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Diversity and Enzyme Activity of Ectomycorrhizal Fungal Communities Following Nitrogen Fertilization in an Urban-Adjacent Pine Plantation

Chen Ning [1,2,3,*], Gregory M. Mueller [2,3], Louise M. Egerton-Warburton [2,3], Andrew W. Wilson [2,3,4], Wende Yan [1] and Wenhua Xiang [1]

1 Faculty of Life Science and Technology, Central South University of Forestry and Technology, Changsha 410004, China; csfuywd@hotmail.com (W.Y.); xiangwh2005@163.com (W.X.)
2 Program in Plant Biology and Conservation, Northwestern University, Evanston, IL 60208, USA; gmueller@chicagobotanic.org (G.M.M.); LWarburton@chicagobotanic.org (L.M.E.-W.); andrew.wilson@botanicgardens.org (A.W.W.)
3 Chicago Botanic Garden, Glencoe, IL 60022, USA
4 Denver Botanic Gardens, Sam Mitchel Herbarium of Fungi, Denver, CO 80206, USA
* Correspondence: chenning2012@u.northwestern.edu; Tel.: +1-312-404-7136

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Abstract: Rapid economic development and accelerated urbanization in China has resulted in widespread atmospheric nitrogen (N) deposition. One consequence of N deposition is the alteration of mycorrhizal symbioses that are critical for plant resource acquisition (nitrogen, N, phosphorus, P, water). In this study, we characterized the diversity, composition, and functioning of ectomycorrhizal (ECM) fungal communities in an urban-adjacent *Pinus elliottii* plantation under ambient N deposition (~24 kg N ha^{-1} $year^{-1}$), and following N fertilization (low N, 50 kg N ha^{-1} $year^{-1}$; high N, 300 kg N ha^{-1} $year^{-1}$). ECM functioning was expressed as the potential activities of extracellular enzymes required for organic N (protease), P (phosphomonoesterase), and recalcitrant polymers (phenol oxidase). Despite high ambient N deposition, ECM community composition shifted under experimental N fertilization, and those changes were linked to disparate levels of soil minerals (P, K) and organic matter (but not N), a decline in acid phosphatase (AP), and an increase in phenol oxidase (PO) potential activities. Based on enzyme stoichiometry, medium-smooth exploration type ECM species invested more in C acquisition (PO) relative to P (AP) following high N fertilization than other exploration types. ECM species with hydrophilic mantles also showed higher enzymatic PO:AP ratios than taxa with hydrophobic mantles. Our findings add to the accumulating evidence that shifts in ECM community composition and taxa specialized in organic C, N, and P degradation could modulate the soil nutrient cycling in forests exposed to chronic elevated N input.

Keywords: extracellular enzymes; hyphal exploration strategy; China; atmospheric nitrogen deposition; *Russula*

1. Introduction

Atmospheric nitrogen (N) deposition has more than doubled the inputs of N into many forest systems. One consequence of N deposition has been the increase in forest productivity [1]. Another is the alteration of soil microbial communities, and especially mycorrhizal communities [2–4]. Most forest trees, such as Pinaceae, depend on symbioses with ectomycorrhizal fungi (ECM) for resource uptake (nitrogen, N, phosphorus, P, water) from soil, and different ECM taxa appear to specialize in various forms of organic resources (reviewed in Lilleskov et al. [5]). As a result, mycorrhizal diversity may underpin many forest ecosystem services including nutrient cycling and water use efficiency. Although

studies have widely demonstrated declines in ECM diversity and changes in ECM community composition following N-enrichment [3,6–12], the extent to which these changes influence the functional capacity of ECM is less well understood [13–16]. In this study, we examined the diversity and functioning in ECM communities under ambient N deposition and following N fertilization in an urban-adjacent *Pinus elliottii* plantation.

Enzymatic activities comprise one type of ECM functional trait that can be directly linked to ecosystem nutrient cycling [17]. Most ECM fungal taxa produce a diversity of extracellular and cell wall-bound hydrolytic and oxidative enzymes that mobilize the release of smaller organic molecules (potential C, N, or P sources) from soil organic matter (SOM) [17], including polyphenol–protein complexes. Studies have revealed substantial interspecific differences in ECM enzymatic activities [18–28]. Such differences can be predicted in part by ECM life history strategies. Key among these is the abundance and morphology of external hyphae among ECM taxa, also referred to as "hyphal exploration strategy" [29]. Each exploration type can vary in its capacity for enzymatic mobilization, uptake, and transfer of nutrients to the host. For example, long-distance exploration types form extensive networks of hyphae and rhizomorphs, are typically abundant in N-limited soils, and appear to be specialized in N-acquisition from complex organic substrates. Conversely, contact-types are more frequently detected in mineral soils, show lower proteolytic capabilities, and access inorganic N sources that are more readily assimilated [6,29]. Differences in ECM taxa and their exploration strategies could therefore have an impact on tree nutrition through changes in their morphological and functional (enzymatic) traits.

