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Nitrogen fixation ability explains leaf chemistry and arbuscular mycorrhizal responses to fertilization

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Abstract

Atmospheric nitrogen (N) and phosphorus (P) deposition rates are predicted to drastically increase in the coming decades. The ecosystem level consequences of these increases will depend on how plant tissue nutrient concentrations, stoichiometry and investment in nutrient uptake mechanisms such as arbuscular mycorrhizal fungi (AMF) change in response to increased nutrient availability, and how responses differ between plant functional types. Using a factorial nutrient addition experiment with seedlings of multiple N-fixing and non-N-fixing tree species, we examined whether leaf chemistry and AMF responses differ between these dominant woody plant functional groups of tropical savanna and dry forest ecosystems. We found that N-fixers have remarkably stable foliar chemistry that stays constant with external input of nutrients. Non-N-fixers responded to N and N+P addition by increasing both concentrations and total amounts of foliar N, but showed a corresponding decrease in P concentrations while total amounts of foliar P stayed constant, suggesting a 'dilution' of tissue P with increased N availability. Non-N-fixers also showed an increase in N:P ratios with N and N+P addition, probably driven by both an increase in N and a decrease in P concentrations. AMF colonization decreased with N+P addition in non-N-fixers and increased with N and N+P addition in N-fixers, suggesting differences in their nutrient acquisition roles in the two plant functional groups. Our results suggest that N-fixers and non-N-fixers can differ significantly in their responses to N and P deposition, with potential consequences for future nutrient and carbon cycling in savanna and dry forest ecosystems.

Keywords: Plant functional group; Nutrient deposition; Stoichiometry; Mycorrhizae; Savanna; Global change

Introduction

Atmospheric nitrogen (N) and phosphorus (P) deposition are major anthropogenic global change drivers that have resulted in drastic increases in the amounts of key plant nutrients cycling through land and water (Galloway et al. 2008; Mahowald et al. 2008). Estimates suggest that anthropogenic N inputs have increased from ~15 Tg yr^{-1} in 1860 to ~187 Tg yr^{-1} in 2005, and the amount of reactive nitrogen has increased 120% from the Holocene and late Pleistocene baseline with nitrate levels recorded post 1980 being higher than anything in the last 100,000 years (Galloway et al. 2008; Waters et al. 2016). The amount of P cycling through ecosystems has also doubled compared to pre-industrial baselines, with much of the additional N and P coming from fertilizer application and fossil fuel combustion (Filippelli 2002; Waters et al. 2016). Rates of N and P deposition are predicted to further increase in the coming decades (Galloway et al. 2008; Mahowald et al. 2008, Bobbink et al. 2010). Given that N and P limit plant growth in most ecosystems, soil nutrient enrichment has the potential to have effects that cascade through the ecosystem. Increased soil N and P availability can alter plant leaf N and P concentrations, which in turn can affect plant photosynthetic rates, competitive ability, community composition, litter quality and processes such as decomposition (e.g., Allison & Vitousek 2004, Wardle et al. 2004, Wright et al. 2004, Reich 2014).

The ecosystem level consequences of anthropogenic N and P deposition depend on how plant tissue nutrient concentrations and stoichiometry change in response to increased availability of N and P, and how these differ between plant species and functional types. Whether plants change their nutrient uptake and tissue nutrient concentrations is, in turn, influenced by whether the plant is limited by the added nutrient or not, the plant's capacity for luxury consumption and storage of the added nutrient (Sistla et al. 2015) and unique mechanisms improving the plant's ability to access nutrients, such as symbiotic N fixation.

Besides foliar chemistry, soil N and P enrichment can also affect plant investment in nutrient uptake mechanisms such as arbuscular mycorrhizal fungal (AMF) symbioses. AMF play a major role in the cycling and storage of carbon (C) and nutrients in ecosystems (Treseder & Allen 2000) with about 70-90% of all land plants investing up to 20% of their photosynthates in AMF for better access to N and P (Parniske 2008). Changes in plant investment in AMF, therefore, can also affect nutrient and C cycling and storage in ecosystems (Rillig 2004). Increased soil nutrient availability is expected to divert investment away from nutrient acquisition structures such as AMF (Johnson 2010), but previous studies have reported highly variable AMF responses to increased nutrient availability, especially in terms of AMF colonization levels (Treseder 2004).

