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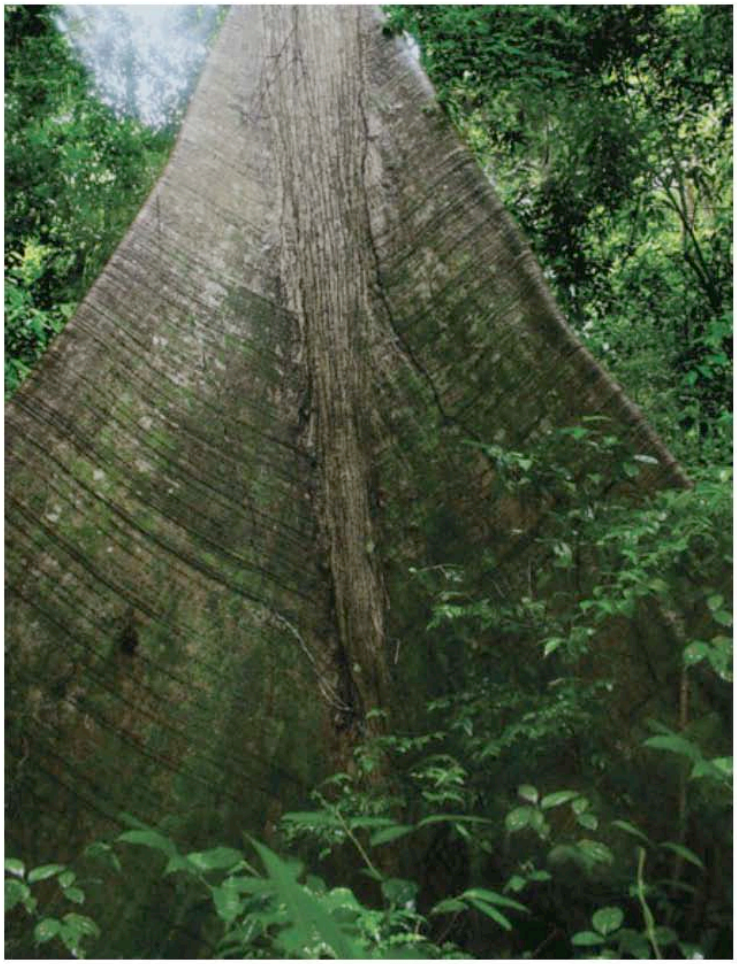

Biological nitrogen fixation limits plant growth and carbon exchange at local-to-global scales. Long-term nutrient manipulation experiments in forests and short-term manipulation experiments in microcosms suggest that the micronutrient molybdenum, a component of the nitrogen-fixing enzyme nitrogenase, limits nitrogen fixation by asymbiotic bacteria in tropical soils in Panama. This image was taken by Lars Hedin in the dense tropical rainforest of the Barro Colorado Nature monument, Panama and shows a tree of the species *Ceiba pentandra* in the family Bombacaceae.

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LETTERS

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Molybdenum limitation of asymbiotic nitrogen fixation in tropical forest soils

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Nitrogen fixation, the biological conversion of di-nitrogen to plant-available ammonium, is the primary natural input of nitrogen to ecosystems[1], and influences plant growth and carbon exchange at local to global scales[2–6]. The role of this process in tropical forests is of particular concern, as these ecosystems harbour abundant nitrogen-fixing organisms[1,4] and represent one third of terrestrial primary production[4,7,8]. Here we show that the micronutrient molybdenum, a cofactor in the nitrogen-fixing enzyme nitrogenase, limits nitrogen fixation by free-living heterotrophic bacteria in soils of lowland Panamanian forests. We measured the fixation response to long-term nutrient manipulations in intact forests, and to short-term manipulations in soil microcosms. Nitrogen fixation increased sharply in treatments of molybdenum alone, in micronutrient treatments that included molybdenum by design and in treatments with commercial phosphorus fertilizer, in which molybdenum was a 'hidden' contaminant. Fixation did not respond to additions of phosphorus that were not contaminated by molybdenum. Our findings show that molybdenum alone can limit asymbiotic nitrogen fixation in tropical forests and raise new questions about the role of molybdenum and phosphorus in the tropical nitrogen cycle. We suggest that molybdenum limitation may be common in highly weathered acidic soils, and may constrain the ability of some forests to acquire new nitrogen in response to CO_2 fertilization[9].