Biotic (host C allocation) and abiotic (climate, soil nutrients, pH) factors can also influence ECM enzyme activities. Ectomycorrhizal fungi may respond to shortages in host C allocation by up-regulating the activity of enzymes used to obtain labile carbohydrates [13], while changes in the relative availabilities of N and P are known to modify the activity of extracellular N- and P-mobilizing enzymes. For example, N fertilization could accelerate the degradation of easily decomposable litter and reduce the activity of extracellular ECM enzymes targeting recalcitrant litter with high levels of lignin and complex organic forms of N [24–28]. Both outcomes may reflect the stimulation or repression of different sets of enzymes. In addition, the activity of P-mobilizing enzymes has been shown to increase following N fertilization as a way to offset plant P demand [7]. However, neutral and negative effects have also been noted [7]. Any changes in the activity of these enzymes may reflect alterations in the ECM community and the physiological functioning of their constituent species. Such shifts, in concert with declines in ECM root colonization following N fertilization [30], could feedback to impact plant nutrient uptake.

Much of our knowledge of N enrichment effects on ECM communities has been obtained from studies in North American and European forests. However, forests in China have also experienced increasing inputs of anthropogenic N deposition owing to rapid economic development, urbanization, and intensified agricultural activities [31–34]. In the forests of south-central China, dry N deposition contributes ~24 kg N ha^{-1} $year^{-1}$ (as NH_4-N) derived from power generation, traffic, and intensive fertilizer applications. In this region, forest plantations are comprised of a fast-growing non-native pine (*Pinus elliottii*, slash pine) that was planted to ameliorate land degradation. Although ECM fungi are critical for the growth and nutrition of *Pinus* species, it is unclear how interactions between N enrichment and a non-native pine could feedback to alter ECM communities and their ecosystem function in soil nutrient dynamics [35,36].

In this study, we examined the link between the ECM community structure and functioning under ambient N-deposition and following N fertilization in a *Pinus elliottii* (slash pine) plantation. To put our study in context with previous research, we first examined the effect of ambient N deposition and N fertilization on soil fertility and ECM community composition, diversity, and root colonization. Next, we tested the capacity of ECM fungal colonized root tips to produce an oxidative enzyme involved in the degradation of recalcitrant plant residues (phenol oxidase), and hydrolytic enzymes for organic N (protease) and P (phosphomonoesterase) mobilization. We used these results to address two questions:

(1) Are there parallel shifts in ECM fungal community structure and functioning with increasing N availability?; (2) Are there ECM fungus species-specific differences in N and P enzyme activity, and if so, do these changes reflect soil fertility or other factors (e.g., hyphal exploration)?

2. Materials and Methods

2.1. Study Site

Our study was undertaken in 35-year-old slash pine (*P. elliottii*) stands at the Hunan Forest Botanic Garden (113°02′03′ E, 28°06′07′ N), Changsha city, Hunan Province, China. The climate is typical subtropical humid monsoon with a mean annual temperature of 17.4 °C and annual precipitation of 1549 mm, most of which occurs between April and October. The soil is classed as an Alliti–Udic Ferrosol (equivalent to Acrisol; IUSS Working Group WRB, 2006), which is generally a clay loam red soil developed from slate and shale parent rock. These soils are acidic (pH = 4.14–4.21 [31]) with deficiencies of SOM and P, and high levels of Fe and Mn (Table 1).

Nine plots (each 10 m × 10 m) enclosing at least five pine trees were established in June 2010 using a completely randomized design. A 3 m buffer zone was installed around each plot to prevent N fertilizer contamination among plots. Three plots were randomly allocated to each of three N fertilizer levels: control (ambient N, no fertilization), low (50 kg N ha^{-1} $year^{-1}$), or high nitrogen (300 kg N ha^{-1} $year^{-1}$). The fertilization rates represent the expected input from N deposition in the near future (low N), as well the potential long-term cumulative N inputs from atmospheric deposition (high N [31–34]). Nitrogen fertilization treatments were applied twice a year (January, June) for three years as a solution of NH_4NO_3 uniformly sprayed across the plot. Control plots were sprayed with a similar volume of deionized water.

Table 1. Mean levels of soil nutrients in control and N-fertilized plots. Data represents mean with the standard error in parentheses.

