineral soil horizon and declined with soil depth. In contrast, available P was greatest in the organic soil horizon and declined steadily with depth. As a result the upper organic soil horizon was characterized by ratios of available P:Mo that were up to five orders of magnitude greater than those of deeper soil layers (Fig. 2e).

Experiments on molybdate and phosphate availability

While high available P has been reported from the O horizon of P-poor tropical soils [28–30], we explored whether interactions with soil organic matter could explain the occurrence of high P:Mo ratios in Panamanian organic soil horizons. First, we found that available Mo constitutes \(2\%\) of total Mo in O horizon samples across our sites, while available P accounts for \(20\%\) of total P \((p<0.0001, t\)-test\). Second, after experimental additions of molybdate and phosphate we could recover only very little \(2\%\) of newly added Mo from the soil matrix in the form of available Mo. In contrast, we could recover substantial amounts \(>60\%\) of newly added P in the form of available P. Third, to verify that soil polyphenols act to reduce Mo availability, we combined solutions of molybdate and tannic acid in the laboratory and observed that the recovery of available Mo decreased with increasing concentrations of tannic acid (Fig. 3c).

Discussion

Soils from our sites in lowland Panama span the range of total P content reported from tropical forests on well-developed soils worldwide (Fig. 1a); from P-rich montane soils [31] (similar to our AVA site) to large areas of P-deficient soils in lowland Amazonia [20] (similar to our Rio Paja site). In contrast, total soil Mo was low compared to the few measures available from natural soils [32] and did not vary systematically across our sites. Therefore, our gradient primarily captures variations in P relative to Mo and provides a localized experimental system for understanding how P and Mo might interact to constrain \(N_2\) fixation in tropical landscapes.

Our vertical sampling of soil profiles revealed that asymbiotic fixation primarily occurred in the O-horizon across all our sites [4,5,15,33]. We infer that asymbiotic \(N_2\)-fixers are stimulated in the O horizon by the continual input of energy-rich plant litter that is poor in N (as indicated by high C:N ratios) relative to the physiological demand of soil microbes. Although a suite of environmental factors (e.g., moisture, temperature) can influence the rate of fixation, our goal here was to examine the specific role of Mo and P in limiting fixation in the O horizon of a given soil.

We predicted that Mo would limit \(N_2\) fixation in P-rich soils while P would limit in P-poor soils. Nutrient amendments stimulated \(N_2\) fixation substantially in four of our six sites following a clear pattern: fixation was limited by Mo alone in P-rich soils but co-limited by both Mo and P in P-poor soils. Contrary to our expectation, we never found exclusive P limitation at any of our sites. The influence of Mo extended even to the most P-poor sites, where additions of both Mo and P were necessary to

Table 2. Nitrogenase activity (Acetylene reduction activity in nmol C\(_2\)H\(_2\) g\(^{-1}\) h\(^{-1}\)) in response to additions of P to soils from six forest sites (mean (s.e.m.); \(n=5\)).

| Site     | control | +2.83 | +28.3 | +2830 |
|----------|---------|-------|-------|-------|
| AVA      | 0.34 (0.08) | 0.24 (0.06) | 0.11 (0.04) | 0.22 (0.04) |
| Fairchild| 6.5 (1.04)  | 5.00 (1.33)  | 5.29 (1.64)  | 5.64 (1.62)  |
| Gigante  | 0.38 (0.06) | 0.17 (0.03)  | 0.28 (0.07)  | 0.24 (0.04)  |
| Barro Verde | 1.84 (0.42) | 1.40 (0.42) | 1.20 (0.53) | 0.92 (0.33) |
| Zetek    | 0.48 (0.21) | 0.59 (0.10)  | 0.54 (0.16)  | 0.36 (0.05)  |
| Rio Paja | 1.57 (0.23) | 1.85 (0.29)  | 1.52 (0.14)  | 1.10 (0.07)  |

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Figure 2. Vertical soil gradients in nitrogenase activity and nutrient abundance averaged across six tropical forests. A) Nitrogenase activity (acetylene reduction activity, ARA). B) Total C to N mass ratio. C) Available (resin-extractable) Mo. D) Available (resin-extractable) P. E) Resin-extractable P to Mo mass ratio. Uppermost soil layer is the organic soil horizon, mineral soil begins at 0 cm. Values (mean ± s.e.m.) represent measurements from six tropical forest sites. Values reported on a soil volume soil to reflect what microbial communities experience, and to minimize the confounding influence of differences in density between organic and inorganic horizons.

