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A phosphorus threshold for mycoheterotrophic plants in tropical forests

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The majority of terrestrial plants associate with arbuscular mycorrhizal (AM) fungi, which typically facilitate the uptake of limiting mineral nutrients by plants in exchange for plant carbon. However, hundreds of non-photosynthetic plant species—mycoheterotrophs—depend entirely on AM fungi for carbon as well as mineral nutrition. Mycoheterotrophs can provide insight into the operation and regulation of AM fungal relationships, but little is known about the factors, fungal or otherwise, that affect mycoheterotroph abundance and distribution. In a lowland tropical forest in Panama, we conducted the first systematic investigation into the influence of abiotic factors on the abundance and distribution of mycoheterotrophs, to ask whether the availability of nitrogen and phosphorus altered the occurrence of mycoheterotrophs and their AM fungal partners. Across a natural fertility gradient spanning the isthmus of Panama, and also in a long-term nutrient-addition experiment, mycoheterotrophs were entirely absent when soil exchangeable phosphate concentrations exceeded 2 mg P kg^{-1} . Experimental phosphorus addition reduced the abundance of AM fungi, and also reduced the abundance of the specific AM fungal taxa required by the mycoheterotrophs, suggesting that the phosphorus sensitivity of mycoheterotrophs is underpinned by the phosphorus sensitivity of their AM fungal hosts. The soil phosphorus concentration of 2 mg P kg^{-1} also corresponds to a marked shift in tree community composition and soil phosphatase activity across the fertility gradient, suggesting that our findings have broad ecological significance.

1. Introduction

The 400-million-year-old symbiotic relationship between plants and arbuscular mycorrhizal (AM) fungi is a fundamental component of terrestrial ecosystems [1,2]. Plants supply the fungi with up to 20% of photosynthetically derived carbon in return for improved access to mineral nutrients, with up to 90% of plant phosphorus being derived from AM fungal partners [3–6]. AM fungi are thus major players in global carbon and nutrient cycles [7–9]. The functioning of plant–AM fungal symbioses in highly diverse tropical forests is poorly understood, partly because tropical plant–AM fungal associations are understudied compared with agricultural and temperate systems [10,11], and partly because tropical plant–AM fungal relations are more complicated than in temperate systems, with the majority of tropical trees depending on AM fungi for establishment and growth [2,12].

Over 400 species of plants are non-photosynthetic and depend entirely on fungi for carbon as well as mineral nutrition [13–16] (figure 1). These plants are known as full mycoheterotrophs because they depend on fungi for their

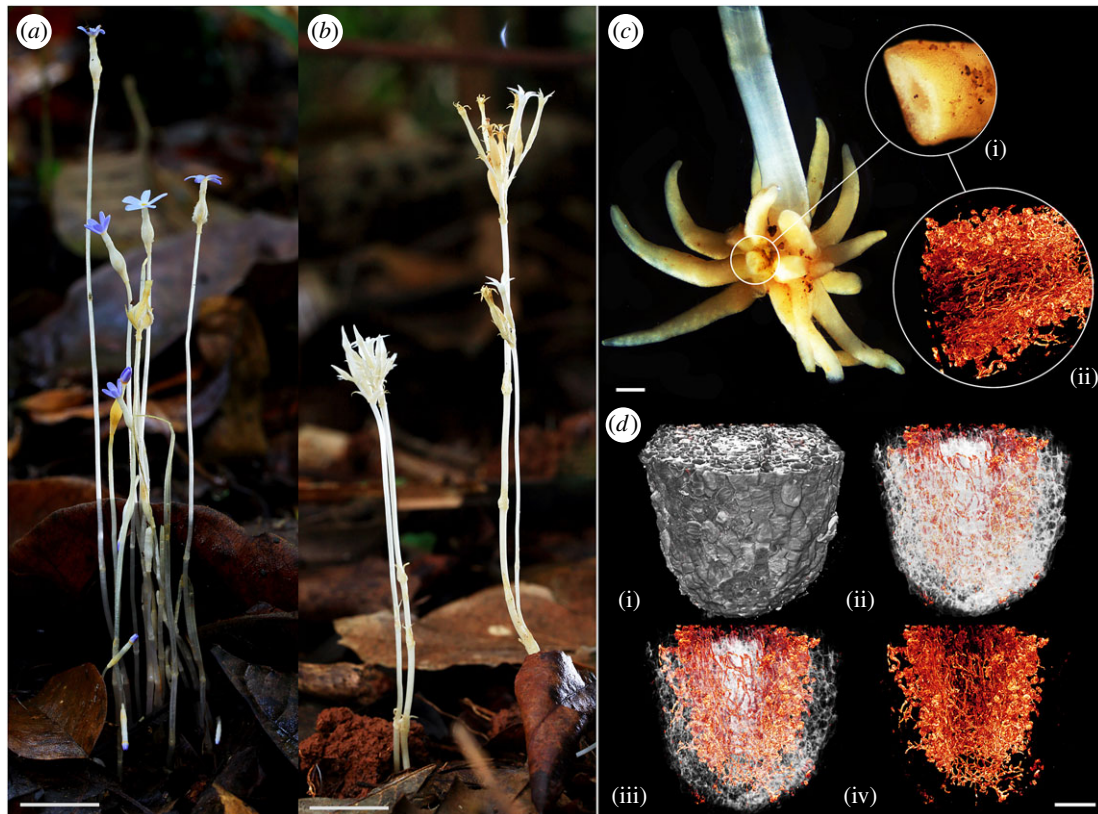


Figure 1. The mycoheterotrophs (a) *Voyria tenella* and (b) *V. corymbosa* in a lowland tropical forest in Panama. The root system of the mycoheterotroph *V. tenella* is intensely colonized by arbuscular mycorrhizal (AM) fungi (d). In (c)(i), fungal material is visible as the light-coloured ring surrounding the central vasculature. In (c)(ii), fungal material (hyphae and coils) is rendered in red and plant material is not shown. In (d), plant material is displayed in grey, and fungal material in red. The same image stack is displayed in (d)(i–iv) with the plant material made increasingly transparent. Confocal micrographs (c)(ii) and (d)(i–iv) were obtained by differential staining of plant and fungal tissues, shown as three-dimensional projections (AMIRA). (a) and (b) Courtesy of Christian Ziegler. (a,b) Scale bar, 20 mm; (c) scale bar, 1 mm; (d) scale bar, 100 μm .