	N Fertilization Level		
Soil nutrient	Control (n = 36)	Low (n = 36)	High (n = 36)
Organic matter (g kg^{-1})	26 (2) ab	21 (2) b	28 (2) a
Total N (g kg^{-1})	1.34 (0.1) a	1.26 (0.1) a	1.48 (0.1) a
Organic C:N	12 (1) a	10 (1) a	12 (2) a
Available N (μg g^{-1} soil)	26 (1.9) a	25 (1.3) a	27 (1.7) a
Total P (μg g^{-1} soil)	110 (4) b	122 (5) a	115 (4) b
Available P (μg g^{-1} soil)	3.5 (0.1) c	4.6 (0.2) a	3.0 (0.1) b
N:P	12 (1) a	11 (1) a	13 (1) a
K (μg g^{-1} soil)	69 (6.8) a	64 (2.7) a	41 (1.8) b
Ca (μg g^{-1} soil)	193 (18) a	257 (15) a	216 (16) a
Mg (μg g^{-1} soil)	896 (22) b	1051 (20) a	977 (23) a
Fe (μg g^{-1} soil)	15,986 (266) b	16,273 (140) b	17,034 (169) a
Mn (μg g^{-1} soil)	80 (7) b	101 (6) a	114 (6) a
CEC (cation exchange capacity)	20 (1.6) a	18 (1.2) a	19 (1.8) a

Means within rows with the same letter do not differ significantly at $p < 0.05$ by Tukey's Honestly Significant Difference (HSD) test.

2.2. Sample Collection

Three slash pine trees in each plot were sampled for ECM fungi in August 2013. Four soil cores (10 cm diameter, 15 cm deep), representing one core from each of the cardinal directions, were collected for each tree (total n = 12 cores per plot). Soil cores were placed in individual plastic bags and then stored at 4 °C until processing (within 7 days). A sub-sample of soil from each core was sieved to 2 mm and analyzed for soil N, P, K, Ca, Mg, Fe, Mn, and organic matter (OM, organic C × 1.724) at the National Engineering Laboratory for Applied Technology of Forestry and Ecology in South China. Analytical methods are detailed in supporting materials (Supplementary S1). Soil cation exchange

capacity (CEC) was calculated as the sum of exchangeable cations (K, Ca, Mg) on an equivalent basis. The remaining soil in each core was sieved over 2 and 0.25 mm sieves. Fine roots collected on each sieve were gently washed to remove adhering soil and pooled for each tree. To quantify ECM root colonization, six roots (~10 cm long) per tree were randomly selected and examined by counting ECM root tip numbers. Every root tip was examined under 40× magnification for the presence of ECM colonization (i.e., turgid, swollen root tips with a well-developed mantle), and then sorted into morphological categories based on mantle color and texture, and the morphology of external hyphae on the root tip [5,29] and the publicly available database DEEMY (http://www.deemy.de/).

2.3. Enzyme Assays

Three extracellular enzymes: acid phosphatase (AP, EC 3.1.3.2), protease (PRO, EC 3.4.23), and phenol oxidase (PO, EC 1.14.18.1) were assayed. The enzyme substrates were 5 mM p-NP (*p*-nitrophenyl phosphate) for AP; 25 mM L-DOPA (L-3, 4-dihydroxyphenylalanine) for PO; and a general proteolytic substrate, Azocoll® (<50 mesh, Calbiochem-Behring Corp. La Jolla, CA, USA) for PRO [37]. All substrates were prepared in 50 mM sodium acetate-acetic acid buffer (pH 5.0, Sigma Chemical, Co. St. Louis, MO, USA). Root tip enzyme activity was assayed using the high-throughput microplate method described by Pritsch et al. [20], with one modification. Instead of a porous microplate, we constructed strips of eight plastic microscopy capsules (diameter 5 mm, height 15 mm) that were perforated at the base (Leica catalog no. 16702738); each strip of capsules fit into eight wells of a 96-well microplate.

For each classified morphotype per tree (generally 3–5 morphotypes), seven active root tips of the same diameter were selected and trimmed to 4 mm length, and one root tip was then placed in an individual capsule. Using ECM root tips with trimmed ends may have introduced intracellular enzymes into the analyses. However, hydrolytic (e.g., AP) and oxidative (e.g., PO) enzymes tend to be unaffected by cell lysis [38], and intracellular phosphatase tends to have an alkaline pH optima [39]. Non-colonized roots were not assayed for enzyme activity, as these frequently host saprophytic fungi with similar enzyme capacities as ECM [37].

Strips of capsules containing root tips were incubated in microplate wells each containing 100 µL of an individual substrate for 1 h at 37 °C (AP, PO) or two-hours (PRO) in the dark. As a control, root tips were bathed in buffer for each incubation step. At the end of each incubation period, capsules were removed from the microplate, and enzyme reactions terminated by the addition of 100 µL of sterile water (PRO, PO) or 100 µL NaOH (pH > 10, 0.2 M; AP) to each well, and absorbance measured at 405 nm (AP), 520 nm (PRO), or 450 nm (PO). After the assays were completed, each root tip was removed from the well, rinsed in deionized water, and three root tips of each morphotype per tree were frozen at −80 °C for later molecular identification, while the remaining root tips were dried to constant weight (65 °C). All measured enzyme activities were calculated per gram dry weight root per hour, and averaged among the weighted root tips of the assay group.