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stimulate rates of fixation (Zetek and Rio Paja in Fig. 1b). We did not observe statistically significant responses to nutrient additions in two locations, perhaps due to the influence of edaphic factors other than Mo and P [34] as suggested by the low fixation rates observed at the AVA site. The slightly weaker response to combined Mo and P addition at Gigante was possibly caused by interference of the macronutrient phosphate on the availability of the micronutrient molybdate.

Our results offer a direct experimental demonstration that asymbiotic N\textsubscript{2}-fixers can be influenced by broad variations in Mo and P availability across forests, and that the influence of Mo can extend (either alone in P-rich soils or in combination with P in P-poor soils) across soil P levels. These findings, together with the lack of P-only limitation across our P gradient raise important questions about how Mo and P interact to constrain fixation in tropical soils.

We next examined why Mo was such a prevalent constraint on fixation in the organic soil layer across our sites. It may be reasonable to expect that molybdate and phosphate behave similarly in soils as they are both oxyanions. However, we found that available forms of Mo and P possessed dramatically different trends with soil depth. Most strikingly, the organic horizon possessed high levels of available P relative to Mo and was characterized by available P:Mo ratios that were up to five orders of magnitude greater than those of deeper soil layers. These contrasting distributions of Mo and P with soil depth create a localized biogeochemical niche (the O horizon) in which high available Mo, high fixation rates, and high available P to Mo ratios could lead to Mo limitation on N\textsubscript{2} fixation.

We next examined whether differences in how Mo and P interact with the plant-soil organic matter cycle can explain whether Mo and/or P emerge as primary constraints on N\textsubscript{2} fixation. Mo is one of the least abundant plant essential elements [35] and is generally scarce relative to P in the organic horizon. For example the mass ratio of total digestible P:Mo averaged \(\sim 5000\) in O horizons across our study sites, which is 6–7 times greater than the optimal P:Mo ratio required by free-living N\textsubscript{2}-fixing microorganisms in our laboratory cultures (680–850 for Azotobacter vinelandii; [27]). This discrepancy was even further magnified when considering available forms of P and Mo, such that P:Mo ratios exceeded the cellular ratio of N\textsubscript{2}-fixers by up to 200 times in the O horizon. These observations offer direct evidence that the availability of P can be abundant relative to Mo in the organic soil layer.

We considered the possibility that the high ratio of available P:Mo in the O horizon is caused by differences in how each element interacts with organic matter. Mo is held strongly by polyphenolic complexes that can diminish bioavailability in soils [17], while most organic P can be hydrolyzed by extracellular phosphatases produced by plants and microbes [36,37]. We found support for the hypothesis that plant organic matter preferentially reduces the availability of Mo relative to P. Not only does a large fraction of P exist as highly available forms relative to Mo, but the addition of Mo and P showed preferential binding of molybdate to natural organic matter and to experimental additions of tannic acid. These results indicate that relative to phosphate, free molybdate is almost entirely consumed by soil even at concentrations much higher than ambient levels.

We speculate that what little molybdate is free in solution in the soil organic layer is likely consumed by biological uptake or lost by leaching to deeper soil horizons. In addition, some organically-complexed Mo can be accessed by free-living N\textsubscript{2}-fixers that have evolved high-affinity systems for Mo uptake [17,38]. The extent of such mining for Mo is poorly known and may depend on the energetic cost and effectiveness of metallophore production in soil environments. The presence of such a specialized uptake mechanism, however, indicates that Mo-poor environments have acted as a strong constraint on asymbiotic N\textsubscript{2}-fixing organisms over evolutionary time.

