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Nitrogen addition alters ectomycorrhizal fungal communities and soil enzyme activities in a tropical montane forest

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ABSTRACT

Long-term increases in nitrogen (N) availability resulting from anthropogenic N deposition can strongly influence ectomycorrhizal (EM) fungal communities with potential consequences for nutrient cycling, forest composition and carbon storage. In a tropical montane forest in Panama, we used Illumina amplicon sequencing to examine how 9 y of experimental N addition has affected EM communities associated with *Oreomunnea mexicana*, and fluorescence assays to measure changes in enzyme activity in the soil. Nitrogen addition significantly reduced EM colonization of *Oreomunnea* roots and altered EM composition. The abundance of *Laccaria* and *Lactarius* increased with N addition, while *Cortinarius* declined. In addition, we found a reduction in soil phosphatase, *N*-acetyl-glucosaminidase, and β-xylanase activity with N addition. We conclude that a reduction in EM fungal taxa specialized in organic N and phosphorus absorption along with a decrease in EM colonization of host plants could decrease soil enzyme activity and therefore have feedback effects on soil nutrient cycling.

1. Introduction

Anthropogenic nitrogen (N) deposition has increased worldwide over the last century (Matson et al., 1999) and is predicted to continue to increase in the next decades, with measurable impacts on low latitude ecosystems (Hietz et al., 2011; Phoenix et al., 2012). The potential effects of excess N on ecosystem processes are diverse. Excess N causes soil acidification, reduces plant productivity, and alters plant species composition, soil carbon (C) storage, and N cycling (Hasselquist and Högberg, 2014; BassiriRad, 2015).

The impact of increasing N availability on tropical forests is relatively poorly understood. Increased N deposition has been linked to increased foliar and wood N concentrations in both the paleo and neotropics (Hietz et al., 2011). In general, both N availability and ecosystem N losses are high in tropical relative to temperate forests (Hedin et al., 2009), suggesting that N deposition effects may be less severe in the tropics. However, tropical montane forests, particularly those dominated by ectomycorrhizal (EM) trees, show greater evidence of N limitation (Tanner et al., 1998; Dalling et al., 2016) and have a tighter N cycle (Corrales et al., 2016a) compared with forests dominated by species with arbuscular mycorrhizal associations. Forests dominated by EM associated tree species might therefore be particularly sensitive to increases in N availability.

Ectomycorrhizal associated tree species are thought to be particularly sensitive to increases in N availability, because EM fungi specialize in N acquisition from soil (Read, 1991) and because host trees strongly reduce C allocation to their root symbionts when there is an excess of readily available N (Högberg et al., 2010; Hasselquist and Högberg, 2014). Long-term increases in N availability in N-limited temperate forests alter species richness,

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community structure, and composition of EM fungi (Peter et al., 2001; Lilleskov et al., 2002; Pardo et al., 2011; Morrison et al., 2016). However, effects of N addition on EM species composition may depend on the functional traits of component EM fungal taxa (see Lilleskov et al., 2011 for review). It has been proposed that differences in sensitivity to N addition are associated with fungal soil exploration type and enzymatic activity. Fungal exploration types are strategies of growth and colonization of the soil environment that can vary considerably among taxa (Agerer, 2001, 2006; Hobbie and Agerer, 2010). Sensitive or 'nitrophobic' species usually have high enzymatic capabilities for organic N uptake, a process that incurs a higher C cost to their hosts (Lilleskov et al., 2002). In contrast, resistant or 'nitrophilic' species are usually limited to the uptake of inorganic N forms, and incur a lower C cost to their hosts (Lilleskov et al., 2002). With increased N availability, nitrophobic EM species may respond by reduced production of sporocarps (Arnolds, 1991; Lilleskov et al., 2011), mycelial growth rate (Wallander and Nylund, 1992), and colonization of root tips (Treseder and Allen, 2000; Peter et al., 2001), while nitrophilic species tend to increase in abundance.

Changes in the taxonomic and functional composition of EM fungi following increases in inorganic N availability are also expected to influence ecosystem processes including soil C storage, N cycling, and plant community productivity (Treseder and Lennon, 2015). Ectomycorrhizal associations play an important role in soil nutrient dynamics due to their capacity to compete for organic N with the decomposer microbial community (Phillips et al., 2013; Averill et al., 2014). Further, because different EM lineages vary in their capacity to take up different N or phosphorus (P) sources, they differentially affect ecosystem level processes (Tedersoo et al., 2012; Koide et al., 2014). Changes in EM fungal community composition could therefore result in changes in enzyme activity, with long-term consequences for N, P and C cycling in the soil.

Many montane forests in Central America are dominated by tree species that form EM associations, such as species of *Quercus* (Halling, 2001; Morris et al., 2008). Large areas of montane forest in western Panama are dominated by *Oreomunnea mexicana* (Juglandaceae) that associates with EM fungal communities that differ according to soil fertility (Corrales et al., 2016a). Thus, there is a critical need to understand the response of EM associations to N addition in this region, if we are to predict the response of this ecosystem to N deposition and global change (Matson et al., 1999; Phoenix et al., 2006; Dalling et al., 2016). Here we take advantage of a 9 y N addition experiment at a site with low background N availability to explore the effect of N on soil enzyme activity and on the composition of the EM fungal community associated with the canopy tree *O. mexicana*. We predicted that an increase in N availability would influence EM fungal community composition and colonization by: (i) shifting the EM fungal community from one characterized by taxa with previously described associations with low inorganic N environments (i.e., nitrophobic taxa) to one increasingly characterized by abundant nitrophilic taxa; (ii) reducing the activity of enzymes associated with organic N acquisition; and (iii) reducing the EM colonization of root tips consistent with a reduction of C transfer from the host tree.

2. Materials and methods

2.1. Study site

The study was located in a primary lower montane forest (1000–1400 m a.s.l.) in the Honda watershed of the Fortuna Forest Reserve in western Panama (hereafter, Fortuna; 8°45′ N, 82°15′ W). Climate records indicate that the mean annual temperature for Fortuna ranges from 19 to 22 °C (Cavelier, 1996) and annual rainfall averages ca. 5800–9000 mm (Andersen et al., 2012). Atmospheric N deposition from rainfall in the area is 5 kg N ha^{-1} y^{-1} (Adamek et al., 2009). Soils at this site are infertile Ultisols derived from rhyolite (Table 1). Plant primary productivity has been described as N limited (Corre et al., 2010) with the plant community showing significant increases in stem diameter growth and production of leaf litter after N addition (Adamek et al., 2009). The focal species, *O. mexicana,* is an EM canopy tree distributed from Mexico to Panama at 900–2600 m a.s.l (Stone, 1972). and forms monodominant stands at the study site (Corrales et al., 2016a,b).