In this study, we focus on N-fixers and non-N-fixers, the two dominant plant functional groups in savanna and dry forest ecosystems. N-fixers and non-N-fixers differ in both their demand for and access to nutrients such as N and P. Symbioses with rhizobia in N-fixers increases their N access, which in turn increases their P demand, as well as their potential to invest in N rich phosphatases that makes P access easier (Vitousek et al. 2002, 2010). Plant N fixation ability can also affect flexibility or stasis of leaf N and P levels, and foliar N:P stoichiometry (Sistla et al. 2015). Further, investment in rhizobia can influence the dependence of N-fixing plant species on other nutrient acquisition mechanisms such as AMF. However, whether N-fixers and non-N-fixers differ in their leaf chemistry and AMF colonization responses to nutrient addition are not known, which we address in this study.

We examined whether N-fixers and non-N-fixers, which differ strongly in nutrient demand and acquisition strategies, also respond differently to nutrient addition. Specifically, we predicted that: (1) N-fixers and non-N-fixers will differ in foliar N and P responses to fertilization, and (2) Plant N fixation ability will influence AMF colonization responses to nutrient addition.

Methods

We conducted a factorial nutrient addition experiment using seedlings of 13 dominant species characteristic of savanna and dry forest ecosystems of southern and central India (Sagar & Singh 2004; Kumar & Shahabuddin 2005; Kodandapani et al. 2008). We focused on seedlings since they are most susceptible to changes in nutrient status of the soil, and altered competitive ability at the seedling stage can influence community composition of forests in the long term (Barbosa et al. 2014; Cárate-Tandalla et al. 2015). Plant species used in the experiment included 7 non-N-fixers (Lagerstroemia indica L., L. speciosa (L.) Pers., Phyllanthus emblica L., Sapindus emarginatus Vahl, Terminalia arjuna (Roxb. ex DC.) Wight & Arn., T. bellirica (Gaertn.) Roxb. and Ziziphus jujuba Mill.) and 6 N-fixers (Acacia catechu (L.f.) Willd., A. ferruginea DC., A. leucophloea (Roxb.) Willd., Albizia amara (Roxb.) B.Boivin, A. lebbeck (L.) Benth. and Dalbergia latifolia Roxb.). N fixation has been reported for all the N-fixers used in the experiment (Germplasm Resource Information Network database (GRIN); http://www.ars grin.gov/).

Plants were grown to seedling stage at the Foundation for Revitalization for Local Health Traditions (FRLHT), Bangalore, India, in uniform, homogenized, unsterilized soil. At four weeks of age, seedlings were transplanted into 20 litre polybags in a 1:1 sand:soil mixture at the experimental site in Hosur village near Mysore, India. Unsterilized soil used in the transplanting medium was collected locally from the experiment site and homogenized prior to mixing with sand. Average C, N and P content per unit weight of the sand:soil medium in which the seedlings were transplanted was 383.4 g kg^{-1}, 24.4 g kg^{-1} and 13.7 g kg^{-1}, respectively (Varma et al. 2017). Each polybag contained an individual seedling. Multiple seedlings of each species were randomly assigned to one of four treatments: N addition (5g N as urea), P addition (0.5g P as single super phosphate (SSP)), combined N and P addition, and a control treatment with no nutrient addition. Fertilizer application (as urea or SSP solution in