Despite the importance of tropical forests in global biogeochemical cycles[4,7,8], little is known about factors that control nitrogen fixation in these systems. The observation that highly weathered tropical soils are poor in rock-derived nutrients has led to the expectation[3,10,11] that nitrogen fixation might be limited by macronutrients in general, and phosphorus (P) in particular. However, rock-derived micronutrients such as molybdenum (Mo) may be an underappreciated factor in such environments. Mo is a key component of the most common nitrogenase enzyme and has been shown to limit nitrogen fixation in agricultural legumes[12,13], heterotrophic nitrogen fixation in some Pacific Northwest soils[14], and has been proposed as a limit on nitrogen fixation under elevated CO_2 (ref. 9). Mo is in particularly short supply in many acidic tropical soils, where weathering and leaching can act to remove trace elements over time. The main inorganic form of Mo in oxic soils is molybdate (MoO_4^{2-}), which can interact with iron oxides and organic matter to drastically reduce Mo bioavailability[15], especially under acidic conditions. The potential for Mo effects is implied by the existence of a high-affinity Mo uptake system in free-living nitrogen fixers[16], and from field additions of micronutrient cocktails, which, besides P additions, increase nitrogenase activity in some Hawaiian soils[11]. Yet, there have been no direct tests of Mo as a limiting factor for nitrogen fixation in tropical soils, nor of why nitrogen fixers may respond positively to both micronutrients and P added separately.

We studied nitrogen fixation by free-living heterotrophic bacteria (hereafter nitrogen fixers) in weathered tropical soils to examine whether Mo and other nutrients limit fixation rates. Our addition experiments were conducted in floristically diverse (300+ tree species), mature, lowland rainforests on the Gigante peninsula in the Barro Colorado Nature Monument, Panama. Soils are highly weathered acidic oxisols[17,18], typical of a broad range of tropical forests worldwide[19]. Our measures showed that heterotrophic fixation almost exclusively occurred in the uppermost organic-rich litter soil layer (O_i horizon), which was characterized by low total contents of nitrogen and Mo relative to carbon (Table 1).

We used two approaches to evaluate potential Mo limitation. First, we examined the nitrogenase activity of field-moist samples of the O_i horizon collected in replicated large forest plots (1,600 m^2) subjected to long-term (7 years) additions of nitrogen (+125 kg N ha^{-1} yr^{-1} as urea), P (+50 kg P ha^{-1} yr^{-1} as triple-superphosphate) or micronutrients (Mo, S, Cu, B, Fe, Mn, Ca and Mg; +0.01 kg Mo ha^{-1} yr^{-1}). Second, we directly manipulated Mo and P levels in the O_i horizon of unamended soils and documented responses in nitrogenase activity 12 h after addition. In all cases, we estimated nitrogenase activity using a standard assay for acetylene reduction activity (ARA) calibrated against $^{15}N_2$ uptake measured at our sites[20,21]. Variations in foliar chemistry, including lignin, have been suggested to influence nitrogen fixation[22], although the exact effect is difficult to quantify, as factors such as nitrogen availability often do not vary independently. In all of our sites, litter originated from a heterogeneous combination of native plant species that ranged widely in leaf chemistry (including lignin) independent of treatment[23].