Table 1
Mean and standard error of soil characteristics in control and nitrogen fertilized (+N) plots at Fortuna Forest Reserve, Panama. The probability value (*P*) was calculated using ANOVA analysis. Bold text indicates statistically significant difference with a *P-value* less than 0.05 (*) or 0.005 (**).

Soil variables	Control	+ N	*P*
Soil nutrients			
pH	4.62 ± 0.09	4.51 ± 0.08	0.3
Resin P (mg P kg^{-1})	1.60 ± 0.42	0.97 ± 0.22	0.6
NH_4 (μg bag^{-1} d^{-1})	29.7 ± 6.9	40.3 ± 7.4	0.9
NO_3 (μg bag^{-1} d^{-1})	16.7 ± 2.8	37.9 ± 6.1	**0.02***
PO_4 (μg bag^{-1} d^{-1})	1.06 ± 0.40	2.53 ± 0.82	0.7
DOC (mg C kg^{-1})	417 ± 23	365 ± 34	0.5
Microbial C (mg C kg^{-1})	1134 ± 170	797.8 ± 94.2	0.4
Microbial N (mg N kg^{-1})	237.3 ± 28.8	180.8 ± 19.6	0.3
Microbial P (mg P kg^{-1})	34.3 ± 4.9	34.5 ± 1.98	0.9
Microbial C:N	4.61 ± 0.31	4.46 ± 0.25	0.7
Microbial C:P	37.0 ± 8.1	24.3 ± 4.3	0.5
Microbial N:P	7.82 ± 1.53	5.38 ± 0.75	0.4
Soil enzymes			
Phosphomonoesterase (MUP, nmol MU g^{-1} min^{-1})	130.33 ± 12.6	68.99 ± 10.48	**0.002****
Phosphodiesterase (BIS, nmol MU g^{-1} min^{-1})	19.73 ± 2.08	9.59 ± 1.64	**0.002****
β-glucosidase (BG, nmol MU g^{-1} min^{-1})	2.97 ± 0.34	2.45 ± 0.61	0.43
N-acetyl-glucosaminidase (NA, nmol MU g^{-1} min^{-1})	4.05 ± 0.69	2.12 ± 0.51	**0.05***
β-xylanase (XYL, nmol MU g^{-1} min^{-1})	2.24 ± 0.92	1.36 ± 0.94	**0.03***
Cellobiohydrolase (CELLO, nmol MU g^{-1} min^{-1})	0.7 ± 0.79	0.73 ± 0.90	0.93
BG:NA (Enzymatic C:N)	0.94 ± 0.19	1.23 ± 0.13	0.28
BG:MUP (Enzymatic C:P)	0.02 ± 0.002	0.03 ± 0.005	0.06
NA:MUP (Enzymatic N:P)	0.03 ± 0.004	0.03 ± 0.005	0.82

2.2. Nitrogen addition experiment

This is an ongoing N addition experiment and consists of a paired-plot design with eight 40 × 40 m plots. Soil characteristics of the plots did not differ prior to N application and were randomly assigned to control or N addition treatments at the beginning of the study (Adamek et al., 2009). The initial soil characteristics of the plots prior to the first N application (January 2006) were reported by Koehler et al. (2009) and added here as supplementary material (Table S1). Four plots have been fertilized with urea ($CO(NH_2)_2$) at an annual rate of 125 kg N ha^{-1} divided into four applications per year. The four remaining plots are untreated controls (Adamek et al., 2009). Even though the N application methods do not simulate current N deposition scenarios for the region (~12 kg ha^{-1} yr^{-1} by 2050 for Mesoamerica, Phoenix et al., 2006) and could increase nutrient leaching (Fransson et al., 2000), the rationale for the study, was to predict the direction of the changes in soil microbial communities induced by high soil inorganic N availability (Corre et al., 2010). The experiment began in February 2006, with 9 y of N addition at the time of the study. Seven of the eight plots (four control and three fertilized) contain individuals of the focal species *O. mexicana*, including seedlings, saplings and adults and therefore are included in this study. *Oreomunnea* individuals >10 cm DBH accounted for 15.5 ± 10.8% of the total number of individuals and 28.2 ± 13.4% of the total basal area of the plots (Dalling et al., *unpublished data*). *Oreomunnea* is the dominant EM host in the plots and, only three other individuals >10 cm DBH of EM tree species in the Fagales occur in the plots (one *Quercus* cf *lancifolia* and two *Alfaroa costaricensis*).

2.3. Soil data collection

Samples of the surface 0–10 cm of mineral soil were collected at nine points separated by 10 m inside each plot (nine internal interception points of 10 × 10 m subplots) using a soil corer, and were pooled to generate a single composite sample per plot prior to analysis. Ammonium, nitrate, and phosphate were measured using resin bags. Four resin bags containing 5 g of mixed-bed anion and cation exchange resins (Dowex Marathon MR-3) sealed inside 220 μm polyester mesh were buried 2 cm beneath the soil surface in the central point of four 20 × 20 m subplots and in the central point of the plot. Bags were buried during October 20 to November 9 of 2013. After incubation *in situ* for 21 d the resin bags were collected, rinsed with deionized water to remove adhering soil, extracted with 75 mL of 0.5 M HCl, and then nitrate (+nitrite), ammonium, and phosphate were determined by automated colorimetry on a Lachat QuikChem 8500 (Hach Ltd., Loveland, CO, USA). In addition, readily exchangeable phosphate (Resin P) was also determined by extraction with an anion exchange membrane in the laboratory based on the method described by Turner and Romero (2009). Microbial P was determined by hexanol fumigation and extraction with anion-exchange membranes following Kouno et al. (1995) as modified in Turner and Romero (2010). Microbial C and N were determined by chloroform fumigation and extraction in K_2SO_4 by standard procedures (Brookes et al., 1985; Vance et al., 1987) following Turner and Wright (2014). Dissolved organic C (DOC) and total dissolved N were determined simultaneously in K_2SO_4 extracts on a TOC-V_{CHN} analyzer (Shimadzu, Columbia, MD) after five-fold dilution of the extracts.

2.4. Sampling of ectomycorrhizas

To assess whether N addition had an impact on the EM fungal community, fine roots from *Oreomunnea* were collected from N fertilized and control plots in August of 2015. From each plot, fine roots were collected from three *Oreomunnea* adult trees, five saplings (40–100 cm height), and five seedlings (5–20 cm height) per plot for a total of 91 individuals. Two lateral roots were excavated 2–3 m from the trunk of each adult tree until fine roots that were clearly connected to the tree were found. The entire root system of each focal seedling and sapling was collected.