2.4. Identification of ECM Fungi on Root Tips

DNA from ECM root tips was extracted using DNeasy Plant Mini Kit (Qiagen SA, Coutaboeuf, France) following the manufacturer's instructions. Genomic DNA was amplified using the ITS1-F/ITS4 primer pair [40,41], after which the PCR products were visualized by gel electrophoresis. Samples with single bands were prepared for sequencing using ITS-4 and Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA), and analyzed on an Applied Biosystems 3130xl Sequencer. PCR products with multiple bands were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Successfully cloned colonies were amplified using primer pair M13F/M13R, screened using gel electrophoresis for the appropriate sized PCR products, and sequenced.

Sequences were manually aligned and edited in CodonCode Aligner 4.2.4 (CodonCode, Co. Centerville, MA, USA) and sequence homologies determined using the Basic Local Alignment Search Tool algorithm (BLAST v2.2.29 [42]) or the UNITE database v7 [43] for operational taxonomic unit

(OTU) clustering. A root tip sample was considered a species match to a database taxon if their sequences had 97% or greater similarity and were aligned over at least 450 base pairs. If no match could be made, a taxonomic placement was made by aligning the sample sequence with representative sequences of fungi from the major ECM clades. The same criteria for BLAST species matching were used to assign a taxonomic identity. Sequences from this study have been deposited in NCBI with access numbers KP866117–KP866136.

2.5. Data Analyses

All analyses—except species richness and diversity estimates—were completed in R 3.1.2 (R Project for Statistical Computing; http://www.R-project.org) with the "vegan" package [44]. Differences in the levels of soil nutrients between each treatment were analyzed using a one-way analysis of variance (ANOVA), followed by pairwise comparisons using the Tukey's Honestly Significant Difference (HSD) test. Data sets were cube root transformed before ANOVA to meet the assumptions of normality. Estimates of ECM species richness (Mao Tau, $Chao_2$, $Jackknife_2$) and diversity (Shannon-Wiener; Simpson) were calculated for each treatment in EstimateS using 50 randomizations with replacement [45]. Differences in ECM species richness and diversity and ECM root colonization among treatments were analyzed using one-way ANOVA and Tukey's HSD.

The effect of N fertilization on ECM community composition was tested using non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarities followed by permutational analysis of variance (PERMANOVA) to test for ECM compositional differences between treatments (999 permutations). We then used vector fitting to the NMDS ordination to determine the effects of soil nutrients; significance values were generated using 999 random permutations. The effect of N fertilization on ECM community enzyme activity was similarly analyzed using NMDS and PERMANOVA.

Potential enzyme activities were used to calculate the relative contribution of each ECM species to community enzyme activity as well as changes in enzyme stoichiometry (also known as enzyme acquisition ratios) between N fertilization treatments. The relative contribution (RC) of each ECM species to community enzyme activity in each N fertilization treatment was calculated as

$$(\text{activity}_{species} \times \text{root tip abundance}_{species})/\text{total activity of the ECM community}$$

where *species* represents an individual ECM species, and total activity of the ECM community was calculated as [13,46]

$$\sum (\text{activity} \times \text{root tip abundance})_{species}.$$

Enzyme data were also used to calculate enzyme stoichiometry (also known as enzyme acquisition ratios) in each N fertilization treatment as PRO:AP, PO:PRO, and PO:AP.

Differences in potential enzyme activity (AP, PRO, PO) and stoichiometry (PRO:AP, PO:PRO, PO:AP) between N fertilization treatments and between ECM exploration and mantle types in response to N fertilization treatments were analyzed using mixed-effect ANOVA with N-treatments (control, low N, high N) and mantle type (hydrophilic, hydrophobic) or exploration type (contact, short-, and medium-distance) [5,29] as fixed effects and plots as random effects. ANOVA were followed by comparisons using Tukey's Honestly Significant Difference (HSD) test for significant variables. Relationships between the enzyme activity and ECM root colonization or soil factors were tested using Spearman's r correlation test. The relative enzyme activity in each ECM species was compared and analyzed against the community average in each enzyme and N fertilization level using *t*-tests. Data sets were square root (enzyme potential) or arcsine square root transformed (root colonization, enzyme ratios) before analyses to meet the assumptions of normality.

3. Results

3.1. Soil Fertility

Nitrogen fertilization resulted in significant increases in levels of Mn and Mg, and declines in available P and K relative to control plots. Levels of soil OM and Fe were highest in the high N fertilization treatment (Table 1). However, there was no significant effect of N fertilization on total or available soil N, C:N, total P, Ca, or CEC (18–20 ± 1.5 Meq).