126 water) was carried out in three instalments: 2, 4 and 6 weeks after the start of the experiment.
127 Seedlings were watered with tap water regularly to prevent drought induced mortality, and
128 above- and belowground biomass were harvested after 6 months from the start of the
129 experiment. At the time of harvest, there were 5-14 seedlings per species per treatment, for a
130 total of 624 seedlings. For more details of the experiment, see Varma et al. (2017).
131
132 Foliar chemistry analysis
133 Foliar %N and %P were estimated from ground, oven dried (at 60 °C for 48 hr) leaves of a
134 subset of the samples (2-7 replicates per species per treatment, median = 5, for a total of 245
135 samples). Foliar %N was determined by combustion, using a LECO TruSpec CN analyser
136 (LECO Corporation), and %P was determined from acid digested samples (treated with 70%
137 HNO_3 and 30% H_2O_2) using inductively coupled plasma (ICP) spectrophotometry (ICP 6300,
138 Thermo Fischer Scientific). Total leaf weight per plant was measured from oven dried plant
139 samples (2-6 replicates per species per treatment, median = 4, for a total of 222 samples), and
140 whole plant leaf N and P were calculated for these samples as the product of leaf dry weight
141 and %N and %P, respectively.
142
143 Mycorrhizal colonization estimation
144 Fine roots from individual seedlings were collected at the time of harvest, stored in FAA
145 (10% formaldehyde, 5% acetic acid, 50% ethanol), and transported for laboratory analyses to
146 the National Centre for Biological Sciences, Bangalore. AMF colonization was estimated for
147 3-18 individuals per species per treatment (median = 13) for a total of 600 samples. For
148 estimating AMF colonization levels, we used the ink and vinegar method (Vierheilig et al.
149 1998, 2005). Briefly, 1-2 cm pieces of the fine roots of each individual were treated with 10%
150 KOH at 90 °C for 1 hr, washed, incubated for 15 min at 90 °C in a 5% solution of black ink

(Parker Quink, Bangalore, India) in 5% acetic acid, and then destained with distilled water acidified with 1-2 drops of acetic acid. AMF colonization was estimated using the grid line intersection method (Giovannetti & Mosse 1980). On average, ~25 intersections for each plant root sample were observed under 400× magnification, and scored for presence of arbuscules, vesicles and/or aseptate hyphae. AMF colonization was calculated as the ratio of the number of these intersections that showed AMF presence to the total number of intersections observed. Preliminary analyses using resampling based simulations suggested that our sampling effort was sufficient to arrive at accurate AMF colonization measures, with AMF colonization estimates stabilizing at ~10 total intersections observed (data not shown).

Data analysis

We used linear mixed effects models, with individual plants as the sampling unit, to analyse fertilization treatment and functional group effects on leaf chemistry and AMF colonization. Our objective was to identify patterns in responses at the functional group level, and host plant species identity was included as a random factor in all analyses to account for intrinsic variation in leaf chemistry and AMF colonization between plant species within functional groups.

In the linear mixed effects models to assess leaf chemistry (%N, %P, N:P ratios, total foliar N and total foliar P) responses, we used nutrient treatments (Control, N addition, P addition, and N+P addition), functional group (N-fixer and non-N-fixer) and their interaction as fixed predictors. Percent N, %P, total N and total P were log transformed before the analyses to meet assumptions of normality.

To assess functional group differences in AMF responses to nutrient addition, we used a generalized linear mixed effects model with binomial errors, given the non-normal nature of the data (AMF colonization data are in the form of proportions) (Bolker et al. 2009).

In a framework similar to the previous analysis, we used treatments, functional groups and their interaction as fixed predictors.

We used the lme4 (ver. 1.1-11), lmerTest (ver. 2.0-30) and car (ver. 2.1-2) packages in R for the mixed effects model analyses and statistical testing of the fixed effects (Bates 2010; Bates et al. 2014, Kuznetsova et al. 2015; Bates et al. 2017). We performed t-tests with Satterthwaite approximations on the degrees of freedom using the lmerTest package, and Wald chi-square tests using the car package to test the statistical significance of predictors (Bates et al. 2014, Bates et al. 2017). We also computed marginal and conditional R^2 values for all our analyses using the R package piecewiseSEM (ver. 1.2.1). The marginal and conditional R^2 values give an indication of the variance explained only by the fixed effects in the mixed effects models, and by the fixed and random effects together, respectively (Nakagawa and Schielzeth 2013). All analyses were carried out using R, version 3.2.4 (The R Foundation for Statistical Computing Platform, 2016).

Results

Marginal and conditional R^2 values computed for all the analyses suggest sizeable inter-species variation (Table 1). However, we found significant functional-group level differences in responses to nutrient addition for the parameters measured.