Fine roots were stored in plastic bags and refrigerated within 2 h of collection. Each sample was carefully cleaned with tap water and cut into 2 cm pieces. Four samples of about 20 cm of total fine root length (each one equivalent to about 1 g of ground root tissue) were collected from each adult, two samples were collected from each sapling, and the entire root system of each focal seedling was collected for a total of 192 samples (Table S2). In a few cases, where 20 cm of root length weighed <0.05 g, two replicates of the same root were pooled together in the same tube prior to DNA extraction. In cases where 20 cm of root length weighed >0.1 g the sample was split in two tubes prior to DNA extraction (Table S2). All roots obtained were included in field collections even if EM fungal colonization was not visible macroscopically. Samples were preserved in 2% CTAB in −20 °C until DNA extraction was performed at the University of Illinois, USA.

2.5. DNA extraction and sequencing of the EM fungi

Prior to DNA extraction root tissue was removed from CTAB, gently dried with a sterile Kimwipe, and ground using a TissueLyser machine (QIAGEN). DNA was extracted from about 0.1 g of ground root tissue from each sample using the MO BIO PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA USA) following the manufacturer's instructions. PCR was performed to amplify the ITS2 region using a mixture of six forward primers (in equimolar concentration) analogous to ITS3 and a degenerate reverse primer analogous to ITS4 (hereafter referred to as ITS4ngs) following Tedersoo et al. (2014; Supplemental methods).

Amplicons were subjected to ligation of Illumina adaptors using two variants of the TruSeq DNA PCR-Free HT Sample Prep kit (Illumina Inc., San Diego, CA, USA). Two libraries were prepared. All samples were sequenced in Illumina MiSeq 2 × 300 bp paired-end run at the Estonian Genome Center at University of Tartu. High-throughput sequences are available in Sequence Read archive (SRA) under accession SRR3944159.

2.6. Bioinformatics

Paired-end sequencing (2 × 300 bp) in the Illumina MiSeq sequencer resulted in 722 649 reads. Quality filtering and selection of representative sequences were done following the methods of Tedersoo et al. (2015) explained in supplementary methods. Representative sequences for each OTU were taxonomically assigned using BLASTn searches (options, word size = 7, gap penalty = −1, gap extension penalty = −2 and match score = 1) retrieving the 10 best BLASTn matches. The UNITE 7.0 beta data set (https://unite.ut.ee/repository.php) and Sanger sequences from fruiting bodies and root tips collected in previous studies at the study area (including sequences from Corrales et al., 2016a) were used as a reference for BLASTn taxonomic assignment of ITS.

Only OTUs with a match of >80% with families previously reported as EM by Tedersoo et al. (2010) in the BLASTn taxonomic assignment were included in the final database for analysis. For the results and discussion we will refer to OTUs as 'species' since 97% is a broadly accepted cut off that usually represents the species concept in EM fungi (Kõljalg et al., 2013).

2.7. Enzyme data collection

Samples of the surface 0–10 cm of mineral soil were collected <1 m from the trunk of three adult *Oreomunnea* trees per plot (same individuals sampled for EM fungi) for a total of 21 individual samples, 12 in control and 9 in N addition plots in May of 2016. The activities of six hydrolytic enzymes were determined using fluorogenic substrates as described previously (Turner, 2010; Turner and Romero, 2010). The enzymes and substrates were: (i) acid phosphomonoesterase (Enzyme Commission (EC) number 3.1.3.2) assayed with 4-methylumbelliferyl phosphate; (ii) phosphodiesterase (EC 3.1.4.1) assayed with bis-(4-methylumbelliferyl) phosphate; (iii) β-glucosidase (EC 3.2.1.21) assayed with 4-methylumbelliferyl β-D-glucopyranoside; and (iv) *N*-acetyl β-D-glucosaminidase (EC 3.2.1.52) assayed with 4-methylumbelliferyl *N*-acetyl β-D-glucosaminide; (v) β-xylosidase (EC 3.2.1.37) assayed with 4-methylumbelliferyl β-D- xylopyranoside; and (vi) cellulose 1,4- β-cellobiosidase (EC 3.2.1.91) assayed with 4-methylumbelliferyl β-D-cellobiopyranoside.

Substrates were purchased from Glycosynth Ltd (Warrington, UK) and dissolved in 0.4% methylcellosolve (2-methoxyethanol; 0.1% final concentration in the assay) and then processed following Turner and Wright (2014). For each sample, fresh soil was suspended in a 1:100 soil to water ratio (1 g on a dry weight basis) and 100 mL of 1 mM NaN_3 solution (to inhibit microbial activity) by stirring on a magnetic stir-plate for 15 min. Plates were incubated for between 30 min and 4 h (depending on the enzyme) at 30 °C. The reaction was terminated by adding 50 μL of 0.5 M NaOH (final solution pH > 11) and the fluorescence determined immediately on a FLUOstar Optima multi-detection plate reader (BMG LABTECH, Offenburg, Germany) following Turner and Wright (2014). All enzyme activities are expressed as nmol MU g^{-1} soil (dry weight) min^{-1}.

2.8. Root staining and frequency of colonization

Twenty cm of fine root of three adults, five samplings and the entire root system of five seedlings was preserved in 96% ethanol for quantification of EM fungal colonization. Roots cleared in 10% KOH and stained with trypan blue. Colonization of four randomly chosen 2 cm root sections per seedling was assessed for 84 individual samples (Table S3) using the grid-line intersect method as modified by McGonigle et al. (1990). A positive encounter was recorded when either the presence of mantle or Hartig net was observed under 40x magnification.

2.9. Statistical analysis

Soil nutrients and enzyme activity were compared between treatments using ANOVA. Phosphate, microbial N, P, cellobiohydrolase, and enzyme C:N were log transformed to improve normality; other variables were normally distributed.

OTUs with fewer than 10 sequences (n = 655) were excluded from alpha and beta diversity analyses to provide a robust data set to compare EM fungal communities among N treatments and host developmental stages (Smith and Peay, 2014; Brown et al., 2015). In addition samples were rarefied to an equal number of sequences prior to diversity analysis. Species accumulation curves with the remaining 376 OTUs were used to compare richness among developmental stages, and between the N addition treatment and control. Curves were rarefied based on the smallest number of sequences per developmental stage and per treatment (Table S2). Alpha diversity indexes (Pielou evenness index and Fisher alpha) were calculated per plot based on an equal number of samples and compared using ANOVA including treatment (N addition and control) and developmental stage as fixed effects and plot as a random effect after checking for equality of variance and residuals using Levene's and Shapiro-Wilk tests. The Pielou index was calculated following Pielou (1966).

Non-metric Multidimensional Scaling (NMDS) was used to visualize differences among EM fungal community composition among N treatments and developmental stages. NMDS analyses were based on Bray-Curtis dissimilarity matrices using abundance data for individual roots, and for samples pooled per plot. The significance of differences between N treatments and developmental stages was determined using permutational analyses of dissimilarity (ADONIS) using 1000 permutations and a Euclidean distance matrix (Oksanen et al., 2008).

