Responses of arbuscular mycorrhizal fungi to long-term inorganic and organic nutrient addition in a lowland tropical forest

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ABSTRACT

Improved understanding of the nutritional ecology of arbuscular mycorrhizal (AM) fungi is important in understanding how tropical forests maintain high productivity on low fertility soils. Relatively little is known about how AM fungi will respond to changes in nutrient inputs in tropical forests, which hampers our ability to assess how forest productivity will be influenced by anthropogenic change. Here, we assessed the influence of long-term inorganic and organic nutrient additions and nutrient depletion on AM fungi, using two adjacent

experiments in a lowland tropical forest in Panama. We characterised AM fungal communities in soil and roots using 454-pyrosequencing, and quantified AM fungal abundance using microscopy and a lipid biomarker. Phosphorus and nitrogen addition reduced the abundance of AM fungi to a similar extent, but affected community composition in different ways. Nutrient depletion had a pronounced effect on AM fungal community composition, affecting nearly as many OTUs as phosphorus addition. The addition of nutrients in organic form (leaf litter) had little effect on any AM fungal parameter. Soil AM fungal communities responded more strongly to changes in nutrient availability than communities in roots. This suggests that the 'dual niches' of AM fungi in soil versus roots are structured to different degrees by abiotic environmental filters, and biotic filters imposed by the plant host. Our findings indicate that AM fungal communities are fine-tuned to nutrient regimes, and support future studies aiming to link AM fungal community dynamics with ecosystem function.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are an ancient, major group of plant symbionts that facilitate the uptake of limiting soil nutrients by plants in exchange for plant carbon (C) (Smith and Read, 2008). The majority of tropical trees—which make up 59% of global forest vegetation—depend on AM fungi (Dixon *et al.* 1994; Alexander and Lee, 2005; McGuire *et al.*, 2008; Averill *et al.*, 2014). This may help to explain how tropical forests account for nearly 40% of terrestrial net primary productivity, while occupying only 12% of the Earth's land surface and frequently occurring on infertile soils (Townsend *et al.*, 2011; Camenzind *et al.*, 2017). Although most lowland tropical soils are strongly weathered and were thought to be P-limited, recent evidence suggests that multiple limiting nutrients interact to limit forest

productivity and function (Kaspari *et al.*, 2008; Wright *et al.*, 2011; Camenzind *et al.,* 2017). To anticipate future effects of anthropogenic change on tropical forest systems, an understanding of how nutrients limit forest productivity is required (Townsend *et al*., 2011; Bonan *et al*., 2012). However, AM fungi are severely understudied in tropical forests (Alexander and Selosse, 2009; Mohan *et al.*, 2014), and despite the well-established role for AM fungi in improving plant access to P (Smith and Read, 2008), their roles in lowland tropical forests remain unclear.

There are two main mechanisms by which changes in nutrient availability could affect AM fungi. Nutrient addition may alleviate direct nutrient limitation of fungal growth, particularly where the background availability of nutrients is low. Conversely, nutrient addition could alter the symbiotic exchange of resources between plant and fungal partners, particularly where background nutrient availability is higher (Treseder and Allen, 2002; Johnson *et al.*, 2010; Hodge *et al.*, 2010): AM fungi incur a substantial C cost to their plant partners (Smith and Read, 2008), and plants are therefore likely to reduce their C investment in AM fungi when nutrients are readily available (Johnson *et al.* 2010). Furthermore, plants may preferentially allocate C to AM fungal partners that supply required nutrients under more favourable 'terms of trade' (Bever *et al.*, 2009; Kiers *et al.*, 2011; Zheng *et al.*, 2014; Bever, 2015).

AM fungi are major actors in global C and nutrient cycles (Johnson *et al.* 2013; Rillig, 2004), and even small changes in the regulation of C flux into AM fungi could have a large global impact (Orwin *et al*., 2011). This is particularly true of tropical forests, which are responsible for at least one third of terrestrial C flux (Cleveland and Townsend, 2006). The availability of nutrients regulates the allocation of plant C to AM fungi (Johnson, 2010), and the addition of

nutrients in inorganic or organic form can have quite different effects on nutrient pools in tropical forests and elicit markedly different responses from plants (Sayer *et al.*, 2012). However, few studies have compared the relative effects of inorganic and organic nutrient additions on AM fungal communities, and to our knowledge, no such studies have taken place outside temperate agricultural settings. This type of comparison is important because experimental inorganic and organic nutrient additions can reveal different aspects of AM fungal ecology. On the one hand, organic matter inputs are the primary route for the cycling of nutrients under natural conditions (Attiwill and Adams, 1993), and simulate the conditions under which the regulatory behaviours governing plant-AM fungal relations have evolved. By contrast, inorganic nutrient additions can highlight the role of specific limiting nutrients, and provide insight into possible ecosystem responses to anthropogenic nutrient deposition.