N-fixers had 59% higher foliar %N (~2.82% in N-fixers versus ~1.77% in non-N-fixers), but 44% lower %P than non-N-fixers (~0.15% in N-fixers versus ~0.22% in non-N-fixers) in the control treatment (Fig. 1a, b). Fertilization affected foliar nutrient concentrations and stoichiometry in the two functional groups differently (Table 2). N-fixers were remarkably stable in their foliar chemistry, and none of the nutrient treatments influenced leaf %N or %P in this functional group (Fig. 1a, b). Non-N-fixers showed increases in %N with N and N+P addition (by 22% and 19% on average, respectively; $P <$

0.001 and P = 0.0019, respectively), while there was no change with P addition (Fig. 1a). Interestingly, in non-N-fixers, there was a concomitant decrease in %P with N and N+P addition (by 61% and 51% on average, respectively; $P < 0.001$ in both cases), to levels comparable to the N-fixers, while P addition elicited no change (Fig. 1b).

In the control treatment, N-fixers had higher N:P ratios compared to non-N-fixers (mean: 19.4 vs. 9.7, respectively). N:P ratio in N-fixers was not affected by any of the nutrient treatments, while in the non-N-fixers it increased to ~18.5 with N addition ($P < 0.001$) and to ~15.2 with N+P addition ($P = 0.0026$), but was unaffected by P addition (Fig. 1c).

Total foliar N and P in N-fixers did not differ from control levels with nutrient addition (Fig. 2a, b). Total foliar N in non-N-fixers increased significantly with N (by 114% on average) and N+P addition (by 98% on average; $P < 0.001$ in both cases), but not with P addition (Fig. 2a). However, total foliar P did not change with nutrient addition in the non-N-fixers, suggesting redistribution of existing P into newly produced foliage when N was added (Fig. 2b).

AMF colonization levels in non-N-fixers were 28% greater than N-fixers in the control treatment on average (Fig. 3). Fertilization affected AMF colonization levels in the two functional groups differently (Table 2). AMF colonization in non-N-fixers remained unchanged with N or P addition alone, but decreased by 15% on average with N+P addition ($P = 0.007$). In N-fixers, AMF colonization levels increased by 20% and 31.5% on average, respectively, with N and N+P addition ($P = 0.007$ and $P < 0.001$, respectively), but remained unchanged with P addition.

Discussion

In this study, we experimentally tested whether seedlings of N-fixing and non-N-fixing dry forest tree species respond differently to soil N and P enrichment, given their distinct nutrient demand and acquisition strategies. We specifically focused on leaf chemistry and AMF colonization responses, given their potential to further affect nutrient and carbon cycling and storage in these ecosystems. Our results suggest that N-fixers have remarkably stable foliar chemistry and N:P stoichiometry that remains unchanged even with external input of nutrients. Unlike N-fixers, non-N-fixers responded to N addition (both N and N+P) by increasing concentrations and total amounts of foliar N, as well as N:P ratios, but showed no responses to P addition. Interestingly, with N addition (both N and N+P) non-N-fixers decreased their leaf P concentrations, while total amounts of foliar P stayed constant. AMF colonization levels decreased with N+P addition in non-N-fixers and increased with N addition (both N and N+P) in N-fixers, suggesting differences in their nutrient acquisition roles in the two plant functional groups.

The remarkable stability of foliar N and P in N-fixers and comparatively greater stoichiometric flexibility in non-N-fixers suggest that foliar chemistry mediated ecosystem level consequences of nutrient deposition will be different for N-fixer dominated and non-N-fixer dominated communities. This result also supports the idea that while the nature of nutrient limitation (or soil N and P availability, and plant nutrient uptake) can drive foliar stoichiometry in many cases, like in the non-N-fixers in our study (Koerselman & Meuleman 1996; Aerts & Chapin 1999; Güsewell et al. 2003; Cleland & Harpole 2010; Huang et al. 2012), foliar stoichiometry can be independent of nutrient availability in others, like the N-fixers in our experiment (Sistla et al. 2015; Koufali et al. 2016). There is also evidence from other systems that suggest that the direction of change of foliar nutrient concentrations could be unrelated to the nature of nutrient limitation. For example, Ostertag (2010) reported an

increase in P content with nutrient addition as a consistent response across 13 plant species in Hawaiian islands, irrespective of whether N or P was limiting in the ecosystem. Functional group level differences in foliar chemistry responses that we found appear to reflect a widespread pattern: a meta-analysis of vegetation responses to N addition suggests that across the globe, foliar N concentration responses of N-fixers are associated with lower effect sizes than non-N-fixers (Xia & Wan 2008). Interestingly, studies have also reported similar stability in foliar nutrient content in other plant groups such as palms (Mayor et al. 2014).