Principal component analysis (PCA) was applied separately on two soil datasets (nutrients and enzymes), resulting in two axes of variation for soil nutrients (describing 78.77% and 17.31% respectively of the total variance) and one axis for soil enzymes (describing 98.63% of the total variance). Given the low level of replication of soil samples only seven soil variables were included in the principal components analysis. The soil nutrient dataset included a subsample of the measured soil variables that showed less than 50% correlation with any other variable in the data set. The soil variables included were, resin P (mg P kg^{-1}), NH_4 (μg bag^{-1} day^{-1}), NO_3 (μg bag^{-1} day^{-1}), PO_4 (μg bag^{-1} day^{-1}), DOC (mg C kg^{-1}), microbial N (mg N kg^{-1}), and microbial C:N (Table 1). The enzyme dataset was averaged per plot prior to the principal component analysis and all the measured enzymes were included in the analysis. The enzyme variables were measured in nmol MU g^{-1} min^{-1} and included phosphomonoesterase (MUP), phosphodiesterase (BIS), β-glucosidase (BG), *N*-acetyl β-D-glucosaminidase (NA), xylanase (XYL), cellobiohydrolase (CELLO), and enzyme C:N (Table 1). Enzyme C:N (Enz C:N) ratio represents the ratio between β-glucosidase (an enzyme targeting C) to *N*-acetyl β-D-glucosaminidase (an enzyme targeting N). The first axis of the resulting PCA for the soil nutrient dataset (PC1.nut) and the first axis of the soil enzyme dataset (PC1.enz) were used in the NMDS analysis of samples pooled per plot to find correlations between soil variables and changes in the EM community composition with N treatments (Ter Braak, 1995). To further explore the relationship between changes in EM community composition and enzyme activity, a permutational analysis of dissimilarity (ADONIS, Oksanen et al., 2008) was used with 200 permutations using the species abundance matrix per plot and a subset of independent enzymes variables. All statistical analyses were carried out using the package *vegan* 2.0–6 in R 2.15.1 (R Development Core Team, 2011).

A GLM with Poisson error distribution was used to compare number of OTUs between treatments, while negative binomial errors were used to test the effect of N addition on the relative abundance of the eight most abundant individual genera, including treatment as a fixed effect. A GLM with Poisson error distribution was used to test whether enzyme activity was associated to the abundance of each of the five most abundant genera after correcting for a treatment effect. Each GLM was run using each enzyme as the dependent variable and each individual genus and treatment (N addition *vs.* control) as predictor variables. The significance level (alpha = 0.05) was adjusted using the Bonferroni correction for five comparisons (adjusted alpha = 0.01).

To test for the effect of N addition, developmental stage and their interaction on the abundance of the 100 most abundant species, a multivariate GLM analysis was performed using *manyglm* in *mvabund* 3.10.4 package in R using a negative binomial model and sequence count data. This analysis runs a univariate analysis to check for responses of individual species to treatment effects (Wang et al., 2012). The univariate ANOVA analysis was run using the 'adjust' option that corrects probability values for multiple

testing using a step-down resampling procedure (Wang et al., 2012). Species with probability values lower than 0.07 were considered significant due to the lower power of the univariate ANOVA compared to multivariate ANOVA (Wang et al., 2012).

Finally, two-way ANOVA including treatment (N addition and control) and developmental stage (seedling, sapling, and adult) as fixed effects and plot as a random effect was used to assess the effect of developmental stage and N addition treatment on EM colonization frequency.

3. Results

3.1. Changes in soil fertility and enzyme activity with N addition

Among soil chemical variables, only nitrate differed significantly between treatments, with two-fold greater turnover in N addition plots (Table 1). The activity of phosphomonoesterase, phosphodiesterase, *N*-acetyl-glucosaminidase, and β-xylanase were significantly greater in control plots than in N addition plots (Table 1). There were significant positive pair-wise correlations among the activities of phosphomonoesterase, phosphodiesterase, *N*-acetyl-glucosaminidase, β-glucosidase and β-xylanase. Also, the activity of cellobiohydrolase was positively correlated with β-xylanase (Table 2).

3.2. Diversity patterns

Illumina sequencing of *Oreomunnea* roots revealed high EM species richness for both the four control plots (291 OTUs) and three N addition plots (293 OTUs) with 210 OTUs shared between treatments for a total of 376 OTUs in the community. The community was dominated by Basidiomycota with 341 species and 19 genera; Ascomycota was represented by 35 species and 3 genera. *Russula* was the most species-rich genus with 124 species, followed by *Lactarius* (45 spp.), *Tomentell*a (44 spp.) and *Cortinarius* (35 spp.). Overall, 212 of the OTUs obtained by Illumina sequencing (56%) had as their closest match Sanger sequences from fruit body voucher specimens or root tips from the study area. Thirty-two OTUs belonging to Cantharellaceae, Elaphomycetaceae, Russulaceae, and Thelephoraceae with a percentage of match >80% were left as 'unidentified' species. In terms of abundance, *Russula, Tomentella, Cortinarius, Cenococcum, Lactarius, Clavulina, Elaphomyces*, and *Laccaria* were the most abundant genera that accounted for 87% of the total number of reads.

Species diversity and evenness were not significantly different between control and N fertilized plots (Fig. 1, Table 3). In contrast, there were significant differences when comparing across developmental stages, driven by significantly higher species diversity and evenness in saplings compared to seedlings and adults based on Tukey HSD, $P < 0.05$ (Fig. 1, Table 3).

EM fungal community composition from *Oreomunnea* root tips

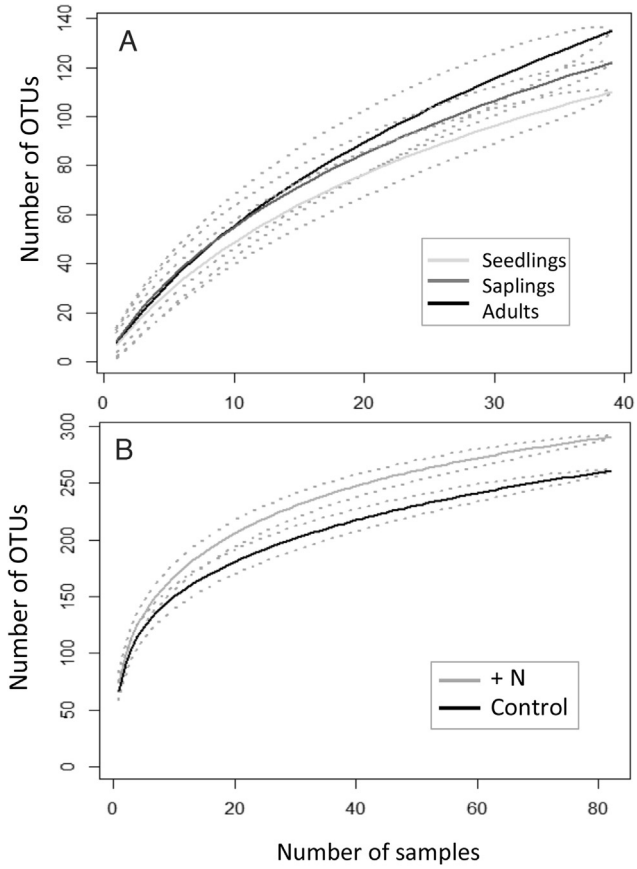

Fig. 1. Species-accumulation curves of EM fungi for (A) developmental stages and (B) N treatments including OTUs with >10 reads.