entire carbon and nutrient supply over their lifecycle. The mycoheterotrophic habit has evolved independently more than 40 times across many plant phyla [17]. Because AM fungi are themselves obligate symbionts and derive all of their carbohydrates from photosynthetic plants, the carbohydrates acquired by mycoheterotrophs are ultimately derived from other plants via common mycorrhizal networks, thus demonstrating that AM fungi can facilitate biologically significant plant-to-plant carbon transfer, a hotly contested topic [6]. Mycoheterotrophs also demonstrate that plant–AM fungal relations are not always based on the reciprocal exchange of resources. In the light of recent efforts to portray plant–AM fungal relations as a ‘biological market’ in which plant–AM fungal relations are determined by the mutual evaluation of a range of trading partners [18], mycoheterotrophs thus represent important exceptions.

Little is known about factors, fungal or otherwise, that determine mycoheterotroph distributions [17]. Changes in the relative availability of resources, notably nitrogen and phosphorus, strongly determine the operation of AM fungal symbioses [19], affecting AM fungal abundance, community composition and diversity, resource allocation, and mutualistic quality of plant and fungal partners [20–23]. Here, we performed the first systematic investigation into the effects of abiotic factors on the abundance and distribution of mycoheterotrophs to ask whether the relative availability of nitrogen and phosphorus altered the occurrence of mycoheterotrophs and their AM fungal partners.

2. Material and methods

We investigated the most common mycoheterotrophic species—*Voyria tenella* and *V. corymbosa* (figure 1)—across (i) a well-characterized fertility gradient across the isthmus of Panama [24,25] and (ii) a fully factorial NPK nutrient-addition experiment that had been running for fifteen years (electronic supplementary material, figure S1) [26]. We asked which environmental factors determined mycoheterotroph abundance by combining mycoheterotroph census data and mycoheterotroph tissue nutrient data with a range of environmental metrics along the fertility gradient. We then used the nutrient-addition experiment to ask whether (i) the patterns of mycoheterotroph occurrence across the gradient could be experimentally recreated, (ii) experimental nutrient addition affected the net abundance of AM fungi in both soil and in the roots of autotrophic plants, and (iii) experimental nutrient addition affected the specific AM fungal hosts of the mycoheterotrophs in the soil and in the roots of autotrophic plants.

(a) Fertility gradient

(i) Site descriptions

The natural fertility gradient consisted of 37 sites near the Panama Canal in closed canopy forest, including undisturbed old growth and secondary stands (60–100 years old) [27]. Sites spanned a rainfall and edaphic gradient at low elevation (less than 200 m above sea level; electronic supplementary material, supplementary methods). Concentrations of readily exchangeable phosphate extracted by anion exchange membranes—a measure of biologically available phosphorus—vary more than

300-fold across these sites and represent a range comparable with that of the entire lowland tropics [25]. Most of the sites were 1 ha permanent census plots [26].

(ii) Mycoheterotroph census and sampling

We censused all sites over a two-week period between 4 and 18 October 2013. This fell in the middle of the wet season and the peak of mycoheterotroph flowering (based on our observations and a preliminary census between August and December 2012 at the Barro Colorado Nature Monument; BCNM). Counts for each plot are the average of three independent counts by the same three-person team who combed each plot in 3 m-wide bands (electronic supplementary material, supplementary methods). In 1 ha plots, we surveyed a randomly selected 40×40 m area. When we found no mycoheterotrophs in the 40×40 m area, we combed the entire 1 ha plot to confirm their absence. Plots where mycoheterotrophs were entirely absent were rechecked in late October, mid-November and mid-December.

Mycoheterotroph samples from the fertility gradient were collected for tissue phosphorus analysis. We sampled 6–10 individuals of the common *V. tenella* and 3–6 individuals of the less common *V. corymbosa* (figure 1) at each site, where present. Plants were located away from gaps and separated from each other by at least 3 m. Roots and stems for tissue nutrient analysis (fertility gradient samples) were dried at 60°C and stored at room temperature.

(iii) Soil chemistry

The fertility gradient is naturally occurring, not experimental, and multiple variables were required to model mycoheterotroph occurrence. All soil chemistry data used was obtained by the methods described by Condit *et al.* [25] (electronic supplementary material, Supplementary methods).

(iv) Tissue nutrient analysis

We asked whether increasing levels of soil phosphorus corresponded to an increase in mycoheterotroph tissue phosphorus across the fertility gradient. Mycoheterotroph stem and root tissue was ground to a fine powder in a homogenizer (Tissue-Lyser II, Qiagen) and then pooled by plot, each individual contributing an equal mass to the composite sample. Samples were ashed at 550°C and dissolved in 1 M HCl, and phosphorus was detected by automated molybdovanadate colorimetry on a Lachat Quikchem 8500 (Hach, Loveland, CO, USA). Values are expressed on a 60°C dry mass basis.

(b) Nutrient-addition experiment

(i) Site descriptions

The nutrient-addition experiment is a factorial NPK fertilization experiment with eight treatments (N, P, K, NP, NK, PK, NPK and unfertilized controls) and a ninth micronutrient and dolomitic limestone treatment, each replicated four times across the 38.4 ha study site (a total of 36 plots). The plots measured 40×40 m. Starting in 1998, fertilizers were applied by hand in four equal doses a year, equally spaced across the wet season. The annual doses were 125 kg N ha⁻¹ yr⁻¹ as urea, 50 kg P ha⁻¹ yr⁻¹ as triple superphosphate, 50 kg K ha⁻¹ yr⁻¹ as potassium chloride and a mixture of micronutrients (B, Ca, Cu, Fe, Mg, Mn, Mo, S, Zn, dolomitic limestone; electronic supplementary material, supplementary methods). The nutrient-addition experiment is located within the BCNM in Panama [26] (see electronic supplementary material, supplementary methods for a detailed site description).