Long-term fertilization with nitrogen, P and micronutrients caused clear shifts in the abundance of nitrogen, P and Mo in soil O_i horizons, with increased Mo levels in both the micronutrient and P treatments (Table 1, $p < 0.001$; t-test; $n = 8$, 16). These treatments significantly affected nitrogenase activity (Fig. 1a; $p < 0.0002$, analysis of variance (ANOVA); 4 plots per treatment; 10 measures per plot), with two-to-threefold increases above background levels occurring in plots that received micronutrient or P treatments ($p < 0.013$ and $p < 0.007$, respectively; post-hoc t-test; d.f. = 78). In addition, fertilization with nitrogen caused a ~75% decrease in nitrogenase activity ($p < 0.001$; post-hoc t-test; d.f. = 78), indicating that nitrogen fixation is suppressed when

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Table 1 | Total carbon and nutrient contents, and molar ratios for soils.

Treatment	%C	%N	P (p.p.m.)	Mo (p.p.b.)	C:N:P:(Mo x 10^6) [s.e.m. C:P, N:P, Mo:P, *n*]
Control (LT)	40.99 (±1.87)	1.44 (±0.07)	397 (±28)	53 (±7)	2,698:81:1:43 [±117, ±2, ±5, 8]
+Nitrogen (LT)	44.82 (±2.77)	1.66 (±0.11)	497 (±47)	55 (±9)	2,425:76:1:39 [±199, ±5, ±9, 8]
+Phosphorus (LT)	35.20 (±2.36)	1.35 (±0.08)	907 (±76)	118 (±12)	1,116:36:1:44 [±133, ±3, ±4, 16]
+Micronutrient (LT)	38.51 (±2.56)	1.32 (±0.09)	469 (±43)	215 (±28)	2,242:65:1:283 [±241, ±6, ±22, 8]
Control (for Mo addition)	52.25 (±0.54)	1.69 (±0.05)	559 (±19)	69 (±39)	2,431:67:1:36 [±68, ±3, ±18, 10]
+Low level Mo	57.02 (±4.78)	1.86 (±0.17)	507 (±24)	94 (±5)	2,976:74:1:60 [±321, ±12, ±2, 10]
+High level Mo	51.05 (±0.57)	1.56 (±0.04)	527 (±21)	660 (±42)	2,545:66:1:406 [±130, ±3, ±22, 10]
+Micronutrient control (LT)	51.17 (±0.63)	1.53 (±0.05)	490 (±40)	456 (±92)	2,896:72:1:283 [±287, ±5, ±42, 10]

Total nutrient content for O_i horizon (litter layer) samples in this experiment. LT: litter from long-term fertilization plots. Values are means (±s.e.m.); sample sizes are listed in the final column.

sufficient soil nitrogen is available. In addition to these treatment effects, significant variability could be explained by between-event differences in fixation rates ($p < 0.001$; ANOVA; d.f. = 2). These clear responses suggest that limitation by nutrients is stronger than the natural variation in soils and litter chemistry within the forest plots.

Although both Mo and P have been suggested as potential limiting factors on nitrogen fixation[3,11,14], we recognized that a parsimonious explanation for our finding is that Mo was present in both long-term fertilizer treatments. Not only was Mo applied as part of the micronutrient treatment, but Mo typically occurs as a trace contaminant in phosphate fertilizers[11,24]. In addition to directly testing for the role of Mo, we evaluate below whether the small 'unintended' amounts of Mo present in the P fertilizer could explain the observed fixation response to long-term +P fertilization.

We manipulated Mo at levels equivalent to roughly 1/2 month versus 1/2 year ('low Mo' versus 'high Mo') of the field micronutrient fertilizer application. We used a diverse mix of litter from a common source (control plot O_i horizon). Replicates were subjected to low versus high Mo in distilled water (+42 versus +504 μg Mo kg^{-1} soil or +0.4 versus +4.7 g Mo ha^{-1}) and a control addition of distilled water alone (0.5 kg H_2O kg^{-1} litter, equivalent to <0.5 mm rainfall). To compare responses between short- versus long-term micronutrient additions, we also treated litter from O_i horizons in the long-term micronutrient fertilization plots with distilled water alone.