3.2. ECM Community Composition and Diversity

Root tips (742; Control: 294; Low N: 224; High N: 224) were sorted into morphological groups, and from these tips, and we submitted 318 root tips for molecular analysis and successfully recovered 350 sequences, including several clones from double bands PCR products. Using a 97% sequence similarity cut-off, we identified 257 sequences representing 24 unique OTUs (hereafter referred to as species) that were ECM (Table 2). The remaining fungi were taxa traditionally considered as saprotrophic (*Paecilomyces, Sphaeropsis, Penicillium*) or of uncertain mycorrhizal status (e.g., *Basidiodendron*, *Mycena*).

Members of the Helotiales (Ascomycota) and Thelephoraceae (Basidiomycota) dominated the ECM community. Many ECM species were detected in both the control and N-fertilized plots, including *Tylospora* (Atheliaceae), *Lactarius* (Russulaceae), and members of the Ascomycota (e.g., *Helotiales* spp.). Nine ECM fungi were absent in control plots (e.g., *Scleroderma*) while an additional three taxa were N-sensitive and recovered only in the control plots (e.g., *Cenococcum* sp. 2). Levels of species richness and diversity did not differ significantly between N-fertilized and control plots (Table 3). Trees in Control (42 ± 9%) and Low N plots (44 ± 10%) showed significantly higher levels of ECM root colonization relative to high N plots (21 ± 6%; $p = 0.039$).

NMDS showed that ECM communities from N fertilization treatments were separated from one another in ordination space (Figure 1a; $p < 0.05$ for PERMANOVA among all three treatments). ECM community composition was significantly correlated with mineral nutrients (Mg, K, P) and organic C, as these resources decreased (mineral nutrients) or increased significantly (soil organic matter) in high N plots (Table 1).

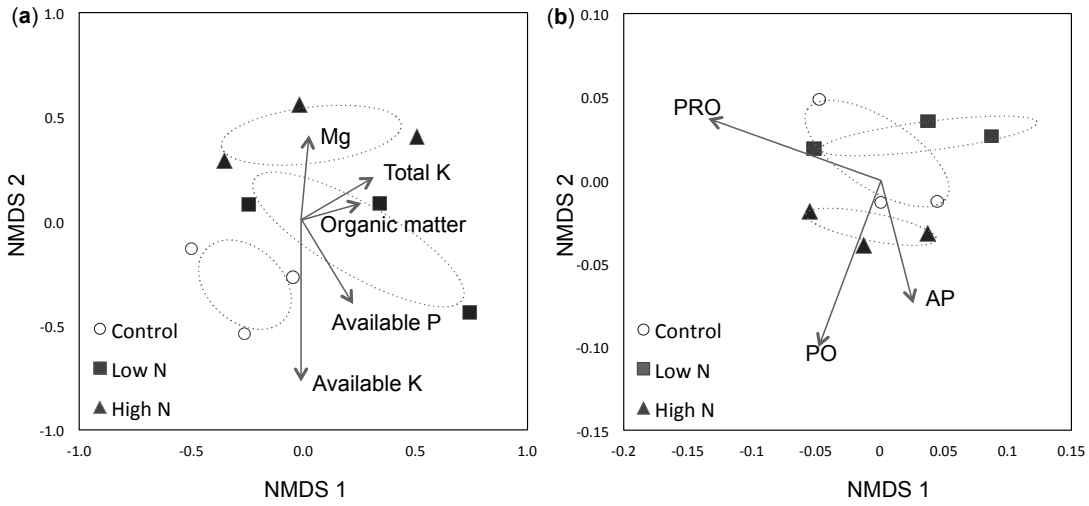

Figure 1. Non-metric multidimensional scaling (NMDS) ordination of ectomycorrhizal (ECM) fungal communities in control and N-fertilized plots based on: (**a**) the ECM root tip community, (**b**) the activity of extracellular enzymes. Each point represents the fungal community composition in each plot. Significant environmental variables are shown: P-phosphorus; K-potassium; Mg-magnesium. AP: acid phosphatase; PO: phenol oxidase; PRO: protease.

Table 2. Identification of ectomycorrhizal fungal operational taxonomic unit (OTU)s associated with *Pinus elliottii* growing at the study site in Hunan botanic garden, China. Species for group level enzyme analysis were tagged in bold font.