(ii) Mycoheterotroph census and sampling

Across the nutrient-addition experiment, we surveyed entire 40×40 m experimental plots (total area = 1600 m²) across all treatments (a total of 36 plots). In all other ways, the mycoheterotroph census and sampling was performed as described for

the fertility gradient. Since mycoheterotroph samples from the nutrient-addition experiment were collected for 454 sequencing of AM fungal communities in their roots, we wiped down and flame-sterilized all equipment in between samples, handled all samples with fresh latex gloves and double-bagged samples in sealed Ziploc bags. We rinsed root systems in filtered deionized water, and removed soil particles with a fine brush. All brushes and containers were sterilized with boiling water between samples to prevent cross-contamination. Roots were surface-sterilized by immersion for 1 min in 70% ethanol and then 1 min in 1% bleach (NaOCl), rinsed with sterile deionized water, dried separately over silica gel in 4 ml tubes and stored at -20°C.

(iii) Soil sampling

We collected soil samples for (i) AM fungal DNA extraction and (ii) lipid analysis across the control, N, P, K, NP and NK treatments (24 plots). We collected 81 soil samples (9 × 9 grid) from 0 to 10 cm depth within the inner 20 × 20 m of each plot, using a volumetric spoon to ensure that there were equal volumes of soil in each subsample. We chose the surface layer (0–10 cm) because the majority of fine roots are located in this layer [28,29]. We composited and thoroughly mixed the samples for each plot, sieved the soil to remove large roots and subsampled the mixed soil 10 times to make two replicate samples (approx. 10 g per sample), for DNA extraction and lipid analysis. We froze these samples at -80°C for 12 h, lyophilized them and stored them dry at -80°C until further processing.

(iv) Autotrophic seedling sampling

We harvested autotrophic seedlings to analyse (i) AM fungal community composition and (ii) AM fungal root colonization. We sampled seedlings of seven of the most common tree species across the plots: *Alseis blackiana* Hemsl. (Rubiaceae), *Desmopsis panamensis* (B. L. Rob.) Saff. (Annonaceae), *Heisteria concinna* Standl. (Olacaceae), *Sorocea affinis* Hemsl. (Moraceae), *Simarouba amara* Aubl. (Simaroubaceae), *Tetragastris panamensis* (Engl.) Kunze. (Burseraceae) and *Virola sebifera* Aubl. (Myristicaceae). These species span a range of life-history strategies and maximum adult heights [30] (electronic supplementary material, supplementary methods).

We harvested four to six seedlings of each of the seven species from each plot. Seedlings were sampled from the same treatments as soil with the exception of NK, due to time constraints (a total of 20 plots). This resulted in approximately 35 seedlings for each of the 20 plots, making a total of approximately 700 seedlings. Seedlings were 15–20 cm tall, were located away from gaps and separated from each other by at least 3 m. From each seedling, we subsampled healthy fine roots for DNA extraction as described above, and stored subsamples in 70% ethanol for microscopic analysis.

(v) Arbuscular mycorrhizal fungal abundance

We used the neutral lipid fatty acid (NLFA) 16 : 1ω5 as a biomarker for extra-radical AM fungal biomass in soils from the nutrient-addition experiment. We performed lipid extraction and analysis following Frostegård *et al.* [31], with modifications described by Nilsson *et al.* [32] (electronic supplementary material, Supplementary methods). The mean NLFA : PLFA ratio across samples was 2.3, indicating that NLFA 16 : 1ω5 is an effective AM fungal biomarker in these soils [33].

We quantified root colonization to assess intra-radical AM fungal abundance in the roots of autotrophic seedlings sampled from the nutrient-addition experiment. To measure AM fungal colonization of roots, we observed cleared and stained roots (electronic supplementary material, Supplementary methods) using a compound light microscope at 200× magnification, and quantified AM fungal colonization following McGonigle *et al.* [34], with at least 100 intersections for one sample per seedling. Mycorrhizal

colonization was expressed as the percentage fine root length colonized by AM fungal hyphae, vesicles or arbuscules.

(vi) DNA extraction and sequencing

Mycoheterotroph and autotrophic seedling root samples were individually pulverized in a homogenizer prior to DNA extraction (TissueLyser II, Qiagen), and an equal mass of each root sample was pooled to make one composite sample per species per plot. Soil samples were pulverized by the same method. We extracted DNA from 50 mg of pulverized root and 25 mg of pulverized soil using MoBio PowerPlant and PowerSoil DNA isolation kits and manufacturer's instructions (MoBio Laboratories, Carlsbad, CA, USA).

We amplified the partial small subunit region of 18S ribosomal DNA (approx. 550 bp) with universal eukaryotic primer NS31 [35] and the AM fungal-specific primer AM1 [36]. The primers were modified by the addition of the 454 pyrosequencing adaptors A and B, in addition to a 10 bp multiplex identifier (MID) on the forward primer (NS31). We conducted duplicate polymerase chain reactions (PCRs) in 25 μ l sample volume using Phire hot start II DNA polymerase (Life Technologies, Paisley, UK). Conditions were 98°C for 1 min; 32 cycles of 98°C for 10 s and 72°C for 15 s; and a final extension phase of 72°C for 2 min. We gel-purified PCR products using MinElute PCR purification kits (Qiagen, West Sussex, UK) and pooled the samples in equimolar concentrations.

Amplicon libraries were sequenced by the Cambridge DNA Sequencing Facility (Department of Biochemistry, University of Cambridge, UK) on an FLX Titanium system (Roche, Basel, Switzerland). No sequences were detected in the blanks included as negative controls at each of the extraction, PCR, gel purification, quantification and sequencing stages.

(vii) Bioinformatic analyses

All bioinformatic analysis was performed using the software mothur [37] unless otherwise stated. Reads were removed from the dataset if they did not contain the 10 bp MID, had more than 1 error in the barcode sequence or more than 2 errors in the forward primer, or were shorter than 200 bp in length.