Two parallel, long-term field experiments in a lowland tropical forest in Panama provided a unique opportunity to unravel the relative importance of the form (organic versus inorganic), amount, and balance of nutrients (the bulk addition of litter versus single or paired inorganic nutrients) on AM fungal ecology. The Gigante Fertilisation Project (GFP) is a factorial NPK addition experiment that allowed us to evaluate AM fungal responses to the addition of inorganic nutrients alone or in factorial combination. The Gigante Litter Manipulation Project (GLMP) at the same site consists of control, litter addition, and litter removal treatments, which allowed us to evaluate AM fungal responses to both a doubling, and the removal of organic matter – a nutrient depletion treatment. Nutrient depletion is an important but rarely performed approach to understand nutrient limitation patterns in ecosystems (Sullivan *et al.* 2014).

Together, these experiments allowed us both to investigate the primary nutrients driving plant-AM fungal interactions and assess the degree to which AM fungal communities are structured by resource-based environmental filters in both components of their 'dual niche': plant roots and soil (Valyi *et al.*, 2016). Specifically, we hypothesised:

1) Given the well-established role of AM fungi in plant P acquisition (Smith and Read, 2008), the low availability of P in weathered lowland tropical soils (Vitousek, 1984), and the role of P in limiting tree distributions in this region (Condit *et al.*, 2013), P addition should cause the strongest changes in AM fungal abundance and community composition.
2) Nutrient addition should alter the ecological processes structuring AM fungal communities, leading to changes in the degree of relatedness (or phylogenetic dispersion) of AM fungal communities.
3) Given the different roles played by intra- and extra-radical AM fungal phases in acquiring C and nutrients respectively, AM fungal communities in the soil should be more sensitive to nutrient additions than those in roots.
4) The addition of single inorganic nutrients—which can create nutrient imbalances—should have a greater effect on AM fungal metrics than the simultaneous addition or removal of all nutrients with litter manipulation.

METHODS

Site description and experimental design

We sampled roots and soil in two parallel long-term experiments in a lowland tropical forest in Panama. The GFP was established in 1998, and had been running for 15 years at the time of sampling (Wright *et al.*, 2011). The GLMP was started in 2003, and had been running for nine years at the time of sampling (Sayer *et al.*, 2010).

We sampled from five treatments across the GFP (N, P, K, NP, and unfertilised controls). Each treatment was applied to four replicate 40 m × 40 m plots across the 38.4-ha study site. Annual doses are 125 kg N ha^{-1} yr^{-1} as coated urea, 50 kg P ha^{-1} yr^{-1} as triple superphosphate, and 50 kg K ha^{-1} yr^{-1} as potassium chloride (SI methods; Figure S1; Wright *et al.*, 2011). Phosphorus addition increased soil phosphate availability by 2800%; K-addition increased K availability by 91%; N-addition increased inorganic N availability by 120% and reduced pH from 5.25 to 4.47 (Mirabello *et al.*, 2013; Yavitt *et al.*, 2011; Turner *et al.*, 2013).

The GLMP consists of fifteen 45 m × 45 m plots. The leaf litter in five litter removal plots is raked up monthly (L-), distributed across five litter addition plots (L+), with five plots left as controls (Sayer and Tanner, 2010). Litter addition increased soil phosphate and calcium (Ca) availability by 47% and 57% respectively, and did not significantly alter inorganic N. Litter removal reduced soil P, inorganic N and Ca availability by 35%, 43%, and 53%, respectively. Neither litter treatment had significant effects on K (Sheldrake *et al.*, 2017a).

The GLMP litter addition and the GFP inorganic nutrient addition treatments supplied similar amounts of N and K to the plots as the inorganic N- and K-addition treatments (143 vs. 125 kg N ha^{-1} y^{-1} and 39 vs. 50 kg K ha^{-1} y^{-1} for the GLMP and GFP, respectively). In contrast, the litter addition treatment added only 12% of the P added in the GFP (5.8 kg ha^{-1} y^{-1} vs. 50

kg ha^{-1} y^{-1}; Sayer *et al.* 2012), because greater inputs of inorganic P were necessary to overcome the P-sorption common to the soils at the study site.

Sampling

We sampled soil and roots from the four replicate N, P, K, NP and control plots in the GFP and from the five replicate L-, L+ and control plots in the GLMP (total of 35 plots) over two weeks in September 2012, at the peak of the growing season. In each plot, we collected 81 soil samples (9 × 9 grid) at 0-10 cm depth, and composited them to make one sample per plot. To control for the effects of host identity on AM fungal parameters, we sampled roots from seedlings of seven of the most common tree species at the study site, harvesting 4-6 seedlings per species per plot (*c.* 1300 seedlings in total; SI methods). In using seedlings, this study differs from previous studies at this site that used mixed root samples from cores (Wurzburger and Wright, 2015; Sheldrake *et al.*, 2017a). In this study, we do not provide an analysis of individual seedling species.