Table 3
Total number of OTUs, and mean value of diversity indexes for EM fungi associated with the roots of *Oreomunnea mexicana* for N addition treatments and *Oreomunnea* developmental stages. The *P* value and degrees of freedom of an ANOVA analysis comparing diversity between treatments and among developmental stages independently are included. Values for N treatments were computed based on plot averages pooling samples from all individuals in each plot. Developmental stages means were computed using individual samples. Values in bold are statistically significant.

	Total OTU	Fisher's alpha	Pielou Evenness
+N	293	50.72	0.6
Control	291	47.5	0.57
df		6	6
P		0.26	0.1
Adults	152	4.96	0.51
Saplings	130	5.85	0.58
Seedlings	116	4.38	0.52
df		2	2
P		**<0.001**	**<0.01**

Table 2
Correlation analysis of soil enzymes. Values in bold represent significant correlations with a *P*-value < 0.05.

Enzyme	MUP	BIS	BG	NA	XYL	CELLO	EnzC.N	EnzC.P	EnzN.P
MUP	1.00								
BIS	**0.96**	1.00							
BG	**0.62**	**0.61**	1.00						
NA	**0.70**	**0.67**	**0.61**	1.00					
XYL	**0.68**	**0.73**	**0.73**	**0.73**	1.00				
CELLO	0.26	0.24	0.59	0.25	**0.44**	1.00			
EnzC.N	−0.08	−0.10	0.09	**−0.57**	−0.13	0.13	1.00		
EnzC.P	−0.21	−0.17	0.57	0.06	0.22	**0.44**	0.28	1.00	
EnzN.P	0.10	0.12	0.37	**0.72**	0.38	0.12	**−0.60**	0.42	1.00

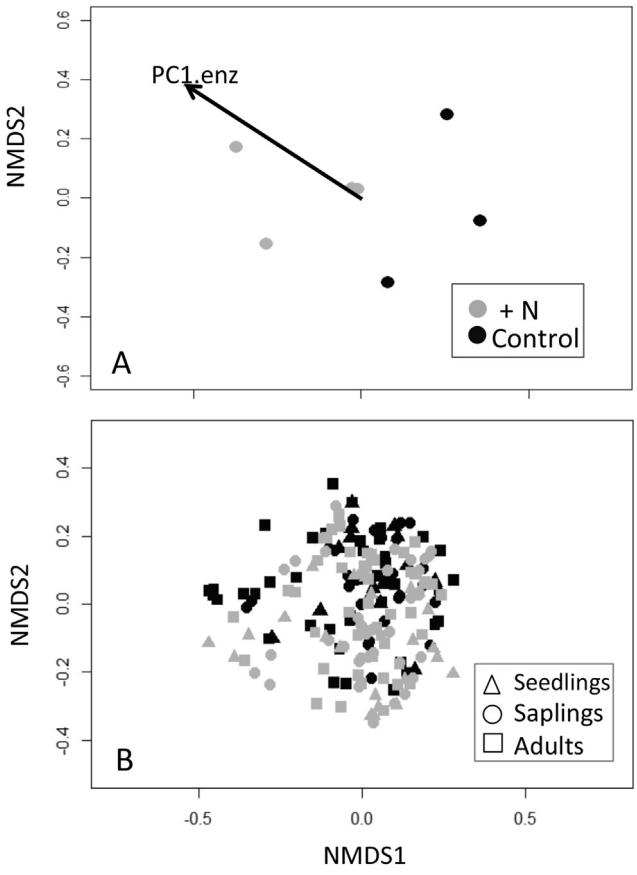

Fig. 2. NMDS of fungal communities associated with *Oreomunnea mexicana* fine roots. A) NMDS of fungal communities based on relative abundance sequence data pooled per 40 x 40 m treatment plot (stress = 0.03), and showing significant correlation with the first axis from a PCA of enzyme activities (PC1.enz). B) NMDS of fungal communities related to different developmental stages of *O. mexicana* (stress = 0.09), colors correspond to treatment as in Fig. 2A legend.

differed between control and N fertilized plots when pooling samples per plot (Adonis $R^2 = 0.22$, $F_{1,6} = 1.42$, $P = 0.04$). This difference between treatments was also reflected in the NMDS ordination where control plots grouped separately from N fertilized plots (Fig. 2A). In contrast, species composition did not differ among developmental stages based on ADONIS analysis (Adonis $R^2 = 0.01$, $F_{2,\ 189} = 1.03$, $P = 0.34$; Fig. 2B).

None of the soil nutrient PCA axes showed a significant correlation with axes of EM fungal community composition derived from the NMDS ordination ($R^2 = 0.5$, $P = 0.49$). However, the first PCA axis of the soil enzyme dataset (PC1.enz) was significantly correlated with the first two axes of the NMDS ($R^2 = 0.83$, $P = 0.02$; Fig. 2A). PC1.enz accounted for 98.6% of the variation in soil enzyme variables. The ADONIS analysis including individual soil variables and the species abundance matrix showed that changes in phosphomonoesterase activity (MUP) was significantly correlated with changes in EM species composition ($R^2 = 23.2$, $P = 0.02$).

3.3. Changes in abundance and species richness with N addition

Nitrogen addition did not affect species richness for any of the EM genera based on the GLM results (Table 4). When the same analysis was done using abundance data, *Cortinarius* showed a significant reduction from 15% of sequence reads in control plots to 6% with N addition ($z = -3.57$, $P < 0.001$; 21 OTUs showed a negative response and 15 OTUs a positive response). In contrast, *Laccaria* and *Lactarius* responded positively to N addition (Fig. 3, Table 4). *Laccaria* increased from 1% to 10% ($z = 3.43$, $P < 0.001$; 1 OTU showed a negative and 5 OTUs a positive response); *Lactarius* increased from 5% to 9% ($z = 2.27$, $P = 0.02$; 21 OTUs showed a negative and 21 OTUs a positive response).