Clustering was performed using the algorithm Clustering 16S rRNA for Operational Taxonomic Unit (OTU) Prediction (CROP). Sequence alignment was performed with the software MAFFT v. 7.149b [38] and improved with MUSCLE [39] using the refine option. Trees were built using RAxML v. 8.0 [40] with GTR GAMMA implementation, and bootstrap values based on 1000 runs. We used the Basic Local Alignment Search Tool (BLAST [41]; minimum e -value 10^{-30}) on one representative sequence from each cluster iteratively against three databases in the following order of preference: (i) sequences from Krüger *et al.* [42]; (ii) all virtual taxa (VT) from the MaarjAM AM fungal sequence database (www.maarjam.botany.ut.ee); and (iii) all 18S Glomeromycotan sequences from the SILVA database. Non-glomeromycotan clusters were removed when the highest blast match did not correspond to an AM fungal sequence in any of the three datasets.

Clusters were named based on matches to database entries at more than 97% covering a minimum of 80% of the query sequence. We used the generic names from Krüger *et al.* [42], and VT numbers from the MaarjAM database. Where clusters did not match a VT at more than 97%, we assigned a name based on the highest VT match and phylogeny (e.g. *Glomus_OTU1*). Clusters that occurred in fewer than two samples or with fewer than five reads in total were removed from the dataset. A breakdown of the sequencing results is provided in electronic supplementary material, supplementary discussion. For a detailed description of bioinformatic procedures, see electronic supplementary material, supplementary methods.

(c) Statistical analyses

All statistical analysis was conducted in R v. 3.1.2 [43].

(i) Mycoheterotroph census (fertility gradient and nutrient-addition experiment)

We analysed the results of mycoheterotroph censuses across the fertility gradient and nutrient-addition experiment using generalized linear models (GLMs) with negative binomial error structures with `glm.nb` from the package MASS [44]. We analysed only the most abundant species (*V. tenella* across the gradient, and *V. tenella* and *V. corymbosa* across the nutrient-addition experiment) and built separate models for each species. Significance of model terms was assessed using likelihood-ratio χ^2 -tests.

The fertility gradient is not a controlled experiment. Consequently, multiple regression was required to model mycoheterotroph occurrence. We worked with a subset of environmental variables selected and described by Condit *et al.* [25] (dry-season moisture, inorganic N, P, Ca, Zn, K; see electronic supplementary material, supplementary methods). P, Ca, Zn and K had extreme values and were log-transformed to reduce the influence of outliers. All predictors were standardized to zero mean and unit variance.

Across the nutrient-addition experiment, we modelled counts of mycoheterotrophs in NPK factorial models and tested for all two-way interactions. The experimental design of the nutrient-addition experiment includes four replicates of an incomplete block design, and 'replicate' was used as a spatial blocking term to control for natural variation across the site [26].

(ii) Mycoheterotroph tissue phosphorus (fertility gradient)

We used linear models to analyse the relationship between soil exchangeable phosphorus and root and stem tissue phosphorus concentration of *V. tenella*.

(iii) Arbuscular mycorrhizal fungal abundance in roots and soil (nutrient-addition experiment)

We used linear models to analyse (i) the concentration of NLFA 16:1w5 (a proxy for AM fungal biomass) in the soil and (ii) the percentage of tree seedling root length colonized by AM fungi, pooling six of the seven seedling species (*Tetragastris* roots were damaged during the clearing process and omitted from analysis). In both cases, we tested for N \times P interactions (omitting the K treatment) using factorial ANOVA and for the significance of K in a separate model with a single 'treatment' term. The spatial blocking term was included in all models.

(iv) Arbuscular mycorrhizal fungal taxa in roots and soil (nutrient-addition experiment)

We used the number of DNA sequences as a measure of relative abundance of OTUs (electronic supplementary material, supplementary discussion). We analysed the relative abundance, in the soil, of the five AM fungal taxa dominating (constituting more than 97%) the AM fungal communities in the roots of *V. tenella* and *V. corymbosa* using factorial GLMs with negative binomial errors (using `glm.nb`), building a separate model for each fungal taxon, including the spatial blocking term, and testing for N \times P and N \times K interactions using likelihood-ratio χ^2 -tests (soil samples were not collected from PK and NPK plots).

We analysed the relative abundance of the AM fungal taxon most strongly associated with the roots of *V. tenella* and *V. corymbosa* (constituting more than 90% of their AM fungal taxa; *Sclerocystis_VTX00126*) in the roots of the seven autotrophic plant species with generalized linear mixed models (GLMMs; `glmer.nb` from the package `lme4`) with 'P' (0 versus 1), 'species', and the spatial blocking term as fixed effects, and 'plot' as a random effect to control for the pseudoreplication arising from having seven species per plot. Main treatment effects (P, 'species' and P \times 'species' interaction) were assessed by comparing nested models using likelihood-ratio χ^2 -tests.