OTU	Accession Number [a]	Closest Blast Match in Genbank [b]	Query/Aligned length (bp) (similarity %) [c]	Closest UNITE Species Match	No. Of root tips/Frequency [d]		
					Control	Low N	High N
***Tylospora* sp.**	KP866117	HM189733 Corticiaceae sp. BB-2010	632/637 (99)	SH192265.07FU	29/2	13/1	21/3
***Atheliaceae* sp.**	KP866118	AB839405 Uncultured ECM fungus	475/512 (93)	SH193510.07FU	0	1/1	0
***Cenococcum* sp.1**	KP866119	JQ347051 Uncultured *Cenococcum*	567/571 (99)	SH214459.07FU	7/1	28/2	0
***Cenococcum* sp.2**	KP866120	JX456699 Uncultured fungus	486/497 (98)	SH214466.07FU	14/3	0	0
***Helotiales* sp.1**	KP866121	KF007259 Uncultured ECM fungus	608/618 (98)	SH214286.07FU	18/4	24/5	40/6
***Helotiales* sp.2**	KP866122	AB571492 Uncultured ECM fungus	637/639 (99)	SH023418.07FU	16/2	7/1	15/2
***Helotiales* sp.3**	KP866123	AB769894 Uncultured Helotiales	550/551 (99)	SH201717.07FU	14/2	7/1	27/2
Helotiales sp.4	KP866124	HM208727 Fungal sp. Phylum141	560/562 (99)	SH196495.07FU	1/1	0	0
Helotiales sp.5	-	FN397286 Uncultured fungus	499/536 (93)	SH211375.07FU	0	0	1/1
Lactifluus parvigerardii	KP866125	JF975641 *Lactifluus parvigerardii* XHW-2011	571/574 (99)	SH012454.07FU	7/1	7/1	7/1
Phialocephala fortinii	KP866127	KF313098 *Phialocephala* sp. YJM2013	562/564 (99)	SH204999.07FU	1/1	0	2/1
***Russula* sp.1**	KP866128	JX457011 Uncultured fungus	683/696 (98)	SH017121.07FU	0	7/1	7/1
Russula virescens	KP866129	KM373243 *Russula crustosa*	669/716 (93)	SH179774.07FU	0	1/1	0
***Russula* sp.2**	KP866130	AB597671 Fungal sp. JK-02M	580/582 (99)	SH017122.07FU	0	7/1	0
Scleroderma yunnanense	KP866131	JQ639046 *Scleroderma yunnanense* XEX-2012	584/588 (100)	SH189277.07FU	1/1	0	1/1
Scleroderma citrinum	KP866132	AB769913 Uncultured *Scleroderma citrinum*	561/569 (99)	SH008294.07FU	0	0	1/1
Sebacinaceae sp.	KP866133	KF000673 Uncultured *Sebacina* clone	628/657 (96)	SH214656.07FU	0	0	1/1
Thelephora terrestris	KP866134	KJ938034 Uncultured fungus	677/706 (96)	SH184510.07FU	7/1	14/2	0
***Tomentella* sp.1**	KP866135	AB769927 Uncultured Thelephoraceae	663/665 (99)	SH177859.07FU	14/2	7/1	0
***Tomentella* sp.2**	KP866136	JX456648 Uncultured fungus	705/706 (99)	SH189353.07FU	37/5	2/1	7/1
Ascomycota sp.1	-	EF619719 Uncultured Orbiliaceae	525/612 (86)	SH015725.07FU	2/2	0	1/1
Ascomycota sp.2	-	KP323399 Uncultured fungus	208/226 (92)	SH469383.07FU	2/2	0	0
Ascomycota sp.3	-	KP689247 Ascomycota sp.	618/625 (99)	SH181934.07FU	0	1/1	3/3
Meliniomyces sp.	-	FJ440931 Uncultured ectomycorrhiza	553/574 (96)	SH181081.07FU	0	2/1	0

[a] Accession numbers of sequences from this study deposited in NCBI; -, sequence not deposited. [b] Closest matched BLAST results with informative species and genera. [c] Similarity values were computed from the percent match between the portion of the query aligned and its reference sequence. [d] Frequency refers to presence/absence of ECM in each focal tree and treatment (n = 9 trees per treatment).

Table 3. Estimators of operational taxonomic unit (OTU) richness and diversity of ECM fungi in different treatments. Data represents mean with the standard error in parentheses per plot; n = 3 (3).

Sites	Rarified Species Richness		Estimators of Expected Total Species Richness		Diversity Indices	
	Mao Tau	Mao Tau (50 runs mean)	Chao 2	Jackknife 2	Shannon's H'	Simpson's 1/D
Control	5.67 (1.67)	6.41 (1.58)	8.54 (1.20)	8.78 (2.19)	2.79 (0.96)	5.89 (1.31)
Low N	4.33 (1.45)	4.5 (1.08)	5.1 (1.24)	6.28 (1.53)	1.35 (0.25)	4.06 (0.98)
High N	4.33 (0.67)	5.1 (0.37)	5.99 (0.32)	6.80 (0.12)	2.84 (1.40)	4.59 (0.43)
p [a]	0.729	0.508	0.119	0.52	0.519	0.444

[a] p-value for effect of fertilizer treatment.