At the species level, the multivariate GLM showed a significant effect of N addition ($P = 0.001$), developmental stage ($P = 0.001$), and their interaction ($P = 0.001$) on the abundance distribution of the 100 most abundant species. The univariate ANOVA revealed a significant effect of treatment on the abundance of eight species. *Laccaria* sp. (OTU 20- KY548915), *Cortinarius* sp. (OTU 87- SH009469.07FU), *Tomentella* sp. (OTU 126- SH177859.07FU), and an unidentified Cantharellaceae (OTU 45- SH481710.07FU) showed higher abundance in N addition plots. *Cortinarius obtusus* (OTU 137- KY548880), two *Cortinarius* (species both affiliated to *Cortinarius junghuhnii* OTUs 18- KY548874 and 85- KY548874), and *Tomentella* sp. (OTU 22- KM594933) showed lower abundance in N addition plots (Table 5). None of the genera showed a significant correlation with any of the soil nutrients measured except for *Lactarius* that showed a positive association with nitrate ($R^2 = 0.52$, $t = 2.7$, $P = 0.04$; data not shown).

The univariate ANOVA also revealed changes in abundance of eight species associated with different plant developmental stages. Three species of *Cortinarius* (OTUs 18- KY548874, 28- JX989852, and 85- SH218435.07FU), and an unidentified Cantharellaceae (OTU 45- SH481710.07FU) showed significantly higher abundance in seedlings compared with saplings and adult individuals.

Table 4
The eight most abundant ectomycorrhizal fungal genera found in roots of *Oreomunnea mexicana* and their distribution among nitrogen fertilized (N) and control plots (C) at the Fortuna Forest Reserve, Panama. The number of OTUs in each genus (No. OTUs) represents the total number of different OTUs identified for each genera ('species richness') and was quantified based on OTUs with more than 10 reads in the total database. The relative abundance of each genus (RA) was calculated as the percentage of the total sum of reads of the OTUs in the genus divided by the total number of reads in the plot. The last two columns show probability values for significant differences between N fertilized and control plots using OTU counts and relative abundance. Significant effects ($P < 0.05$) are shown in bold.

Genus	**No. OTUs**					**RA**				
	Control		**N**		*P*	**Control**		**N**		*P*
	$\bar{x}$	*SE*	$\bar{x}$	*SE*		$\bar{x}$	*SE*	$\bar{x}$	*SE*	
Cenococcum	12	1.19	11	0.76	0.48	8.97	1.32	5.58	1.41	0.06
Clavulina	5	0.71	7	1.32	0.28	7.65	2.72	4.13	1.34	0.15
Cortinarius	21	1.55	19	3.88	0.54	**14.95**	**2.20**	**6.19**	**1.57**	**<0.001**
Elaphomyces	10	1.47	7	0.58	0.24	5.48	1.76	6.49	1.65	0.87
Laccaria	5	0.63	6	0.50	0.68	**1.42**	**0.67**	**10.09**	**2.99**	**<0.001**
Lactarius	31	2.48	37	1.15	0.15	**5.12**	**0.63**	**9.53**	**0.60**	**0.02**
Russula	67	1.41	75	9.83	0.23	31.06	2.67	26.15	3.84	0.35
Tomentella	22	1.66	24	1.80	0.68	10.51	2.00	19.76	5.65	0.12

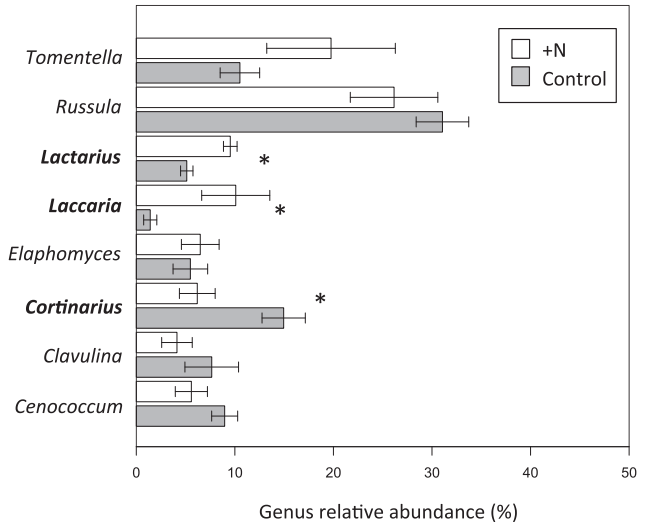

Fig. 3. Changes in relative abundance of the eight most abundant genera associated with *Oreomunnea mexicana* roots in control and nitrogen fertilized plots. Error bars represent standard error of the mean. Stars denote significance based on $P < 0.05$.

Cenococcum sp. (OTU16- SH189430.07FU) and *Tomentella* sp. (OTU22- KM594933) showed a significantly higher abundance in saplings and just one species of *Tomentella* (OTU 61- SH006961.07FU) showed a higher abundance in adult trees (Table S4).

3.4. Association between fungal genera and enzyme activity

At the fungal genus level, the activity of phosphomonoesterase and phosphodiesterase were positively associated with *Cortinarius* and negatively associated with *Russula* and *Tomentella* abundance after correcting for N treatment effect (Table 6). The remaining enzymes were not associated with any of the genera included in the model.

3.5. Effect of N addition and developmental stage in EM colonization

EM colonization of *Oreomunnea* root tips was significantly lower in the N addition treatment (mean 75.5%, sd = 13.6) compared to control plots (mean 86.4%, sd = 14.7; $F_{1,78} = 18.1$, $P < 0.0001$), and differed across development stages ($F_{4,78} = 2.3$, $P = 0.02$; Fig. 4, Table S3) with seedlings showing significantly lower EM colonization (adults 85.1%, saplings 80.7%, and seedlings 75.8%). There was no interaction between N addition treatment and developmental stage ($F_{2,78} = 1.44$, $P = 0.24$).

4. Discussion

To our knowledge, this is the first study to explore the effects of long-term N addition on tropical EM fungal communities. Our results indicate that N addition changes the EM fungal community, with alterations in the relative abundance of fungal genera known to differ in their enzymatic activity. Over the long term, changes in enzyme activity resulting from altered EM communities might be expected to influence key ecosystem processes, including soil C storage and the cycling of N and P.

4.1. Effect of N addition on EM symbiosis

Nitrogen addition did not affect the number of EM species associated with *Oreomunnea* roots. This result is consistent with studies in temperate forest showing no effect of N addition on species richness in short-term N addition experiments (<5 y, Lilleskov et al., 2011). However, a significant reduction in species richness has been found along N deposition gradients in Europe that have been monitored for more than 40 y (Lilleskov et al., 2011; Suz et al., 2014) and therefore a future reduction in richness at our site is possible.