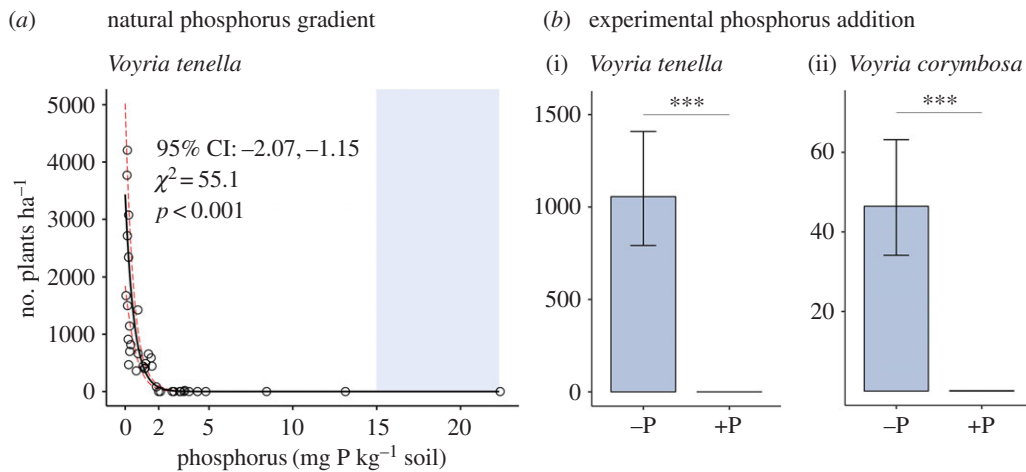


Figure 2. (a) Numbers of the mycoheterotroph *Voyria tenella* sharply decline with increasing soil exchangeable phosphorus (P) across a naturally occurring gradient in lowland tropical forests in Panama. The solid line depicts the fitted response of a GLM with negative binomial errors ($n = 37$). Red dashed lines indicate the 95% CI. The blue shaded region represents the concentrations of soil exchangeable phosphorus found in +P plots in the nearby factorial nutrient-addition experiment (electronic supplementary material, figure S1). (b) The abundant mycoheterotroph *V. tenella* (i) and less common congener *V. corymbosa* (ii) are eliminated by phosphorus (P) addition in a long-term factorial nutrient-addition experiment in a lowland tropical forest in Panama. The figure contrasts 16 no-P plots (control, N, K, NK treatments) with 16 +P plots (P, NP, KP, NPK treatments). Values are fitted responses of a GLM with negative binomial errors and show 95% confidence intervals. The effects of individual fertilization treatments on numbers of *V. tenella* and *V. corymbosa* are presented in electronic supplementary material, figure S3.

3. Results

(a) Fertility gradient

(i) Mycoheterotroph census

Across the fertility gradient, the abundance of *V. tenella* precipitously declined with increasing soil exchangeable phosphorus; numbers of plants fell from 3500 ha⁻¹ at the lowest soil phosphorus concentrations to 0 plants ha⁻¹ above 2 mg P kg⁻¹ ($\chi^2 = 55.1$, $p < 0.001$, figure 2a). The abundance of *V. tenella* was not related to any other environmental variable included in the analysis (dry-season moisture, inorganic N, and exchangeable Ca, K and Zn; electronic supplementary material, figure S2 and table S1). Furthermore, we found no mycoheterotrophs of any other species growing in plots with exchangeable soil phosphorus above 2 mg P kg⁻¹.

(ii) Mycoheterotroph tissue phosphorus analysis

Stem and root tissue phosphorus concentrations of *V. tenella* did not respond to increasing levels of soil exchangeable phosphorus across the network of forest plots at the lower end of the fertility gradient (less than 2 mg P kg⁻¹) where they were found (stem: $F_{1,21} = 1.8$, $p = 0.20$; root: $F_{1,17} = 0.45$, $p = 0.51$; electronic supplementary material, figure S7). Some root samples did not yield sufficient material for analysis, meaning that sample sizes for stem and root analyses differed ($n = 23$ and $n = 19$ for stem and root, respectively).

(b) Nutrient-addition experiment

(i) Mycoheterotroph census

In the nutrient-addition experiment, phosphorus addition in all nutrient combinations (P, NP, PK, NPK) completely eliminated mycoheterotrophs of any species. In the case of *V. tenella*, numbers were reduced from 1000 plants ha⁻¹ (in no-P treatments) to 0 ($\chi^2 = 497$, $p < 0.001$), and in the case of *V. corymbosa* from 45 plants ha⁻¹ (in no-P treatments) to 0 ($\chi^2 = 205$, $p < 0.001$; figure 2b; electronic supplementary material, figure S3). Micronutrient addition did not affect

numbers of either *V. tenella* or *V. corymbosa*, confirming that phosphorus and not the calcium counter ion of the phosphorus fertilizer (triple superphosphate, Ca(H₂PO₄)₂·H₂O) was responsible for the elimination of mycoheterotrophs (electronic supplementary material, figure S8). Numbers of *V. tenella* were reduced by nitrogen addition and restored to control levels when nitrogen and potassium were added together, as indicated by a significant nitrogen × potassium interaction ($\chi^2 = 7.94$, $p = 0.005$; electronic supplementary material, figure S3), while the abundance of *V. corymbosa* was increased by N addition ($\chi^2 = 7.68$, $p < 0.006$; electronic supplementary material, figure S3).

(ii) Arbuscular mycorrhizal fungal abundance

Phosphorus addition reduced the biomass of AM fungi in the soil by roughly 25% ($F_{1,11} = 5.02$, $s = 0.04$; electronic supplementary material, figure S6a) and AM fungal colonization in tree seedling roots by approximately 12% ($F_{1,11} = 9.39$, $p = 0.01$; electronic supplementary material, figure S6b). AM fungal biomass in the soil was also reduced by nitrogen addition ($F_{1,15} = 5.05$, $p = 0.04$; electronic supplementary material, figure S6a), as was AM fungal colonization of tree seedling roots ($F_{1,9} = 18.4$, $p = 0.002$; electronic supplementary material, figure S6b).

(iii) Arbuscular mycorrhizal fungal community composition

Five AM fungal families were represented across root and soil samples (Acaulosporaceae, Archaeosporaceae, Diversisporaceae, Gigasporaceae, Glomeraceae). Rarefaction curves for each sample approached asymptotes, indicating that sequencing intensity was sufficiently high to detect the majority of OTUs and that sampling effort was sufficient to capture the range of AM fungal diversity across the sites (electronic supplementary material, figure S9). Of all the sequences, 95.9% were Glomeromycotan, and 80 OTUs remained after blasting, filtering, merging and trimming, representing a total of 288 139 sequences. Samples contained a mean of 13 OTUs (range: 1–40), and the mean number of sequences per sample was 1055 (range: 201–2442). Sclerocystis_VTX00126 was the most dominant taxon across the dataset.