3.3. Extracellular Enzyme Activity

Although we measured enzyme activities in 742 individual ECM root tips, we used results from only those ECM taxa with well-supported molecular identities in the statistical and comparative analyses. Consequently, we used the results from 426 root tips (Control: 166; Low N: 126; High N: 134), which represented eight ECM fungal taxa. These taxa were recovered in sufficient numbers across all treatments so that at least three individual root tips of each species from each treatment could be assayed (Table 2, species names in bold). Overall, High N fertilization resulted in a significant increase in PO activity (p = 0.004 for ANOVA) and decrease in AP activity (p = 0.035 for ANOVA), but had no significant effect on PRO activity (p = 0.795 for ANOVA) (Figure 2).

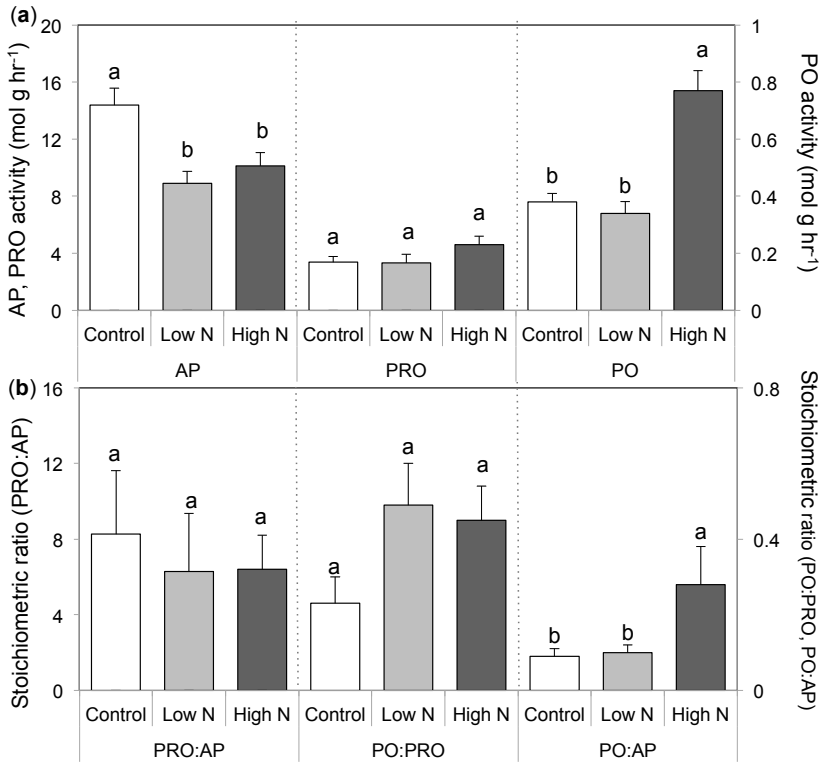

Figure 2. Mean levels of (**a**) acid phosphatase, protease, and phenol oxidase activity; and (**b**) enzymatic stoichiometric PRO:AP, PO:PRO, and PO:AP in ectomycorrhizal communities in control and N-fertilized plots. Vertical bars indicate the standard error of the mean; for each enzyme (or ratio), columns with the same letter do not differ significantly at $p < 0.05$ based on Tukey's HSD test.

Potential AP and PO activity were correlated with levels of soil P and K. Potential AP activity was negatively correlated with total ($r = -0.086$, $p = 0.023$) and available soil P ($r = -0.077$, $p = 0.044$), and total K ($r = -0.082$, $p = 0.030$). Potential PO activity was positively correlated with soil P ($r = 0.154$, $p < 0.001$) and available ($r = 0.109$, $p = 0.004$) and total K ($r = 0.101$, $p = 0.008$). There was no relationship between PRO and any tested soil factor. Potential enzyme activity was also correlated with root tip colonization in low ($r = 0.611$, $p = 0.001$) and high N plots ($r = 0.445$, $p = 0.029$), but not in control plots ($r = 0.229$, $p = 0.281$).

The NMDS ordination showed significant differences in ECM community between N fertilization treatments based on enzyme activity (Figure 1b; $p = 0.001$ for PERMANOVA). This pattern was driven by PO activity, because high N plots had significantly higher levels of PO activity than control or low N plots (Figure 2).

Enzyme activity varied significantly between ECM species (Figure 3; Figures S1 and S2). Overall, *Helotiales* sp.1, and Thelephoraceae were the largest contributors to ECM community enzyme activity in most enzyme systems and N fertilizer treatments, and relative enzyme activity in these taxa was always significantly greater than the community mean (Figure 3a–c). Certain taxa were restricted to a specific enzyme system or N fertilization treatment (e.g., *Lactarius* for PO in N plots; Figure 3c), but for the most part, ECM taxa varied in their relative activity among enzyme systems and N fertilization treatments. For example, the activity of Atheliaceae has greater contribution to the community for AP in high N plots (Figure 3a), and for PRO in control plots (Figure 3b). There was also strong inter-specific variation in relative enzyme activity among the three species of Helotiales.