AM fungal abundance

We quantified colonisation of seedling roots by AM fungi using microscopy (staining with trypan blue), as described in Sheldrake *et al.* (2017b; SI methods); used the neutral lipid fatty acid (NLFA) 16:1ω5 as a biomarker for extra-radical AM fungal biomass in the soil (Olsson, 1999; SI methods); and extracted and counted spores from the soil. We identified spores to family level using morphological characteristics, with reference to the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM; https://invam.wvu.edu; SI methods). The use of the biomarker lipid provides a root length-independent measure of net AM fungal abundance. Sheldrake *et al.* (2017b) previously published the colonisation and NLFA data from the GFP (N, P, NP, and C treatments).

187

188 **DNA extraction and sequencing**

189 Root and soil samples were individually pulverised in a homogeniser prior to DNA extraction
190 (TissueLyser II, Qiagen). An equal mass of each root sample was pooled to make one
191 composite sample per species per plot. We extracted DNA from pulverised roots and soil
192 using MoBio PowerPlant and PowerSoil DNA isolation kits according to the manufacturer's
193 instructions (MoBio Laboratories Inc., Carlsbad, CA, USA). We amplified the partial small
194 subunit (SSU) region of 18S ribosomal DNA (*c.* 550 bp) with the universal eukaryotic primer
195 NS41 (Simon *et al.*, 1992) and the AM fungal-specific primer AM1, which amplifies the
196 major families of the Glomeromycota (Helgason *et al.*, 1998). Amplicon libraries were
197 sequenced on an FLX Titanium system (Roche, Basel, Switzerland) at the Cambridge DNA
198 Sequencing Facility (Department of Biochemistry, University of Cambridge, UK).

199

200 **Bioinformatic analysis**

201 Bioinformatic processing followed Sheldrake *et al.* (2017a; SI methods). Briefly, reads were
202 removed from the dataset if they had > 1 error in the MID barcode sequence, > 2 errors in the
203 forward primer, were shorter than 200 bp, or had an average quality score below 25 over any
204 40 bp portion of the sequence. Clustering was performed using the algorithm Clustering 16S
205 rRNA for Operational Taxonomic Unit (OTU) Prediction (CROP; Hao *et al.*, 2011).
206 Sequence alignment was performed with the software MAFFT v7.149b (Katoh *et al.*, 2002)
207 and improved with MUSCLE (Edgar, 2004). We used the Basic Local Alignment Search
208 Tool (BLAST; Altschul *et al.* 1990; minimum e-value 10^{-30}) on one representative sequence
209 from each cluster iteratively against three databases in the following order of preference: i)
210 sequences from Krüger *et al.* (2012); ii) all virtual taxa (VT) from the MaarjAM AM fungal
211 sequence database (www.maarjam.botany.ut.ee); and iii) all 18S glomeromycotan sequences

212 from the SILVA database. Clusters were named based on matches to database entries at >
213 97% similarity covering a minimum of 80% of the query sequence. Where clusters did not
214 match a VT at > 97%, we assigned a name based on the highest VT match and phylogeny
215 (eg. Glomus_OTU1). Raw sequence data were deposited in the International Nucleotide
216 Sequence Database Sequence Read Archive (accession no. SRP076949). Sequencing data
217 from soil and seedlings in N, P, NP and control plots was previously published (Sheldrake *et*
218 *al.* 2017b).
219
220 **Statistical analysis**
221 All statistical analyses were conducted in R, version 3.1.2 (R Development Core Team,
222 2014).
223
224 We performed separate analyses for GFP and GLMP due to their different designs. The GFP
225 includes four replicates per treatment in an incomplete block design, and 'replicate' was used
226 in all models as a spatial blocking term to control for natural variation across the site (Wright
227 *et al.*, 2011). For GFP data, we tested for N × P interactions (omitting the K treatment) using
228 factorial models, and for the K treatment in a separate one-way model with a single
229 'treatment' term, using treatment contrasts to test the significance of K-addition relative to
230 controls (we did not sample from all treatments so could not use the full factorial design). For
231 the analysis of GLMP data, we built one-way models with a single 'litter treatment' term,
232 using treatment contrasts to compare each treatment with controls. Each experiment had its
233 own set of control plots. We calculated log response ratios and confidence intervals to allow
234 visual comparison between experiments (Nakagawa and Cuthill, 2007). The SI presents
235 figures showing the absolute value of variables.
236

To determine overall root AM fungal responses to treatments, we averaged across seedling species to calculate a pooled root response for each metric and plot. Three of the seedling species were absent from the litter removal treatment. To make results comparable between all treatments, we present only analyses based on the four remaining species. Unless otherwise indicated, analysis of all seven species led to the same conclusions.

Analysis of AM fungal communities

To account for variation in the number of sequences among samples, we used a variance stabilising (VS) transformation of the OTU table, implemented with the DESeq2 package (Anders and Huber, 2010). VS transformations use a mixture model framework based on the negative binomial distribution, and avoid the need for rarefaction, which fails to account for overdispersion, and can bias the results towards false positives (McMurdie and Holmes, 2014; Hart *et al.*, 2015). We performed all subsequent analysis on the VS transformed OTU table, with root values calculated as the mean of individual seedling species, and using the copy number of DNA sequences as a measure of relative abundance of OTUs (SI methods).