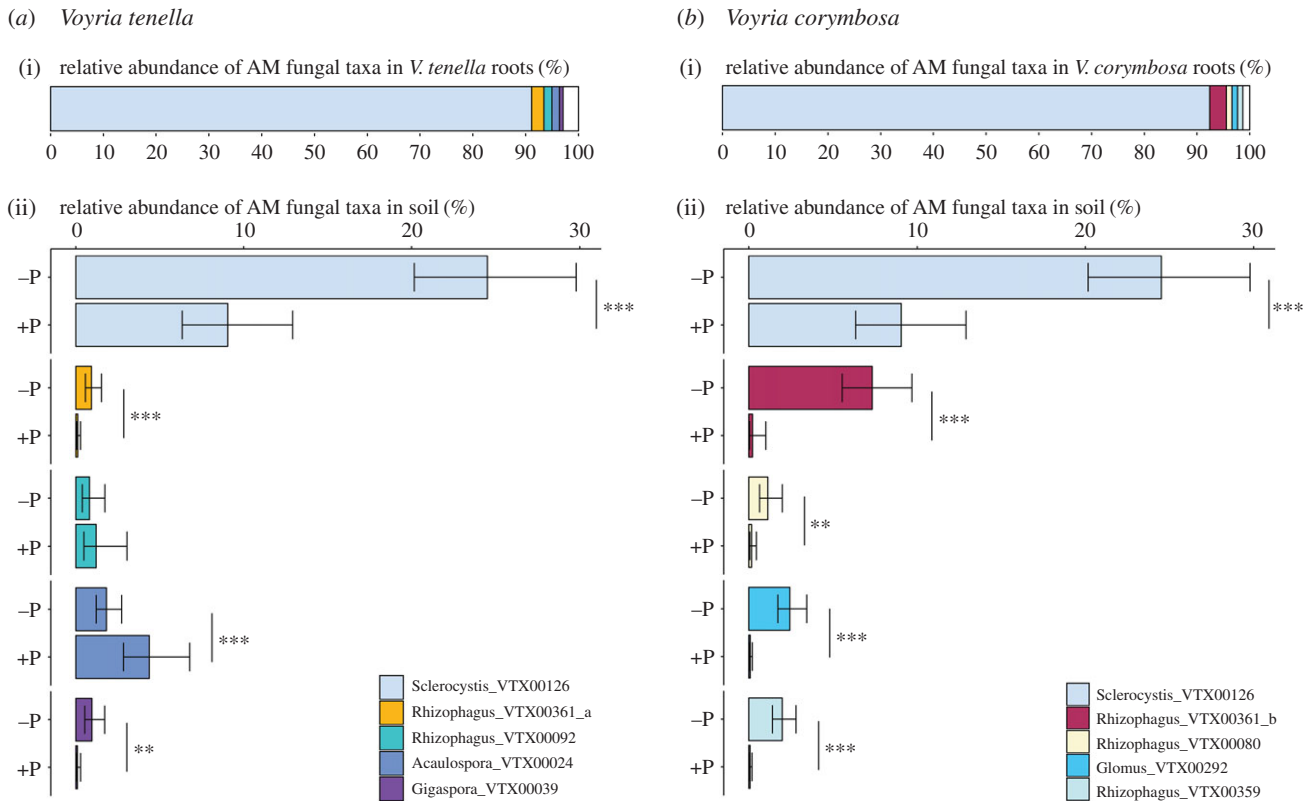


Figure 3. The relative abundance—in the soil—of the AM fungal taxa most strongly associated with (a) *Voyria tenella* and (b) *Voyria corymbosa* are reduced but not eliminated in +P treatments in a long-term factorial nutrient-addition experiment in a lowland tropical forest in Panama. (i) Upper bars represent the relative abundance of AM fungal taxa in the roots of *V. tenella* and *V. corymbosa* (averaged across control, N, K, NK treatments; $n = 16$). (ii) Lower bars illustrate the effect of experimental phosphorus addition on the proportional abundance of AM fungal taxa in the soil. The figure contrasts 16 no-P plots (control, N, K, NK treatments) with eight +P plots (P, NP treatments). Values are fitted responses of GLM with negative binomial errors and show 95% confidence intervals. Significant effects of phosphorus addition are asterisked. Data are based on read counts from 454-sequencing. See electronic supplementary material, figure S4 for the effects of individual fertilization treatments on the relative abundance of AM fungal taxa in the soil.

Experimental phosphorus addition reduced the relative abundance of the AM fungal taxon most strongly associated with the roots of *V. tenella* and *V. corymbosa* in soil communities (constituting more than 90% of their AM fungal communities; Sclerocystis_VTX00126) by approximately 65% ($\chi^2 = 24.8$, $p < 0.001$; figure 3; electronic supplementary material, figure S4). By contrast, in the roots of photosynthetic seedlings the relative abundance of Sclerocystis_VTX00126 was reduced in some species but not others (phosphorus \times species interaction, $\chi^2 = 26.8$, $p < 0.001$; electronic supplementary material, figure S5). Although reduced, the AM fungal taxa colonizing mycoheterotrophs were still present in the soil in P-fertilized plots, and remained part of intact networks with the photosynthetic plant species that they partnered with in unfertilized controls (figure 4).

4. Discussion

We found that the abundance and distribution of *V. tenella* and *V. corymbosa* were strongly dependent on soil phosphorus availability, and that numbers of *V. tenella* increased sharply below a phosphorus threshold of 2 mg P kg⁻¹. Long-term experimental phosphorus addition not only eliminated *Voyria*, but also reduced the net abundance of AM fungi in soil and roots of autotrophic plants (electronic supplementary material, figure S6) and the relative abundance of the specific AM fungal taxa hosting both species of *Voyria* (figure 3). We observed a moderate response of *V. tenella* and *V. corymbosa* abundance to experimental

nitrogen addition, although this was not consistent between the two species (electronic supplementary material, figure S3). The abundance of AM fungi in soil and roots of autotrophic plants was also reduced by nitrogen addition (electronic supplementary material, figure S6).