These direct additions confirmed that the addition of Mo alone can trigger the observed increase in nitrogenase activity in response to micronutrients (Fig. 1b, $p < 0.03$; d.f. = 2; $n = 23$; ANOVA, left three bars). Nitrogenase activity increased as a function of increasing Mo concentrations ($p < 0.02$; $n = 79$; $S = 62{,}578$; Spearman's rank correlation), although side-by-side comparison of low-level Mo versus control was not significant using a statistically less powerful post-hoc *t*-test ($p < 0.36$; $n = 23$). In addition, nitrogenase activity in the high-Mo treatment could not be distinguished from the response observed in the micronutrient-enriched litter treated only with water ($p = 0.420$; $n = 23, 10$; *t*-test). As soil concentrations of Mo were roughly similar in both treatments (micronutrient versus high Mo: $x = 456$ versus 564 μg Mo g^{-1} soil; s.e.m. = 92 versus 35), we conclude that short-term responses to Mo (as measured in the high-Mo treatment) did not differ greatly from the long-term value (as measured in the micronutrient plots). This result supports earlier observations of rapid nitrogenase response to Mo addition[14].

Because P was applied to field plots at loadings three orders of magnitude above trace elements, we examined whether contaminant Mo in the phosphate fertilizer could represent a significant Mo source to nitrogen fixers. First, Mo was present in the P fertilizer (14.1 mg Mo kg^{-1}; s.e.m. = 0.8) at levels consistent with typical triple-superphosphate fertilizers (2.4–18.5 mg Mo kg^{-1}) (ref. 24). Second, Mo was significantly higher in +P soils when compared with controls (Table 1; 118 versus 55 μg Mo kg^{-1}; $p < 0.001$; $n = 16, 8$; Wilcoxon test). Third, we estimate that one year of P fertilizer delivered an amount of Mo five times larger than the increase that we measured in the O_i horizon of the +P treatment. The unaccounted for Mo was probably lost by leaching or plant uptake.

As a final test of whether contaminant Mo could explain the fixation response in long-term +P fertilizer plots, we subjected unamended soils to solutions of distilled water (control), analytical-grade 'clean' P (uncontaminated by Mo) at low, medium and high levels (+2.83, +28.3 and +283 mg P kg^{-1} soil, respectively) or Mo (+667 μg Mo kg^{-1} soil). Whereas the +Mo addition triggered a nearly fivefold increase in nitrogenase activity (Fig. 1c; $p < 0.001$; Tukey–Kramer post-hoc test), each of the three levels of +P addition failed to induce any significant response above background levels (Fig. 1c; $p > 0.17$; Tukey–Kramer post-hoc test). We conclude that fixation rates were limited by Mo but not P, and that Mo probably constituted a 'hidden treatment' sufficient to create the appearance of P-limitation in the +P fertilizer plots. As commercial fertilizer is widely used in nutrient manipulations, previous studies might have misinterpreted such apparent P effects.

When considered in combination (Fig. 2), our different experiments offer consistent evidence that Mo availability alone can limit heterotrophic nitrogen fixation in these tropical soils, and illustrate the dynamic nature of interactions between Mo, nitrogen and nitrogen fixation. Direct additions of either low or high levels of Mo increased nitrogenase activity (squares and triangles in Fig. 2), as did additions of Mo as part of the long-term micronutrient (diamond) and +P treatments (circle). In contrast, long-term addition of nitrogen caused the suppression of nitrogenase activity at native levels of Mo (+N). Although the relative response in fixation increased with the extent of soil Mo enrichment ($p < 0.0001$; $r^2 = 0.80$; $n = 12$), seasonality also affected experimental responses. For example, compared with experiments conducted in the wet season (++Mo square), the relative increase in nitrogenase activity was greatest during the late dry season (++Mo triangle)—a time when ambient fixation rates were at their lowest.