To examine the effect of experimental treatments on AM fungal community composition, we used multivariate generalised linear models (M-GLMs) with negative binomial error structures using the mvabund package (Wang *et al.*, 2012), building separate models for root and soil communities. To compare the relative effects of treatment on root and soil communities, we built an M-GLM to test for the interaction between experimental treatment and 'sample type' (root or soil). We evaluated the degree to which individual OTUs were affected by litter manipulation using DESeq2 (Anders and Huber, 2010), which estimates the effect size of treatments relative to controls (as logarithmic fold change; SI methods).

We asked whether experimental treatments altered the degree of relatedness among taxa in AM fungal communities (or phylogenetic dispersion), using the Net Relatedness Index (NRI) as an index of community phylogenetic structure. Positive values of NRI indicate that taxa in a community are on average more closely related to each other than to members of the regional taxon pool (phylogenetically clustered), and negative values indicate that taxa in a community are less closely related (phylogenetically over-dispersed; Webb, 2000; SI methods).

Univariate analysis of AM fungal abundance and diversity

We used linear models to analyse: i) the concentration of NLFA 16:1ω5 in the soil and, ii) the percentage of seedling root length colonised by AM fungi. We analysed spore counts using generalised linear models (GLMs) with Poisson errors (Venables and Ripley, 2002; Crawley, 2012). We built separate models for the total spore number and the number of spores in each family. Spatial blocking terms were included for the above analyses. We analysed the total number of AM fungal OTUs (richness), the proportional abundance of the most dominant taxon (predominance), and the NRI metric with linear mixed effects models (lme4 package; Bates *et al*., 2015). The significance of fixed effects was assessed using likelihood ratio tests (LRT) and parametric bootstrapping. We modelled the relationship between occurrence frequency (the proportion of plots in which a given OTU is found) of AM fungal taxa in soil and root communities, using fixed dispersion beta regression (SI methods).

Additional details of all procedures and analyses are given in the SI methods.

RESULTS

287

288 **AM fungal abundance**

289 The amount of the AM fungal biomarker (NLFA 16:1ω5) in the top 10 cm of mineral soil
290 was *c.* 30% lower with N-addition and *c.* 25% lower with P-addition ($F_{1,9} = 11.2$, $P = 0.009$;
291 $F_{1,15} = 6.3$, $P = 0.03$, respectively; Figure 1b, S2). There was a significant overall effect of
292 litter manipulation on the amount of NLFA 16:1ω5 in the soil, suggesting a trend towards a
293 positive effect of litter addition and a negative effect of litter removal ($F_{2,8} = 5.4$, $P = 0.03$,
294 Figure 1b, Figure S2), but individual treatment contrasts were not statistically significant.
295 Neither inorganic nutrient addition nor litter manipulation influenced the total number of AM
296 fungal spores in the soil (Figure 2, S3). Across all treatments, we identified spores belonging
297 to three families, Glomeraceae, Acaulosporaceae, and Gigasporaceae. The Glomeraceae
298 constituted *c.* 90% of the total spore pool, Acaulosporacea *c.* 10%, and Gigasporaceae *c.*
299 0.4%. Separate analyses by family showed that Acaulosporaceae spores were more abundant
300 in plots where N and P were added together (N+P) relative to the treatments where either
301 nutrient was added alone (N × P interaction, $\chi^2 = 6.1$, $P = 0.01$; Figure 2, S3). There was no
302 effect of inorganic nutrient addition on spores of the Glomeraceae or Gigasporaceae and no
303 effect of litter manipulation on the number of spores from any family.

304

305 AM fungal colonisation of seedling roots was *c.* 18% lower with both N- and P-addition ($F_{1,9}$
306 $= 6.9$, $P = 0.03$; $F_{1,9} = 7.2$, $P = 0.02$, respectively; Figures 1a, S4) but was unaffected by litter
307 manipulation. When the analysis was repeated with the additional three species (seven in
308 total), there was a marginally significant N × P interaction, whereby AM fungal colonisation
309 of seedling roots was lower with the addition of N and P together compared to either N or P
310 addition alone (N × P interaction*:* $F_{1,9} = 5.0$, $P = 0.05$).

311

K-addition had no significant effects on any of the metrics assessed in this study and is therefore not reported.

AM fungal OTUs and sequencing

Rarefaction curves for each sample indicated that sequencing intensity was sufficiently high to detect the majority of OTUs and that sampling effort was sufficient to capture AM fungal diversity across the sites (Figure S7). A total of 222 748 sequences were retained after quality control and clustered into 226 OTUs, of which 62 OTUs (corresponding to 22 069 sequence reads, 9.9% of total reads) matched either non-glomeromycotan taxa in the sequence databases or failed to match with any accessions in the database. OTUs remaining after blasting, filtering, merging, and trimming (exclusion of OTUs arising from only one sample or with a total of 5 reads or fewer), represented a total of 200 554 sequences. The number of OTUs and sequences per sample averaged 24 OTUs (range: 9 - 45) and 1146 sequences (range: 328 - 2117).