The 9 y of N addition, however, were sufficient to significantly change EM community composition. Both N addition treatment and enzyme activity were significantly correlated with the NMDS axes of the EM community suggesting an effect of N on community composition and function. The significant association of EM fungal community composition and phosphomonoesterase activity could be interpreted as a change in the activity of enzymes associated with access to phosphate (phosphomonoesterase, phosphodiesterase) and N (*N*-acetyl-glucosaminidase) given the high correlation among these enzymes.

Changes in community composition also reflected altered abundance of some EM genera. *Laccaria* and *Lactarius* had significantly higher relative abundance with N addition plots while *Cortinarius* had significantly lower abundance. Likewise, regression analysis showed that *Lactarius* was positively correlated with soil nitrate. These patterns are congruent with broader affinities for N reported for these genera. In studies from temperate forest in Europe and North America *Laccaria* and *Lactarius* consistently

Table 5
Species showing significant changes in abundance across nitrogen addition treatment (N), developmental stage (DS), and their interaction, based on a multivariate GLM analysis using a negative binomial model and sequence count data. Response to N represents the direction of the abundance response to N addition. The closest match in the GenBank database and the species hypothesis from the sequences deposited in UNITE are included. ID% refers to the percentage of sequence match with the closest match in GenBank and the e-value refers to the probability of finding a match by chance when searching the GenBank database. Data are for the 100 most abundant taxa; *P* values are corrected for multiple comparisons using step-down resampling procedure (see methods). *P* value for statistically significant differences was set at 0.07.

Species	OTU	Effect	Response to N	*P*	Accession number	UNITE Species hypothesis	Family	ID%	e-value
Laccaria sp.	**0020**	N	+	0.04	KY548915		Hydnangiaceae	99%	4.29 e−157
Cortinarius sp.	**0087**	N	+	0.04	DQ102685	**SH009469.07FU**	Cortinariaceae	93%	5.37 e−127
Unknown	**0045**	N, N*DS	+	0.07, 0.004	JQ991671	**SH481710.07FU**	Cantharellaceae	89%	1.27 e−132
Tomentella sp.	**0126**	N	+	0.005	AB587786	**SH177859.07FU**	Thelephoraceae	93%	1.10 e−140
Cortinarius sp.	**0137**	N	−	0.01	KY548880		Cortinariaceae	98%	7.35 e−159
Tomentella sp.	**0156**	N	−	0.006	EF411110	**SH189430.07FU**	Thelephoraceae	96%	1.58 e−153
Cenococcum sp.	**0016**	N*DS	ns	0.027	FJ440882	**SH189430.07FU**	Gloniaceae	98%	7.65 e−125
Cortinarius sp.	**0018**	N*DS	−	0.02	KY548874		Cortinariaceae	99%	5.98 e−162
Tomentella sp.	**0022**	N*DS	ns	0.06	KM594933		Thelephoraceae	99%	2.16 e−162
Cortinarius sp.	**0085**	N*DS	−	0.07	KY548874		Cortinariaceae	98%	2.10 e−158
Tomentella sp.	**0183**	N*DS	ns	0.07	KM594933		Thelephoraceae	99%	1.66 e−161
Tomentella sp.	**0061**	DS		0.06	UDB004090	**SH006961.07FU**	Thelephoraceae	92%	2.17 e−131
Cortinarius sp.	**0028**	DS		0.03	KY548889		Cortinariaceae	97%	1.06 e−144

Table 6
Generalized linear models for enzyme activity in the soil and each of the most abundant EM genera found in *Oreomunnea* root tips after after accounting for treatment effect. GLMs were run using a Poisson error distribution for a continuous positive response variable. Variables with stars denote significance based on the > |z| value at $\alpha = 0.01$ after Bonferroni correction for five comparisons with a *P-value* less than 0.01 (**) or 0.001 (***). Only models that were significant are shown.

Enzyme	MUP		BIS	
	Coefficient	*P-value*	*Coefficient*	*P-value*
Cenococcum	3.4 E-05	0.457	8.7 E-05	0.41434
Treatment	**−5.2 E-01**	**6 E-09****	**−6.1 E-01**	**0.004****
Cortinarius	**8.6 E-05**	**0.002****	**2.5 E-04**	**0.0001*****
Treatment	−2.9 E-01	0.012	9.8 E-02	0.721
Lactarius	−8.4 E-05	0.074	−3.2 E-05	0.77
Treatment	**−4.6 E-01**	**1 E-06****	**−6.7 E-01**	**0.003****
Russula	**−4.4 E-05**	**1 E-05*****	**−7.7 E-05**	**0.001****
Treatment	**−6.7 E-01**	**2 E-16*****	**−9 E-01**	**4 E-07*****
Tomentella	**−4.6 E-05**	**0.0004*****	**−9.3 E-05**	**0.004****
Treatment	**−4.3 E-01**	**2 E-08*****	**−4.8 E-01**	**0.008****

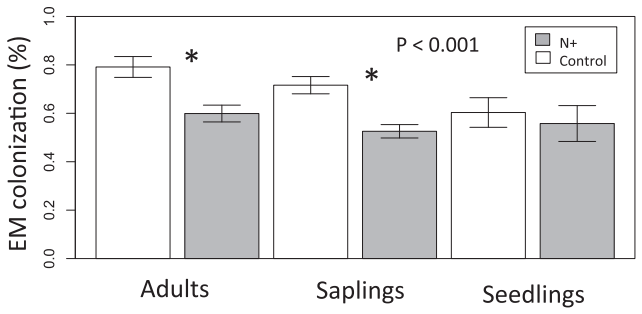

Fig. 4. Mean percent EM colonization (±1 SE) of *Oreomunnea mexicana* roots based on 84 samples of adults, saplings, and seedlings in control and nitrogen addition plots.

exhibit a positive response to high N availability, and therefore represent 'nitrophilic' genera (Lilleskov et al., 2011), while *Cortinarius* has been reported as showing a consistent negative association with N availability and has therefore been considered 'nitrophobic' (Lilleskov et al., 2002, 2011; Suz et al., 2014). In turn, N affinity reflects hyphal exploration traits; *Lactarius, Laccaria,* and *Tomentella* have hydrophilic short and medium distance exploration types and seem to use labile forms of soil N (Lilleskov et al., 2011) while *Cortinarius* has hydrophobic medium-distance fringe exploration and seems to use organic N sources and associates with less fertile soils. Interestingly, *Russula* abundance did not correlate with any of the soil variables included in this study. This likely reflects high variability in resource acquisition traits in this genus, *Russula* species have been reported as showing both negative and positive responses to N availability (Avis et al., 2008; Cox et al., 2010). A study of *Russula* communities across forest sites varying in N availability at the landscape level at Fortuna also showed significant differences in the phylogenetic relatedness of *Russula* between sites with high and low fertility consistent with environmental filtering (Corrales et al., 2016a). Intrageneric variation in N affinity was also observed in the present study. *Cortinarius* sp. (OTU 87, SH009469.07FU) showed a positive association with N addition while *Cortinarius* sp. (OTU 137, KY548880) showed a negative response (Table 5).