Mo limitation of free-living nitrogen fixers may be widespread in ecosystems with acidic soils that are poor in rock-derived nutrients, and in which Mo complexation to iron oxides or soil organic matter is common[13,25]. Although such conditions are prevalent in many lowland tropical regions, low Mo can also be found in extra-tropical locations including highly leached soils in the Pacific Northwest[14] or reclaimed agricultural soils in Australia[15].

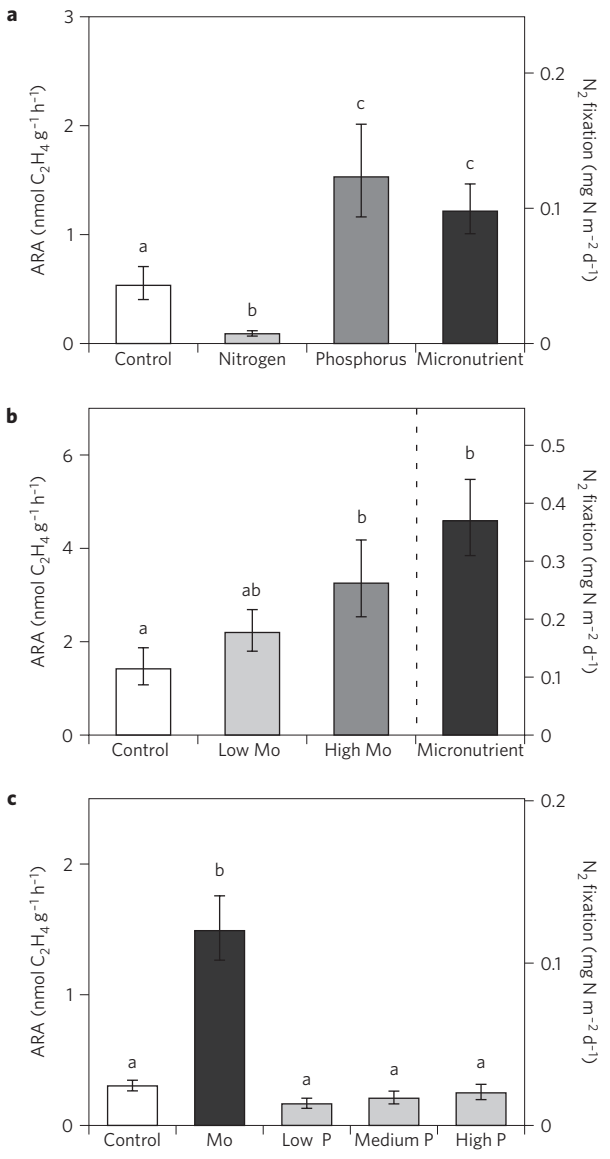

Figure 1 | Nitrogenase acetylene reduction activity (ARA) in response to long-term nutrient fertilization and direct additions of molybdenum and phosphorus. a, ARA in field-moist samples collected from O_i (litter) horizons following long-term fertilization to large forest plots ($n = 4$ replicate plots; 10 samples per plot). **b**, ARA response to addition of Mo solutions to microcosms of soil collected from a control plot. The first three bars represent control (+water alone) and two different Mo treatments ($n = 23$ each). The fourth bar represents samples from the long-term micronutrient fertilization plots treated as the control (+water alone; $n = 10$). **c**, ARA response to additions of water alone (control; $n = 15$), Mo solution ($n = 10$) or analytical-grade phosphorus solutions at three different concentrations ($n = 5$ each) to soil microcosms. All graphs show geometric means (±s.e.m.) and the letters signify statistically different treatments at $p = 0.05$ or less (Tukey–Kramer post-hoc test). See the Methods section for further information.