AM fungal richness and predominance

The total number of AM fungal OTUs (OTU richness) was *c.* 35% higher in soil than in roots in both the GFP and GLMP (LRT = 42.4, $P < 0.001$ and LRT = 35.6, $P < 0.001$ for GFP and GLMP, respectively). The mean number of OTUs was similar between experiments both for soil (GFP: 34 OTUs and GLMP: 35 OTUs) and root samples (GFP: 21 OTUs and GLMP 23). The occurrence frequency of AM fungal OTUs in soil and roots was strongly correlated (beta regression; $\chi^2 = 196.3$, $P < 0.001$; Figure S5), indicating that AM fungal OTUs that were common in soil communities also tended to be common in root communities. However, the proportional abundance of the most dominant taxon at a site (predominance) was higher in

root AM communities than soil communities (LRT = 42.0, $P < 0.001$; Figure S6). The SI discussion provides a full description of sequencing results.

N-addition reduced OTU richness in both soil and roots (LRT = 17.9, $P < 0.001$, Figure 3a, b, S7), but the negative effect of N-addition on OTU richness was stronger in soil than in root samples (N × 'sample type' interaction; LRT = 6.9, $P = 0.03$; Figure 3a, b, S8). Predominance in both sample types was *c.* 27% higher with N-addition (LRT = 17.9, $P < 0.001$; Figure 3c, d, S7). P-addition did not affect OTU richness or dominance; however, P- and N-addition together mitigated reductions caused by N-addition (N × P interaction: LRT = 8.1, $P = 0.007$; Figure 3a, b). In the GLMP, there was a non-significant trend towards lower OTU richness in the soil in both litter manipulation treatments relative to controls ('treatment' term: LRT = 4.4, $P = 0.17$; Figure 3a, b, S8). Predominance increased with litter addition in both soil and root communities (Full model LRT = 9.4, $P = 0.02$; Figure 3c, d) but was unaffected by litter removal.

AM fungal community composition

Within all treatments, soil and root samples had distinct AM fungal community composition (treatment × sample type interaction: Deviance = 960.3, $P = 0.001$; Figure S9) and a greater number of OTUs were affected by nutrient manipulation in the soil than in roots (Figure 4 and Table S1). There was a clear separation of AM fungal communities in plots with P-addition, regardless of sample type (soil: Deviance = 608.3, $P < 0.001$; roots: Deviance = 268.7, $P = 0.002$; Figure 5a, b). In soil samples, the effect of P-addition on AM fungal community composition differed according to whether N was also added (N × P interaction; Deviance = 254.8, $P = 0.001$; Figure 5a, 4). A similar pattern was observed in root communities, although the N × P interaction was only marginally significant (Deviance =

189.3, $P = 0.06$; Figure 5b, 4) and there was no effect of N-addition alone. Litter removal altered AM fungal community composition in both soil and roots (soil: Deviance = 202.3, $P < 0.001$; roots: Deviance = 181.3, $P = 0.007$; Figure 5a, b), whereas litter addition only altered the composition of communities in soil (Deviance = 131.5, $P = 0.01$; Figure 5a, b). Tables S2 and S3 present all OTUs significantly affected by experimental treatments.

AM fungal communities were no more phylogenetically clustered or dispersed than expected by chance (i.e. relative to simulated null communities), and there was no effect of any experimental treatment on the relatedness of taxa in AM fungal communities when the analysis was conducted with four seedling species. However, when the analysis was repeated with the additional three seedling species, N+P reduced the relatedness of taxa in AM fungal communities relative to treatments where they were added separately, across root and soil communities (N × P interaction: LRT = 7.0, $P = 0.01$; Figure S11).

DISCUSSION

Primary nutrients driving plant-AM fungal relations

We found support for our hypothesis that the addition of inorganic nutrients should have a stronger effect than litter addition. Both N- and P-addition reduced AM fungal abundance in roots and soil (colonisation and NLFA 16:1ω5, respectively) in a similar manner (Figure 1), whereas litter addition had no effect. Nutrient addition may affect AM fungi directly by alleviating fungal nutrient limitation (leading to an increase in fungal abundance), or indirectly, by altering plant C investment in their AM fungal symbionts. The second alternative may involve selection for AM fungi that provide more nutritional benefits or are better competitors for plant C, usually leading to a decrease in fungal abundance (Bennett and