Unlike N-fixers, non-N-fixers in our experiment showed an increase in foliar N with N and N+P addition and a concomitant decrease in P concentrations. However, total foliar P content remained unchanged, suggesting a 'dilution' of the nutrient with increased N availability, indicative of luxury consumption of P. In other words, higher concentrations of P that we see in non-N-fixers in the absence of additional N might be indicative of P 'accumulation' in the leaf tissue. This 'dilution' of P could be because the plants are limited by some other nutrient or resource other than P, such as N – biomass responses in this experiment suggest that non-N-fixers are N limited while N-fixers are co-limited by N and P (Varma et al. 2017). N-fixers showed substantial increases in total biomass (by >50%) from control levels with only N+P addition. Non-N-fixers showed similar increases from control levels in total biomass with N and N+P addition, while P addition elicited no significant change (Varma et al. 2017). A meta-analysis of responses of aquatic and terrestrial plants to fertilization reports similar evidence for dilution of P (and increasing C:P ratios) from N limited aquatic systems, where fertilization with N dilutes biomass P across tissue types (Sistla et al. 2015). Biomass C:P across studies conducted in terrestrial ecosystems also show increases with N enrichment, where the systems are co-limited by N and P, or do not have an identified limiting nutrient (Sistla et al. 2015). Limitation by resources other than nutrients, such as water, can also bring about reductions in foliar nutrient concentrations with

fertilization. For example, N and P concentrations in leaves of savanna tree species have been found to decrease with nutrient addition under an uneven water regime (i.e., under natural rainfall with uneven frequency, as opposed to a watering treatment with regular frequency that was part of the study) (Barbosa et al. 2014). Further, the effect was found to be more pronounced in broad leaved, non-N-fixing species than fine leaved, N-fixing species (Barbosa et al. 2014). Increased addition of N can also make P less available in the soil, causing a reduction in P content in the plant (Witkowski 1989).

Plant functional groups in our study differed not only in foliar chemistry responses to fertilization, but in AMF colonization responses as well. Our results suggest that reduction in AMF with greater nutrient availability may not be a general rule that applies to all plants. Host plant differences in nutrient acquisition strategies and nutrient demand such as between N-fixers and non-N-fixers can result in differences in AMF responses to nutrient addition with consequences for nutrient and carbon cycling and storage in ecosystems. In our experiment, AMF colonization levels in non-N-fixers showed decreases with N+P addition, while we observed increases in AMF colonization levels in N-fixers with N and N+P addition compared to control levels. This is contrary to what one would expect based on optimal allocation theory, which suggests that plants reduce resource allocation to nutrient acquisition apparatus such as AMF with increasing soil nutrient availability (Johnson 2010; Johnson et al. 2013). Several studies suggest that in general nutrient enrichment favours phototrophs at the expense of heterotrophs, and carbon dependent mutualists such as AMF are particularly vulnerable (Shantz et al. 2016). While evidence from earlier studies points to nutrient addition mediated decreases in AMF levels in plant roots especially in P rich soils (Antoninka et al. 2011), our results suggest that functional groups of plants might be an important factor to also consider.

Reduction in AMF colonization levels with N+P addition in non-N-fixers, and increases with N addition in N-fixers suggests that while AMF in non-N-fixers in our study may be useful for both N and P uptake, N-fixers might be relying more on AMF-mediated uptake from the soil to meet their N requirement with N addition, rather than investing in atmospheric N fixation. Atmospheric N-fixation is an energy demanding process (Vitousek et al. 2002), and with greater soil N availability, one can expect a shift in N-fixer nutrient access strategy from N fixation to uptake via structures such as AMF. Apart from increases in AMF colonization levels in N-fixers with N addition that we see in our study, significant decreases in nodulation levels in N-fixers in this experiment with N and N+P addition were also observed (Varma et al. 2017). N addition elicited a 67% reduction in nodule dry weight, while N+P addition elicited 88% declines in nodule weights when compared to levels in the control and P addition treatments (Varma et al. 2017). This supports the idea of a possible shift from N fixation to AMF-mediated soil N uptake with greater N availability. Further, there is evidence from laboratory studies – experiments with root organ cultures as well as with some intact plants – for AMF-mediated N transport to host plants, especially under conditions of extra N in the soil, and when N is in less mobile forms such as ammonium, though evidence from studies under field conditions with natural AMF communities is yet to be gathered (Hodge & Storer 2015).