We identify the existence of broad gradients in nutrient limitation on N\textsubscript{2} fixation across tropical forests. Our findings are consistent with the following set of interacting mechanisms: 1) N\textsubscript{2} fixation is favored in the organic soil horizon because the plant-soil cycle creates a vertical gradient in energy-rich and N-poor organic matter; 2) In this specialized niche Mo is complexed strongly while most organic P is readily turned over, favoring the availability of P over Mo and making possible the localized emergence of Mo over P limitation on N\textsubscript{2}-fixers; and 3) N\textsubscript{2}-fixers respond to broad trends...
in soil P availability, such that Mo constrains fixation in P-rich soils while Mo and P together constrain fixation in P-poor soils.

Our results offer a link between Mo and P stoichiometry from the scale of molecular biochemistry to that of landscape nutrient cycles. Sole Mo-limitation indicates deficiency in the FeMo-co-factor of nitrogenase [39], while Mo+P co-limitation suggests deficiency in both the co-factor and the P-rich molecules (e.g., ATP, RNA and phospholipids) that support N₂ fixation and cell growth [6]. These constraints may, in turn, scale up to larger levels of observation if Mo and P interact across soils in general (as we found in lowland Panama).

These results lend support to the idea of an emerging paradigm shift in the way we view nutrient limitation in tropical forests. Our findings suggest that N₂ fixation should not be considered solely constrained by P in conceptual theories or global biogeochemical models (e.g., [40]). The idea of uniform single-element limitation is increasingly challenged as more complex relationships between nutrient cycles emerge. In these Panamanian forests, this complexity is a consequence of the presence of N₂ fixing organisms, inputs of P and Mo (from weathering and dust), and interactions of C, N, P and Mo within different soil layers. It is critical to determine if the soil-based mechanisms observed in this study are pervasive across land ecosystems where N₂ fixation is a critical process.

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Our conceptual model may also apply to N-poor ecosystems in temperate and boreal biomes that have highly leached soils (thereby high Mo availability), distinct organic horizons (thereby high Mo concentration) and where symbiotic N₂ fixation can be the major vector of new N inputs. A previous experiment in temperate rainforests identified a direct influence of Mo on fixation [11], but there exists little to no information on how Mo and P interact across soils of extratropical as well as tropical biomes. A critical issue is whether our results extend also to symbiotic N₂-fixers. Limitation by Mo has to our knowledge not been experimentally evaluated for symbiotic fixers in natural communities including tropical forests. It is plausible that the new concept of Mo+P co-limitation also applies to symbiotic fixers that primarily access nutrients from organic-rich soil horizons. Our findings therefore raise the question of how symbiotic N₂-fixing plants relate to variations in Mo and P distributions in soils.

Here we offer a new mechanistic model against which the diverse responses of tropical N₂ fixation to nutrient additions (including superphosphate fertilizer) can be interpreted [4,5,14–16]. We demonstrate that Mo and P act together to limit fixation across a range of soil P concentrations, from among the highest to the lowest reported for tropical forest ecosystems. In no case did we observe sole P limitation, but this constraint might emerge in soils where Mo is more abundant than it is across our gradient. Re-examining previous studies with our current hindsight is challenging, but we note that the common use of superphosphate fertilizer might confound the very interactions of P and Mo that our present study has uncovered. Since tropical soils vary greatly in distributions of macro- and micronutrients [20] it is likely that the land N cycle depends on P and Mo in ways that are more complex than we have previously considered.

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**Author Contributions**

Conceived and designed the experiments: NW JPB AMLK LOH. Performed the experiments: NW JPB. Analyzed the data: NW LOH. Contributed reagents/materials/analysis tools: NW JPB AMLK. Wrote the paper: NW LOH.

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