386 Bever, 2009). Hence, the observed reductions in AM fungal abundance imply that plants
387 reduced their investment in AM fungi as nutrients became more readily available following
388 N- and P-addition, and suggest a role for AM fungi in both plant N and P acquisition under
389 normal conditions (Johnson, 2010). That no nutrient treatment increased AM fungal
390 abundance suggests that AM fungi at this site are not directly limited by nutrients apart from
391 root-derived C (Treseder and Allen, 2002). These findings are consistent with the results of a
392 global meta-analysis of AM fungal responses to N and P, which found that overall, N and P
393 decreased AM fungal abundance, despite significant variability in AM fungal responses to N
394 (Treseder, 2004).
395
396 Previous studies at this site have found strong evidence for plant K limitation, notably in root
397 responses (Yavitt *et al.* 2011, Wright *et al.* 2011, Wurzburger and Wright, 2015). We
398 observed no significant effects of K-addition on any AM fungal metric. This suggests that
399 AM fungi do not play a role in plant K nutrition in this system, and/or that root C allocation
400 to AM fungi does not vary as a function of plant K status.
401
402 Although N- and P-addition reduced AM fungal abundance by similar amounts (Figure 1),
403 AM community parameters responded quite differently to N- versus P-addition. N-addition
404 reduced OTU richness and increased predominance (Figure 3), whereas P-addition alone had
405 no effect on OTU richness but when added with N (the N+P treatment), alleviated the
406 reduction in OTU richness associated with N-addition (Figure 3). Furthermore, P-addition
407 had much stronger effects on overall community composition than N-addition (Figure 5). The
408 reduction in richness and increase in predominance following N-addition suggests that plants
409 increasingly rely on a subset of AM fungi when P becomes more limiting. By contrast, when
410 P limitation was reduced, AM fungi in soil and roots maintained their diversity despite the

411 decline in abundance, perhaps pointing to a role for the fungal partners in providing other
412 nutrients or benefits to plants. These findings suggest that N- and P-additions affect plant-
413 AM fungal relations in different ways and agree with a previous study at this site which
414 suggested a strong effect of P-addition on plant-AM fungal relations without a concomitant
415 effect of N-addition (Sheldrake *et al.*, 2017b).
416
417 We found that addition of N and P in combination reduced the relatedness of taxa in AM
418 fungal communities relative to treatments where N and P were added separately. Taxa that
419 share a common evolutionary history can also share traits and ecological functions (Maherali
420 & Klironomos 2007; Powell et al. 2009). According to this principle – known as
421 phylogenetic trait conservatism – an increase in phylogenetic dispersion suggests that AM
422 fungal communities in N+P treatments experience increased competitive interactions among
423 taxa, preventing closely related and functionally similar taxa (those sharing a common niche)
424 from co-occurring. This possibility is consistent with a reduction in C supplied by plant hosts
425 in response to N and P addition (as suggested by reduced AM fungal abundance), which
426 would force AM fungi to compete for increasingly limited resources. However, the effect of
427 N+P treatments on phylogenetic structure was weak and should be interpreted with caution.
428
429 Our results contrast with an earlier study at this site which found that AM fungal colonisation
430 in mixed root cores decreased with N-addition but increased with P-addition (Wurzburger
431 and Wright, 2015). Wurzburger and Wright (2015) used mixed cores, dominated by the roots
432 of sun-exposed canopy adults, while we sampled roots from deeply shaded, understory
433 seedlings. Given that photosynthetically fixed C represents the plant currency of symbiotic
434 exchange, different degrees of light limitation could cause adult and seedling plants to adjust
435 their investment in AM fungi in different ways in response to nutrient addition. However, in

436 the present study, AM fungal abundance in the soil (as indicated by the lipid biomarker)
437 shows a similar response to nutrient addition as the AM fungal colonisation of seedling roots,
438 suggesting that seedling colonisation levels reflect response of extra-radical AM fungal
439 abundance to nutrient addition. We lack a good explanation for this discrepancy with our
440 findings.
441
442 Interpretation of the effects of N-addition on AM fungal communities is complicated because
443 ten years of N-addition reduced the pH from 5.25 to 4.47 (Turner *et al.*, 2013). However, the
444 variety of AM fungal responses to reduced pH in the literature makes it difficult to determine
445 which responses can be attributed to the decrease in pH. Low pH has been shown to reduce
446 AMF spore production, colonisation and extra-radical hyphal growth (Daniels and Trappe,
447 1980; Wang *et al.*, 1993; Clark, 1997; van Aarle *et al.*, 2002), and reduce AMF OTU richness
448 (Kohout *et al.*, 2015). Accordingly, many of the effects of N that we observed in this study
449 may be explained by a reduction in pH. Nonetheless, it is unlikely that pH entirely explains
450 the observed effects of N on AM fungal community composition because: i) the N+P
451 treatments clustered far more closely with P treatments than with N treatments (Figure 5),
452 and ii) The addition of N+P did not reduce OTU richness, while the addition of N alone did.
453 If lower soil pH explained the observed N effect, we would expect the AM fungal community
454 in the N+P treatments (soil pH *c.* 4.8) to have a similar community composition and richness
455 to N treatments (soil pH *c.* 4.5).
456
457 **AM fungal responses in both components of their 'dual niche'**
458 Soil and root communities differed from each other across all treatments (Figure S9). This
459 may be because different AM fungal taxa have contrasting life history (Sýkorová *et al.*, 2007)
460 or root-colonisation strategies (Dodd *et al.*, 2000; Hart and Reader, 2002), which can alter the

relative proportion of AM fungal taxa in intra- versus extra-radical phases (Clapp *et al.*, 1995; Hempel *et al.*, 2007).