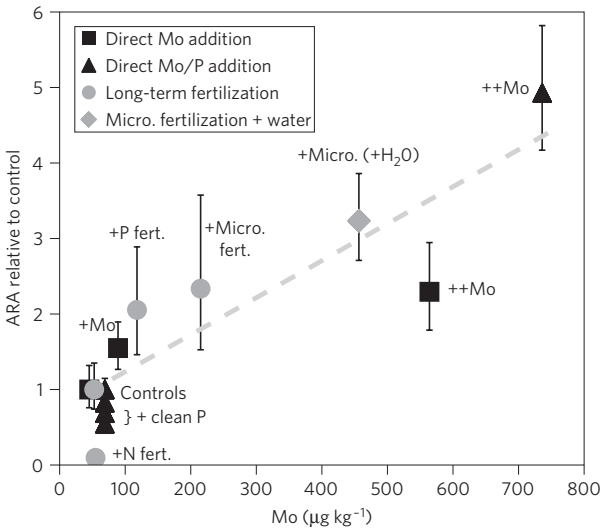

Figure 2 | Relative nitrogenase acetylene reduction activity (ARA) in soil samples as a function of soil Mo enrichment, across all nutrient manipulations. Circles represent field-moist samples collected in the O_i horizon of large, long-term fertilization forest plots ($n = 4$ plots; 4 samples per plot; early wet season): control, nitrogen (+N fert.), superphosphate (+P fert.) and micronutrient (+Micro. fert.). Values differ slightly from Fig. 1a, as soil Mo was analysed on a subset of all samples. Squares represent additions of water alone (control), low (+Mo) and high (++Mo) Mo solutions to soil microcosms from a control plot in early wet season (Fig. 1b). The diamond represents addition of water to soils from the long-term micronutrient plot (+Micro. (+H_2O)). Triangles represent additions of a Mo solution (++Mo) and analytical-grade phosphorus solutions at low, medium and high levels (+Clean P) to control soils in late dry season (Fig. 1c). Values are means (geometric for ARA, arithmetic for Mo; ±s.e.m.). The line represents a linear regression ($p < 0.001$; $r^2 = 0.81$), excluding the +N fertilization.

It is less clear how our findings extend to symbiotic fixers such as epiphytic lichens, which are largely detached from the soil environment, or leguminous plants, which rely on roots and mycorrhizal fungi to acquire soil minerals. Although symbiotic fixers vary from abundant to rare across tropical forests, free-living heterotrophic fixation remains a geographically widespread mechanism of sustained di-nitrogen input to the plant–soil system[4]. In the forests studied here, for example, nodulation and fixation rates among putatively nitrogen-fixing legumes (which make up ~6% of basal area) are exceedingly low, except in locations subject to recent and major disturbance[26]. In our own estimation, and barring recent disturbance, heterotrophic fixation therefore seems to be a significant vector for sustained nitrogen input to these old-growth forests. Although direct measures are scarce[4], rates of symbiotic and free-living fixation probably vary within and across tropical forests, making the issue of controls on fixation a truly critical one.

We show here for the first time that the trace element Mo can alone have a central role for asymbiotic nitrogen fixation in lowland tropical forests that grow on weathered acidic soils. These findings suggest that our general view of tropical nitrogen fixation ought to be broadened, beyond the traditional[3,6,10,11] emphasis on the macronutrients P and nitrogen as master variables. In addition, because most coupled carbon–climate models predict substantially increased nitrogen requirements as forests respond to CO_2 fertilization[27], Mo may represent a current or potential future limit to the ability of at least some nitrogen fixers to supply the extra nitrogen needed for plant growth[5,9]. Most broadly, our findings raise new questions about the potential, yet largely unexplored, role that trace metals may have in terrestrial ecosystems. While we have only recently come to appreciate the apparent importance of Fe limitation of nitrogen fixation in the world's oceans[2,28], we know even less about trace metals in terrestrial ecosystems.