(a) Explaining *Voyria*'s phosphorus sensitivity

Although we cannot resolve the mechanism by which phosphorus impacts *Voyria*, we can evaluate several possibilities.

(i) Phosphorus toxicity

It is unlikely that phosphorus is directly toxic to either species of *Voyria*. Across the plant kingdom, phosphorus toxicity occurs at tissue phosphorus concentrations of 10–40 mg phosphorus g⁻¹ dry mass (from the extremely phosphorus-sensitive Proteaceae to arable crops) [45]. Across the phosphorus gradient where *V. tenella* occurred (from 0 to 2 mg P kg⁻¹ soil exchangeable phosphorus), tissue phosphorus concentrations of *V. tenella* never exceeded 3.2 mg P g⁻¹, well below the documented lower limit of phosphorus toxicity, and did not increase in response to increasing soil phosphorus (electronic supplementary material, figure S7).

(ii) Shifts in plant species distributions

Plant species distributions shift markedly across the fertility gradient [25]. In some cases, mycoheterotrophs can specialize on fungi linked to a specific set of autotrophic host trees [46], and the change in AM fungal host might influence the

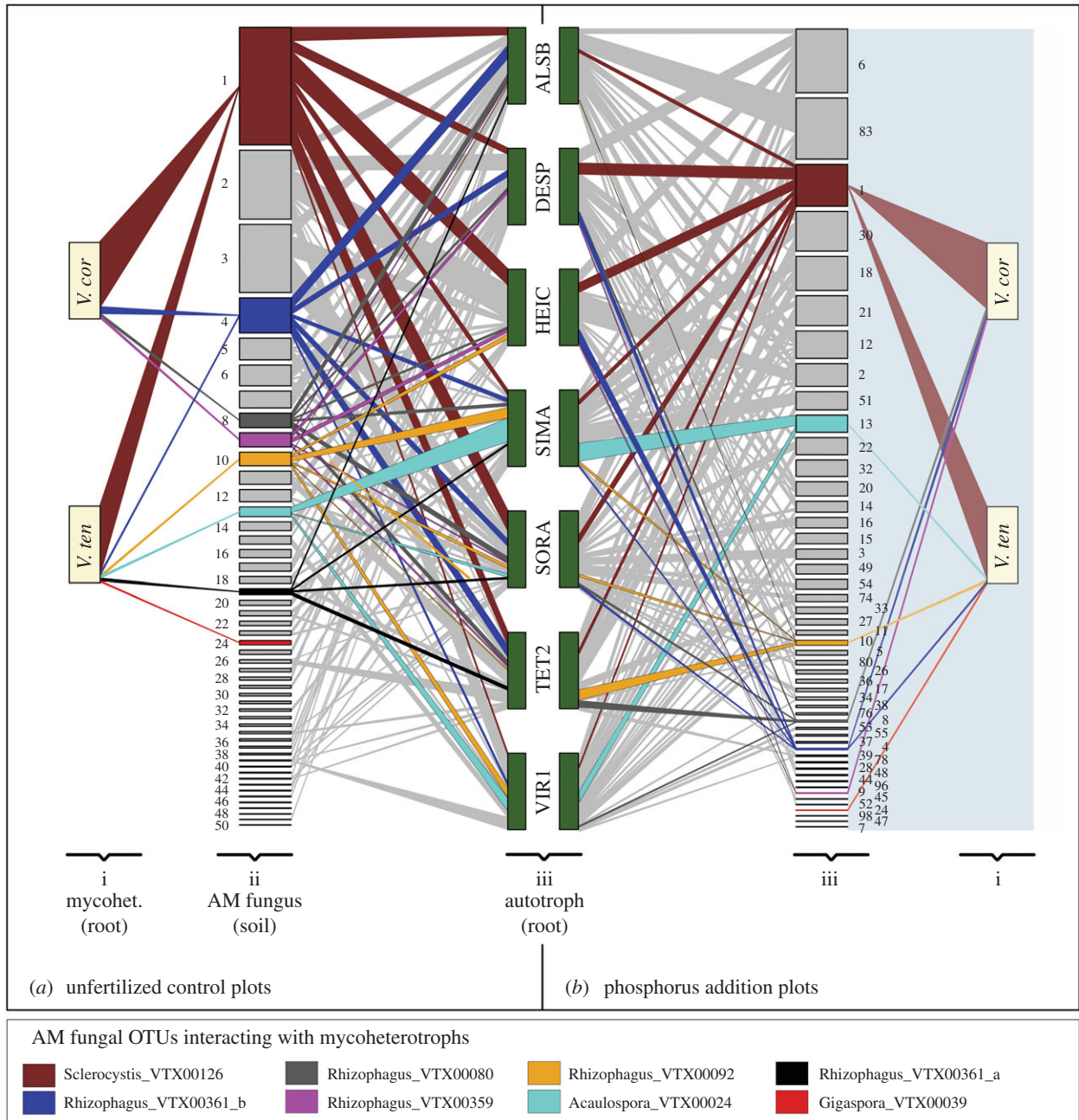


Figure 4. The AM fungal partners of mycoheterotrophs *Voyria tenella* and *Voyria corymbosa* under unfertilized conditions were present both in the soil and in the roots of autotrophic plants when fertilized with phosphorus, although the relative abundance of AM fungal taxa shifted in response to phosphorus addition. Interactions between mycoheterotrophs, AM fungi and autotrophs in unfertilized control plots are represented in (a), and in phosphorus-addition plots in (b). The latter displays *potential* linkages (indicated by shaded blue region) between mycoheterotrophs and AM fungi based on their partners in unfertilized control plots; there were no mycoheterotrophs actually found in phosphorus-addition plots. Values are based on the mean of four unfertilized control plots and four phosphorus-addition plots. The widths of bars representing AM fungal OTUs (ii) are scaled to the relative abundance of OTUs in soil communities in control (a)(ii) and phosphorus-addition (b)(ii) treatments, respectively. The thickness of the linkages is scaled to reflect the proportion of the AM fungal community constituting the linkages. AM fungal OTUs found in the roots of either species of *Voyria* and their linkages are depicted in colour (see legend), and those AM fungal OTUs not interacting with mycoheterotrophs in grey. Full list of OTU codes are given in electronic supplementary material, table S3. Only the 50 most abundant AM fungal OTUs (in the soil) are plotted, and AM fungal OTUs making up less than 1% of the total number of sequences in a sample type are omitted for clarity. Data are based on read counts from 454-sequencing. *V. ten*, *Voyria tenella*; *V. cor*, *Voyria corymbosa*; ALSB, *Alseis blackiana*; DESP, *Desmopsis panamensis*; HEIC, *Heisteria concinna*; SIMA, *Simarouba amara*; SORA, *Sorocea affinis*; TET2, *Tetragastris panamensis*; VIR1, *Virola sebifera*.