We hypothesised that AM fungal communities would be more sensitive to nutrient manipulation in soil than in roots because the intra- and extra-radical AM fungal phases play different roles in nutrient acquisition (the extra-radical phase obtaining nutrients from the substrate, and intra-radical phase obtaining fixed C from the plant). Our results support this, as most treatments (N-addition, P-addition, L+ and L-) altered AM fungal communities more strongly in the soil than roots (Figure 5). Similar effects of P-addition on AM fungi were reported in a recent study in maize fields (Liu *et al.*, 2016), and there is evidence that intra- and extra-radical phases are subject to different degrees of limitation depending on the relative availability of N, P and plant C (Hodge and Fitter, 2010). In this study, the greater sensitivity to experimental treatments of AM fungi in the soil suggests that extra-radical phases may be more sensitive to abiotic environmental filters, and intra-radical phases more sensitive to filters imposed by the plant host (such as preferential allocation; Werner *et al.*, 2015). This intriguing possibility warrants further investigation.

Together with other studies performed at this site, our findings indicate that some treatments caused changes in overall plant belowground allocation (measured as fine root biomass) without appearing to affect plant allocation to AM fungi, and vice versa, suggesting a fine degree of control over C allocation to different belowground structures. K-addition reduced fine root biomass (Yavitt *et al.*, 2011, Wright *et al.*, 2011, Wurzburger and Wright, 2015) while litter addition increased fine root biomass (Sayer et al., 2006), with neither treatment affecting AM fungal abundance or communities (this study). By contrast, N- and P-addition reduced plant belowground allocation to fine roots (Wurzburger and Wright, 2015), while

also reducing AM fungal abundance and altering AM fungal communities and increasing the sporulation of Acaulosporaceae when added together (this study).

Inorganic versus organic nutrient addition

As expected, the effects of litter manipulation on AM fungal abundance and community composition were generally not as strong as the effects of inorganic nutrient addition. This may be because inorganic treatments – particularly P – added a greater amount of fast-release nutrients than the litter addition treatment. As the amount of P added in the inorganic P-addition treatment was much greater than the amount added with litter, the potential influence of nutrient source is confounded by differences in nutrient amount. However, findings from other studies conducted at this site suggest that N and P added as litter were more available (in the case of N) or comparably available (in the case of P) to plants as the inorganic N and P added in the GFP. For example, litter N concentrations increased with litter addition but not with inorganic N-addition, despite the fact that both treatments supplied similar amounts of N. By contrast, although the litter addition treatment supplied only *c.* 12% of the P added as inorganic fertiliser, the estimated additional P-return with increased litterfall was very similar between litter addition and inorganic P treatments (*c.* 1.2 kg P ha^{-1} y^{-1} versus 1.4 kg P ha^{-1} y^{-1}; Sayer *et al.*, 2012). Consequently, if the effects of inorganic N- and P-addition were solely due to plants altering their C investment in AM fungi in response to requirements (according to the trade balance model; Johnson, 2010), we would expect to see comparably large effects of litter addition on AM fungal abundance and AM fungal communities in roots.

However, despite the large amounts of nutrients added with litter, AM fungal abundance in roots was unchanged (Figure 1a), and AM fungal abundance in soil tended to increase in

response to litter addition. These findings suggest that plants may have experienced the increases in N or P from organic versus inorganic sources in different ways.

We propose three possible reasons for the distinct responses of AM fungi to nutrients from organic versus inorganic sources. First, AM fungi are better than plant roots at acquiring nutrients from organic nutrient pools as opposed to inorganic pools, such that plant C allocation to their AM fungal associates were maintained despite the net increases in the amount of nutrients on the forest floor following litter addition. In other words, plants still needed AM fungi to fulfil the same nutritional function even though the supply of nutrients from organic matter had increased (Sheldrake *et al.*, 2017a). This possibility is also raised by Vargas *et al.* (2010), who reported increased AM fungal root colonisation in response to substantial organic matter inputs after a hurricane. A second possibility is that nutrient stoichiometry (as opposed to the absolute quantity of a nutrient) regulates plant-AM fungal relations (Azcón *et al.*, 2003; Blanke *et al.*, 2005; Johnson, 2010), and the addition of one or two inorganic nutrients, such as in the N-, P-, and N+P-addition plots, may have a larger effect on AM fungi than litter addition by creating greater nutrient imbalances (and thus potentially greater plant limitation and demand). Finally, AM fungi may have a 'priming' effect, through which they stimulate other soil microbes in the rhizosphere involved in nutrient cycling via decomposition of organic matter (Herman *et al.*, 2012, Nuccio *et al.*, 2013), providing a net benefit to plants, which could cause them to maintain C allocation to AM fungal symbionts.