Methods

Study site. The Gigante Fertilization Experiment is located in a floristically diverse (300+ species) old-growth forest on the Gigante peninsula in Barro Colorado

Nature Monument, Panama. Precipitation averages 2,600 mm annually[29]. The experiment consists of thirty-six 40 by 40 m plots exposed to one of nine fertilization treatments (control, N/P/K in factorial design, or micronutrients) four times annually during the May–December wet season since 1998. The treatments examined in this letter are nitrogen (urea, 125 kg N ha^{-1} yr^{-1}), P (triple-superphosphate, 50 kg P ha^{-1} yr^{-1}) and micronutrients (Standard Trace Element Mixture at 25 kg ha^{-1} yr^{-1}, including Mo at 0.01 kg ha^{-1} yr^{-1}, as well as S, Cu, B, Fe, Mn and Zn along with $CaMg(CO_3)_2$).

Nitrogen fixation. We collected field-moist O_i horizon litter from randomly located 100 cm^2 areas. Moisture contents did not differ between treatments ($p > 0.53$, Kruskal–Wallis, d.f. = 3, $n = 95$). Samples were placed in clean 480 ml glass canning jars, fitted with butyl rubber septa, and incubated outdoors at ambient temperature; 10% of the headspace was replaced with C_2H_2. Mixed headspace samples were collected at 5 and 10 h, stored in gas-tight vials and measured for C_2H_4 within 48 h on a gas chromatograph equipped with a flame ionization detector and a Poropak N column. We dried and stored samples for moisture content and nutrient analysis. Our tests indicated neither differences in ARA under light versus dark conditions, nor ethylene production in the absence of C_2H_2. We conducted a total of 160 ARA incubations across 16 fertilizer plots subjected to 4 treatments (December 2003 (late wet season, $n = 2$ samples/plot), July and November 2004 (early and late wet season, $n = 4$)). Fixation rates were log transformed before statistical analysis, and rates indistinguishable from zero were assigned the best-case detection limit of 0.05 nmol g^{-1} h^{-1}. We used an ANOVA to contrast the different nutrient treatments, including a second term that captured seasonal differences between individual sample events. We calculated C_2H_4:N_2 conversion ratios by subdividing litter into three replicates: two in C_2H_4 and a third in a 59 at.% $^{15}N_2$ atmosphere[21]. We determined ^{15}N composition by isotope-ratio mass spectroscopy; spiked samples were significantly enriched with respect to reference samples (paired Wilcoxon test, $p < 0.0001$, $n = 18$) and the geometric mean conversion ratio was 7.9 ($n = 18$, 95% confidence interval: 3.1–20.3). This ratio was used to provide an estimate of nitrogen fixation assuming 24 h fixation and a litter density of 950 g m^{-2} (ref. 30). Our estimates of fixation may be conservative as studies typically use a conversion ratio half this value (3.9) (ref. 11).

Mo and P Additions. In July 2005, we collected O_i horizon samples from 12 random locations in the untreated control forest, and the +micronutrient long-term fertilization plots. We used spray bottles to treat 40 g samples (wet weight) of control litter in one of three ways ($n = 23$ each): +14 ml distilled water (control), or +42 versus +504 μg Mo kg^{-1} as Na_2MoO_4 in 14 ml of water (low versus high Mo). In addition, samples from the micronutrient plots were treated with 14 ml of distilled water ($n = 10$). We allowed samples to equilibrate for 12 h at field conditions before commencing ARA measures (as described above). Our own work, and that of others[14], has shown that the physiological response of microbial populations to Mo addition occurs over hours. In May 2008 (late dry season), we collected O_i horizon samples from control plots. Following the above methods for ARA incubations, we applied one of five treatments: +14 ml distilled water (control; $n = 15$), +667 μg Mo kg^{-1} as Na_2MoO_4 in 14 ml of water (+Mo; $n = 10$), and +2.83, +28.3, and +283 mg P kg^{-1} as NaH_2PO_4 in 14 ml of water (+low P, +medium P and +high P, $n = 5$, respectively).

Soil nutrients. Samples were ground, digested in concentrated nitric acid and analysed for Mo (2 μg Mo kg^{-1} detection limit) and P (60 mg P kg^{-1} detection limit). Separate ground samples were analysed for C and nitrogen by infrared gas analysis combustion.

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Additional information

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