Ectomycorrhizal colonization was reduced by N addition as expected (Treseder and Allen, 2000; Peter et al., 2001). This could have been caused by a reduction in the C supply from *Oreomunnea* to its associated ectomycorrhizas or a reduction in mycelium abundance in the soil (Wallander and Nylund, 1992; Peter et al., 2001; Nilsson and Wallander, 2003; Nilsson et al., 2007). In addition EM fungal biomass may also be indirectly impacted via N addition effects on *Oreomunnea* root biomass (not measured in this study). Hölscher et al. (2009) examined fine root mass in the same plots used in this study, and found that *O. mexicana* produced twice as much fine root mass than the other trees in the plots. They also showed that the density of fine root biomass was significantly positively correlated with the thickness of the organic layer and the amount of total N in the organic layer (nitrate was undetectable and organic N was higher than inorganic N in the plots prior to the N treatment application). Given these results, N addition could also have a negative impact on fine root biomass and therefore indirectly affect the EM fungal biomass present in the soil. Lower EM mycelium abundance in the soil could also be an explanation for the lower enzyme activity found in soils in the N addition plots.

The results discussed here are based on comparisons of sequence counts that in some cases may not represent actual number of individuals in the community (Bálint et al., 2016). However, variation of the relative abundance of specific taxa is informative to understand ecological processes when comparisons are made across samples in the same study (Morrison et al., 2016).

4.2. Changes in soil enzyme activity with N addition

Nitrogen addition was associated with significant changes in soil enzyme activity. Notably phosphomonoesterase, phosphodiesterase, *N*-acetyl-glucosaminidase, and β-xylanase were about 50% lower in N addition plots. Lower *N*-acetyl-glucosaminidase activity was expected due to a higher availability of soil inorganic N for plants and microorganisms (Midgley and Phillips, 2014). However, lower phosphatase activity in N addition plots was unexpected since the production of phosphatase requires a high investment of N (Olander and Vitousek, 2000). At Fortuna, organic P is abundant in the soil and organic P turnover is likely to be important for plant and microbial P acquisition. The observed similarity in phosphomonoesterase and phosphodiesterease activity in response to N addition could indicate that phosphatase activity is not being reduced by sorption into organic matter or clay but instead implies a biological mechanism. Marklein and Houlton (2012) in a meta-analysis of the response of phosphatase to N and P addition found that even though the average response of soil phosphatase was positive, 26 of the 85 studies included in the analysis showed a negative response of phosphatase to N addition. These authors hypothesize that soil fertility, duration of the fertilization, soil organic matter content, C: N: P ratio, and microbial community composition could be possible causes for the negative response (Marklein and Houlton, 2012). In the study of Cusack *et al.* (2011), the only N addition study from montane tropical forest included in the Marklein and Houlton (2012) meta-analysis, phosphatase activity decreased marginally, and was associated with an increase in AM fungal biomass.

Lower enzyme activity in N addition plots may indicate a down-regulation of enzyme production from plant roots, a reduction in EM fungal associated enzymes, or may simply reflect the size of the microbial biomass pool in the N addition treatment. Ectomycorrhizal fungi have been shown to release phosphatase in pure culture (Louche et al., 2010) and to actively deplete organic P (Read and Perez-Moreno, 2003). In addition, phosphatase activity has been shown to increase with the formation of EM associations (Ali et al., 2009; van Aarle and Plassard, 2010) and to show a higher activity in the rhizosphere soil around EM root tips (Buée et al., 2005; Courty et al., 2006). Consistent with reduced EM enzyme activity, we observed significantly lower EM colonization of

Oreomunnea roots in N addition plots (Fig. 4).

Although the enzymatic activity of EM taxa was not measured directly, covariation in soil enzyme activity and EM community composition suggests a functional response to N addition. Overall, phosphomonoesterase and phosphodiesterase activity was negatively associated with the abundance of *Russula* and *Tomentella* and was positively associated with the abundance of *Cortinarius.* This supports previous findings of higher enzymatic capability to access organic N and P from soil organic matter and litter reported for *Cortinarius* species (Nygren and Rosling, 2009; Lilleskov et al., 2011; Bödeker et al., 2014) and the lower enzymatic capabilities and affinity for inorganic forms of N reported for some groups of *Russula* and *Tomentella* (Nygren et al., 2008; Lilleskov et al., 2011).

4.3. Implications of anthropogenic N deposition for tropical montane forest

Tropical montane forests have a strong short-term response to moderate nutrient inputs, including a reduction of investment in below-ground biomass and changes in tree species composition (Homeier et al., 2012; Dalling et al., 2016). Consequently, the predicted increase in N availability in tropical ecosystems resulting from urbanization and agricultural intensification (Hietz et al., 2011) could cause significant changes in plant species composition and soil C storage (Homeier et al., 2012; Dalling et al., 2016). With regard to mycorrhizal associations, soils supporting EM dominated forest tend to have a higher soil organic matter content compared to soils supporting arbuscular mycorrhizal dominated forest, probably due to stronger competition of EM fungi with free-living decomposer communities for organic sources of N present in litter (Averill et al., 2014). Changes in the EM community composition and soil enzyme activity with N addition could have important implications for soil C storage and ecosystem N cycling, ultimately affecting forest productivity and diversity. Our results indicate that N addition could reduce the abundance of 'nitrophobic' EM fungal species and the activity of enzymes associated with organic N and P acquisition in tropical montane forests. A reduction in EM fungal taxa specialized in organic N and P acquisition (e.g., *Cortinarius*) along with a decrease in EM colonization of host plants could also negatively impact forest resilience by reducing the abundance of EM associated plants and generating further feedback effects on decomposition rates and soil C storage.

We conclude that an increase in inorganic N availability in tropical montane forest could cause changes in the EM fungal community composition along with a decrease in soil enzyme activity that could have feedback effects on key ecosystem processes, including soil C storage and the cycling of N and P. These responses to N addition are similar to the responses reported for temperate ecosystems. Further research on the interaction of the EM fungi and the decomposer communities under different environmental conditions, and on EM fungal enzyme activity including peroxidase, chitinase, laccase, and leucine aminopeptidase, could improve our understanding of the implications of N addition on soil processes under EM dominated tropical forest.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2017.02.004.

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