The pronounced effect of litter removal on AM fungal community composition also suggests that nutrients from organic sources play an important role in AM fungal nutrition and function. Litter removal differs from all other treatments in that it involves the depletion

rather than the addition of nutrients but it affected nearly as many OTUs as P addition (Figure 5), even though there was no effect on AM fungal abundance in roots, and only a marginal effect in soil (Figure 1). Interestingly, AM fungal community composition in roots in the litter removal treatment was similar to that in the N-addition treatment (Figure 5b) suggesting that both treatments exerted similar selective pressures on the AM fungal communities. We speculate that this could be due to an increase in plant demand for P in both treatments. Although the litter removal treatment reduced litter and foliar N concentrations, there was no reduction in the concentration of P in the litter (Sayer *et al.*, 2010*;* 2012), nor a reduction in seedling foliar P (Sheldrake *et al., Unpublished Data*). This suggests that plants were able to maintain adequate P supply from alternative organic or inorganic sources in the soil, potentially due to a shift towards P-specialist AM fungal taxa (Sheldrake *et al.*, 2017a).

Concluding remarks

We present a large, experimental dataset which helps to elucidate the roles of AM fungi in the lowland tropics, and provides a key reference for future studies hoping to link AM fungal community dynamics with symbiotic function, or integrate AM fungi into ecosystem models, notably those incorporating nutrient limitation (Townsend *et al.*, 2011). We show that P is the primary nutrient driving plant-AM fungal interactions in this lowland tropical forest, suggesting that AM fungi are a key mechanism by which tropical forests maintain productivity on low-P soils. Interestingly, while both N- and P-additions elicited reductions in AM fungal abundance, AM fungal communities showed a pronounced, yet distinct response to N- and P-addition. Our findings suggest that AM fungal interactions with plants are more sensitive to nutrient imbalances than to the bulk addition of nutrients with leaf litter, and suggest that plants depend on AM fungi to acquire nutrients from organic nutrient pools. The finding that soil and root communities differed in their responses to nutrient availability

provides evidence that the 'dual niches' of AM fungi are structured to different degrees by abiotic environmental filters and biotic filters imposed by the plant host, a possibility that warrants further testing. Future work should examine the functional significance of the observed shifts in AM fungal community and abundance in terms of both forest nutrition and C sequestration; the relative importance of AM fungi versus roots in nutrient uptake from different soil pools; and the mechanisms underlying shifts in plant-AM fungal relations in response to nutrient additions and altered nutrient stoichiometry.

SUPPLEMENTARY INFORMATION

Supplementary information is available at ISME's website.

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AUTHOR CONTRIBUTIONS

MS and SM designed the study. EVJT and EJS established the leaf litter manipulation experiment. SJW established the nutrient addition experiment. MS and DR performed the lab and field work. PAO supported the lipid analysis. NR conducted the bioinformatic analysis. MS conducted the statistical analysis and wrote the manuscript with input from all authors.

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FIGURE CAPTIONS

Figure 1. Effect of long-term inorganic and organic nutrient addition on AM fungal colonization in the roots of four seedling species (a) and on the concentration of the AM fungal lipid biomarker in the top 10 cm of forest soil (b). Significance was assessed using separate linear models for GLMP and GFP; significant effects are inset in panels above. Note that the overall effect of litter manipulation on NLFA 16:1ω5 was significant (ANOVA: $F_{2,8} = 5.4$, $P = 0.03$), although neither treatment significantly differed from controls. Values are log response ratios (not the predictions of the statistical models), and error bars represent 95% confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium.

Figure 2. Effect of long-term inorganic and organic nutrient addition on the abundance of AM fungal spores in the top 10 cm of forest soil. Significance was assessed using separate generlised linear models for GLMP and GFP; significant effects are inset in panels above. Values are log response ratios (not the predictions of the statistical models), and error bars represent 95% confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium.

Figure 3. Effect of long-term inorganic and organic nutrient on the AM fungal OTU richness (a, b) and predominance (proportional abundance of the dominant AM fungal OTU; c, d) in

soil and root samples. Significance was assessed using separate linear models for GLMP and GFP; significant effects are inset in panels above (LRT = likelihood ratio test). Values are log response ratios (not the predictions of the statistical models), and error bars represent 95% confidence intervals obtained by bootstrapping with 9999 replicates. L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium.

Figure 4. Number of AM fungal OTUs significantly affected by long-term inorganic and organic nutrient addition. Significance was ascertained based on negative binomial Wald tests using standard maximum likelihood estimates for generalised linear models with *P*-values ($\alpha = 0.05$) adjusted for multiple comparisons, as implemented in the DESeq2 package. L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium. Colours correspond to AM fungal genera.

Figure 5. Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in AM fungal community composition in response to inorganic and organic nutrient-addition in soil (a) and root (b) samples in a lowland tropical forest in Panama. 'Site' scores are shown and ellipses describe 95% confidence limits. Ordinations are based on Bray-Curtis dissimilarity. Axes are scaled to half-change (HC) units, by which one HC unit describes a halving of community similarity. C1 = control treatment in GFP; C2 = control treatment in GLMP; L- = litter removal; L+ = litter addition; N = nitrogen; P = phosphorus; NP = nitrogen + phosphorus; K = potassium. Colours and symbol shapes correspond to different treatments.