While the role of AMF in N transport is still unclear, studies on rhizobia-AMF interactions suggest that rhizobia and AMF colonizing the same host plants could have either facilitative or competitive interactions (Larimer et al. 2010, 2014; Lin et al. 2015). AMF colonization in legumes can improve N fixation since AMF make P, which the N-fixers have a high demand for, easier to access. Studies have also suggested that co-infection with AMF can reduce the negative effect N addition has on nodulation in N-fixers (Larimer et al. 2014). However, AMF and rhizobia can also compete for the C pool of their common host plant,

whereby increases in host plant investment in one symbiont can reduce investment in the other, which could be one possible mechanism for the increases in AMF colonization and concomitant decreases in nodulation (Varma et al. 2017) with N and N+P addition observed in this study. Alternately, increases in AMF levels could also indicate an increase in more pathogenic forms of AMF under high nutrient levels, rather than changes in plant strategy or competition between symbionts (Johnson 1993; Johnson 2010; Powers et al. 2015). Several studies have shown that nutrient addition can change the AMF species pool in the soil, both the quantum of inoculum in the soil and AMF species composition (Johnson 1993; Egerton-Warburton et al. 2007; Ochoa-Hueso et al. 2013). N-fixers and non-N-fixers may also host different AMF communities, which can also potentially explain differences in AMF responses that we see (Scheublin et al. 2004, Ochoa-Hueso et al. 2013).

Our study focused on N-fixer and non-N-fixer responses to fertilization at the seedling stage, but whether similar responses are likely to be observed at the adult tree stage is unclear and can depend on several other factors. For instance, adult trees capable of N fixation may not be actively fixing N in forests (Vitousek et al. 2002), since the nature of N and P limitation depends on plant age among other factors (Güsewell 2004). Further, how results in this study scale up to influence processes at the ecosystem level is also open-ended, and can be influenced by factors such as neighbourhood competition for soil resources which were not part of this study, given that we used individual plants. Greater nutrient availability can also make other factors limiting and thus influence plant responses; for instance, high nutrient conditions might make plants limited by light (and consequently carbon) as a consequence of greater shading due to increased aboveground growth of neighbouring plants, and this can affect the direction and magnitude of plant biomass allocation, stoichiometry and AMF colonization responses. Plant and AMF responses to N and P deposition can also be influenced by factors such as plant species composition of the ecosystem, the ability of N-

fixing and non-N-fixing trees to modify the biochemistry of the soil patch in which they grow, land use history, disturbances such as fire, intra- and inter-annual variability in other resources such as water, and increasing atmospheric CO_2 (Allison & Goldberg 2002; Matson et al. 2002; Novotny et al. 2007; Yang et al. 2011; Chimphango et al. 2015; Powers et al. 2015; Varma 2016). Nevertheless, this study provides several indications of how dry forest and savanna ecosystems might respond to enhanced N and P availability. For one, our results suggest that dominant functional groups of woody plants in tropical savannas and dry forests, i.e., N-fixers and non-N-fixers, are likely to differ fundamentally in their responses to enhanced future atmospheric deposition of N and P. Our data also suggest that savanna communities dominated by N-fixers are likely to be more 'resistant' to foliar chemistry induced shifts in ecosystem processes than communities dominated by non-N-fixers. Further, while stoichiometric ratios might provide a useful index for evaluating responses of some functional groups to changes in nutrient availability (e.g., non-N-fixers), they are unlikely to be a useful index in other cases (such as N-fixers, palms, etc.). However, further experiments, including long term studies, of nutrient addition effects in tropical dry forests are needed for elucidating the individual and combined effects of several nutrients on vegetation, AMF, and carbon storage potential of these ecosystems, especially in the context of global change.