occurrence of mycoheterotrophs across this gradient. However, both species of *Voyria* were eliminated by experimental phosphorus addition, whereas tree species distribution has not been altered by phosphorus addition, strongly suggesting that the sensitivity of mycoheterotrophs to phosphorus is not mediated by autotrophic plant host identity.

(iii) Reduced abundance of mycoheterotrophs' arbuscular mycorrhizal fungal partners

Both the overall abundance of AM fungi in soil and roots, and the relative abundance of the AM fungal taxa required by *V. tenella* and *V. corymbosa* in soil, were reduced by phosphorus addition (figures 3 and 4). This is likely to contribute to reduced *Voyria* abundance with increasing soil phosphorus, and

strongly suggests that the phosphorus sensitivity of *Voyria* can be explained in terms of the phosphorus sensitivity of *Voyria*'s AM fungal symbionts. The impact of phosphorus availability on AM fungal abundance and function is well documented and is caused by changes in the exchange relationships between AM fungi and their autotrophic hosts [19]. This finding raises the interesting possibility that changes in the pattern of resource exchange between autotrophs and AM fungi may affect the abundance and distribution of mycoheterotrophs.

(iv) Shift in arbuscular mycorrhizal fungal function

Although the AM fungal taxa required by both species of *Voyria* were reduced by phosphorus addition across the nutrient-addition experiment, these taxa were not eliminated (figures 3 and 4). Furthermore, in three of the +P plots, where mycoheterotrophs were absent, the proportional abundance of the dominant *Sclerocystis_VTX00126* and net AM fungal biomass in the soil was comparable or exceeded that in three no-P plots where mycoheterotrophs were present (electronic supplementary material, table S2). This indicates that, at least in some cases, phosphorus addition eliminated mycoheterotrophs without reducing the prevalence of their preferred fungal partners in the soil or reducing net soil AM fungal biomass. We speculate that this could indicate a phosphorus-dependent shift in the underlying exchange relationships between photosynthetic plants and their AM fungal partners. Although elucidation of the exact mechanism falls outside the scope of this study, we hypothesize that such a functional shift could be underpinned by a reduction in carbon allocation from photosynthetic plants to AM fungal partners at increasing concentrations of soil phosphorus [47], either inhibiting the release of chemical factors from the fungi that stimulate the germination of mycoheterotrophs [48], or causing the fungi to restrict carbon flow to mycoheterotrophs [49]. Alternatively, it could be that changes in AM fungal community dynamics (such as competition) at elevated soil phosphorus (indicated by changes in soil AM fungal community composition; figure 4) may affect mycoheterotroph abundance. These possibilities merit further investigation.

(b) Broader implications

Mycoheterotrophs are necessarily connected to other plants via common mycorrhizal networks and cannot exist without fungal support [17]. If, as seems likely, the sensitivity of *Voyria* to phosphorus is due to the sensitivity of their AM fungal symbionts to phosphorus, we might expect to see phosphorus-dependent responses in not only mycoheterotrophic plant species, but in autotrophic species too.

Indeed, soil exchangeable phosphorus has been identified as the most important mineral nutrient driving tree species distribution across the fertility gradient, with more than half of the 550 measured tree species showing significant affinity with either high- or low-phosphorus soils [25]. The point at which

species with high or low phosphorus affinity dominate the tree community shifts at a concentration of 2.2 mg P kg⁻¹ exchangeable soil phosphorus. Strikingly, this concentration corresponds to a marked shift in tree growth rates and the activity of soil phosphatase enzymes (responsible for the hydrolysis of the majority of organic phosphorus compounds in soil [24]); below 2 mg P kg⁻¹ tree growth responses to phosphorus increased markedly, and phosphatase activity increased exponentially (B.L.T., T. Brenes-Arguedas & R. Condit 1996–2015, unpublished data). This suggests that the mycoheterotrophic response we describe here corresponds to an ecosystem-wide threshold below which phosphorus demand increases markedly above and below ground.

5. Conclusion

We show that (i) the occurrence of two species in the mycoheterotrophic genus *Voyria* is strongly determined by levels of soil phosphorus, and (ii) the effects of phosphorus on *Voyria* are likely to be underpinned by the phosphorus sensitivity of their AM fungal symbionts. We identify a critical concentration of soil phosphorus for *V. tenella* (2 mg P kg⁻¹), which corresponds to broad shifts in plant species distributions and growth responses. Our findings suggest that the well-documented effects of phosphorus availability on plant species distributions [25,50] may act by altering the dynamics of resource exchange in mycorrhizal networks, and highlight the importance of further investigation into the functioning of plant–AM fungal relationships under natural conditions.

Data accessibility. Supporting data are available in the electronic supplementary material. Raw sequence data are available in the International Nucleotide Sequence Database Sequence Read Archive (accession no. SRP076949).

Authors' contributions. M.S. conceived and carried out the study, analysed the data, and drafted the manuscript with input from B.L.T., N.P.R., P.A.O. and S.J.W. N.P.R. conducted bioinformatic analysis. D.R. provided laboratory support. P.A.O. oversaw lipid analysis. S.J.W. established the nutrient-addition experiment and supported the fieldwork. B.T. conducted nutrient analyses and supported the fieldwork.

Competing interests. We declare we have no competing interests.

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