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Figure legends

Fig. 1 (a) Average foliar %N (b) %P and (c) N:P ratio responses to nutrient addition in N-fixers and non-N-fixers. Bars are species averages and error bars represent 1SE, predicted from their respective mixed effects models. Asterisks indicate mixed effects model output of statistically significant within functional group differences from the control (** $P < 0.01$, *** $P < 0.001$). Sample sizes for non-N-fixers = 36, 33, 36 and 32, and for N-fixers = 27, 30, 27 and 24, for the Control, N, P and N+P treatments, respectively, in each of the above graphs.

Fig. 2 (a) Total foliar N and (b) total foliar P responses to nutrient addition in N-fixers and non-N-fixers. Bars are species averages and error bars represent 1SE, predicted from their respective mixed effects models. Asterisks indicate mixed effects model output of statistically significant within functional group differences from the control (*** $P < 0.001$). Sample sizes for non-N-fixers = 31, 31, 31 and 27, and for N-fixers = 26, 27, 26 and 23, for the Control, N, P and N+P treatments, respectively, in each of the above graphs.

Fig. 3 AMF colonization responses to nutrient addition in N-fixers and non-N-fixers. Bars are species averages and error bars represent 1SE, predicted from their respective mixed effects models. Asterisks indicate mixed effects model output of statistically significant within functional group differences from the control (** $P < 0.01$, *** $P < 0.001$). Sample sizes for non-N-fixers = 97, 83, 98 and 87, and for N-fixers = 62, 51, 61 and 61, for the Control, N, P and N+P treatments, respectively.

Table 1 Marginal and conditional R^2 values for all the response variables analyzed using mixed effects models. These values give an indication of the variation explained by the fixed effects only, and the fixed and random effects together, respectively. AMF – Arbuscular mycorrhizal fungi.

Response variable	$\mathbf{R^2_{marginal}}$	$\mathbf{R^2_{conditional}}$
Foilar N (%)	0.31	0.65
Foliar P (%)	0.23	0.51
Foliar N:P	0.27	0.38
Total foliar N (g)	0.12	0.27
Total foliar P (g)	0.10	0.16
AMF colonization	0.005	0.08

Table 2 Summary of mixed effects model results of leaf chemistry and arbuscular mycorrhizal fungal (AMF) colonization responses to factorial N and P addition.

Response	**Effect**	**Wald chi-square**	**df**	**P**
Foilar N (%)	Treatment	14.2992	3	0.0025
	Functional group	10.2346	1	0.0014
	Interaction	8.3413	3	0.039
Foliar P (%)	Treatment	57.2565	3	< 0.001
	Functional group	2.0061	1	0.16
	Interaction	27.2621	3	< 0.001
Foliar N:P	Treatment	30.758	3	< 0.001
	Functional group	13.953	1	< 0.001
	Interaction	13.179	3	0.0043
Total foliar N (g)	Treatment	27.4464	3	< 0.001
	Functional group	0.2807	1	0.60
	Interaction	8.4222	3	0.038
Total foliar P (g)	Treatment	1.3985	3	0.70
	Functional group	9.6718	1	0.0019
	Interaction	1.4220	3	0.70
AMF colonization	Treatment	2.9627	3	0.40
	Functional group	0.1426	1	0.70
	Interaction	35.8166	3	< 0.001

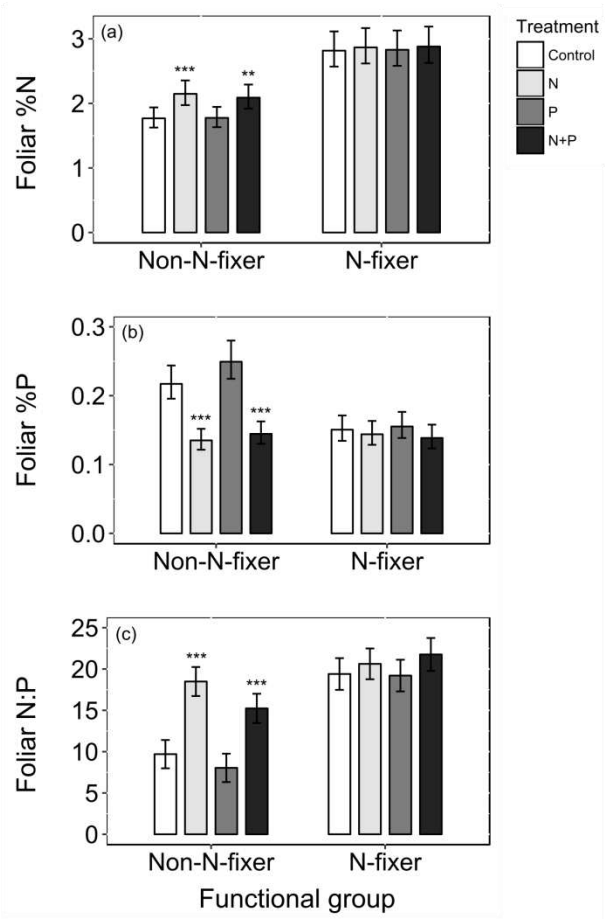
(a)
Foliar %N
Treatment
Control
N
P
N+P
***
**
Non-N-fixer
N-fixer
(b)
Foliar %P
***
***
Non-N-fixer
N-fixer
(c)
Foliar N:P
***
***
Non-N-fixer
N-fixer
Functional group

Fig. 1

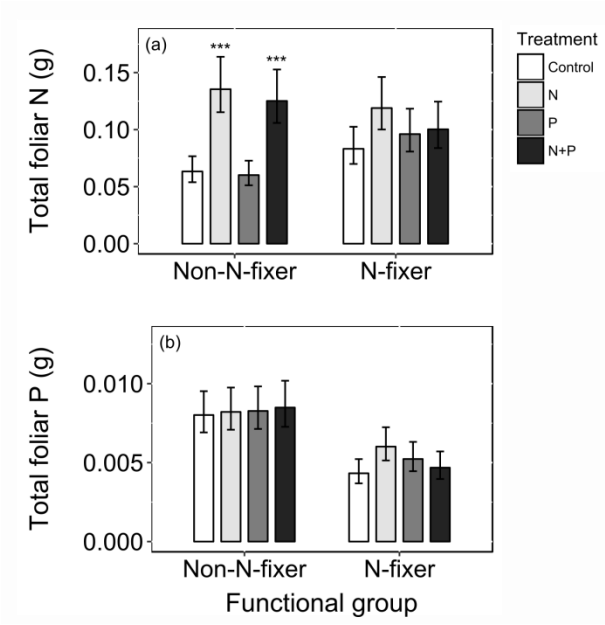
(a)
Total foliar N (g)
Treatment
Control
N
P
N+P
***
***
Non-N-fixer
N-fixer
(b)
Total foliar P (g)
Non-N-fixer
N-fixer
Functional group

Fig. 2

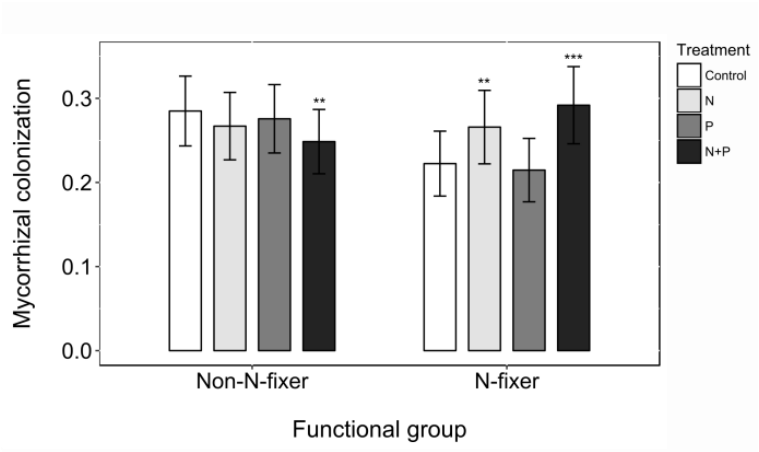
Mycorrhizal colonization
0.0
0.1
0.2
0.3
**
**
***
Non-N-fixer
N-fixer
Functional group
Treatment
Control
N
P
N+P

Fig. 3