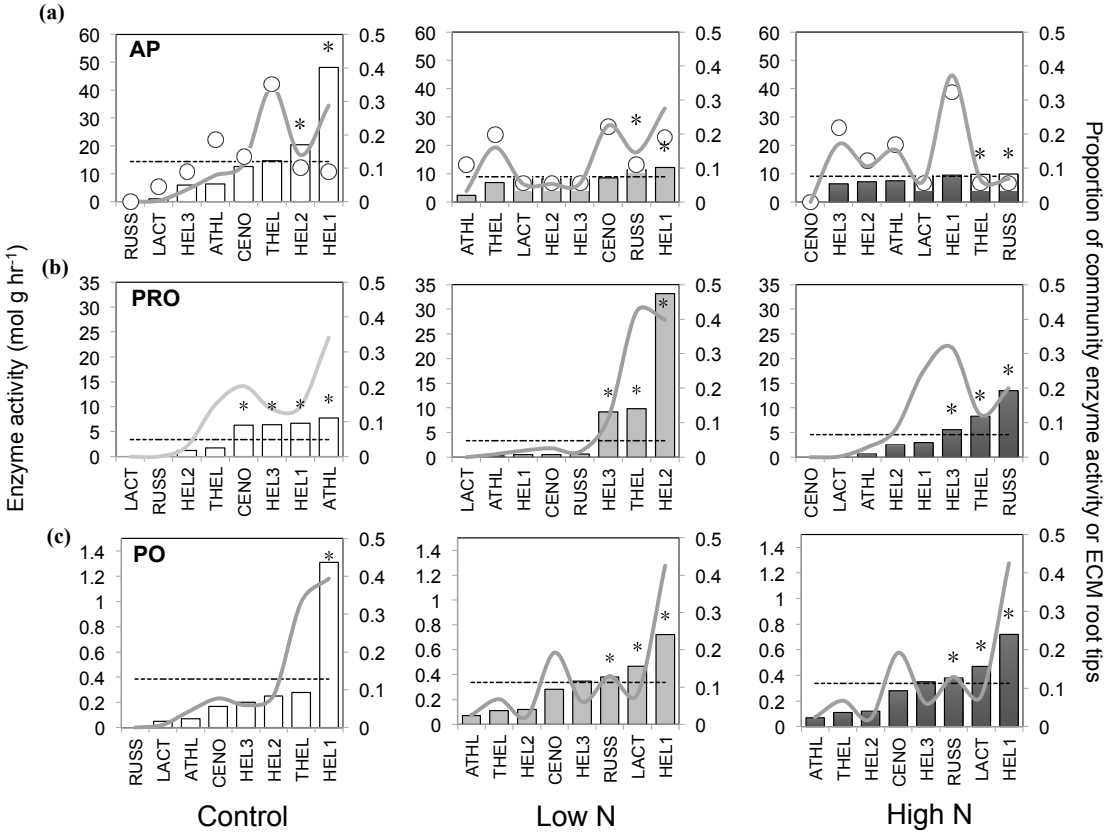

Figure 3. Levels of ectomycorrhizal (ECM) root tip relative abundance (open dot) and the relative contribution (grey curve) of individual ECM group to overall ECM community activity of (**a**) acid phosphatase (AP), (**b**) protease (PRO), and (**c**) phenol oxidase (PO) in response to N fertilization. Within each panel for enzyme and N fertilization treatment, broken lines within each panel indicate the mean community level of enzyme activity. Columns denoted with an asterisk (*) denote ECM species in which enzyme activity was significantly greater ($p < 0.05$) than the mean community value.

Fertilization altered the stoichiometry of enzyme activity by significantly increasing the depletion of AP (P-cycling) relative to PO and PRO (C-, N-cycling, respectively; Figure 2b). In N-fertilized plots, contact (Russulaceae) and medium-fringe (Atheliaceae) types showed a significant increase in the PRO:AP ratio, whereas short (*Cenococcum* and Helotiales) and medium-smooth (Thelephoraceae) types showed a decline (Figure 4a). Conversely, the PO:AP ratio increased in medium-smooth types and declined in medium-fringe types (Figure 4c). The PO:PRO ratio declined significantly (contact, medium-distance types) or did not differ significantly between treatments (short-distance; Figure 4b). Although the PO:AP ratio was higher in ECM species with hydrophilic mantles (groups of *Cenococcum*, Thelephoraceae, Russulaceae, and *Helotiales* spp.) compared to those with hydrophobic mantles (group of *Atheliaceae* spp.) (Figure 5), this difference was not statistically significant ($p = 0.223$). Similarly, PO:AP activity was greatest in high N plots, but again, this difference was not statistically significant (hydrophilic $p = 0.746$; hydrophobic $p = 0.391$).

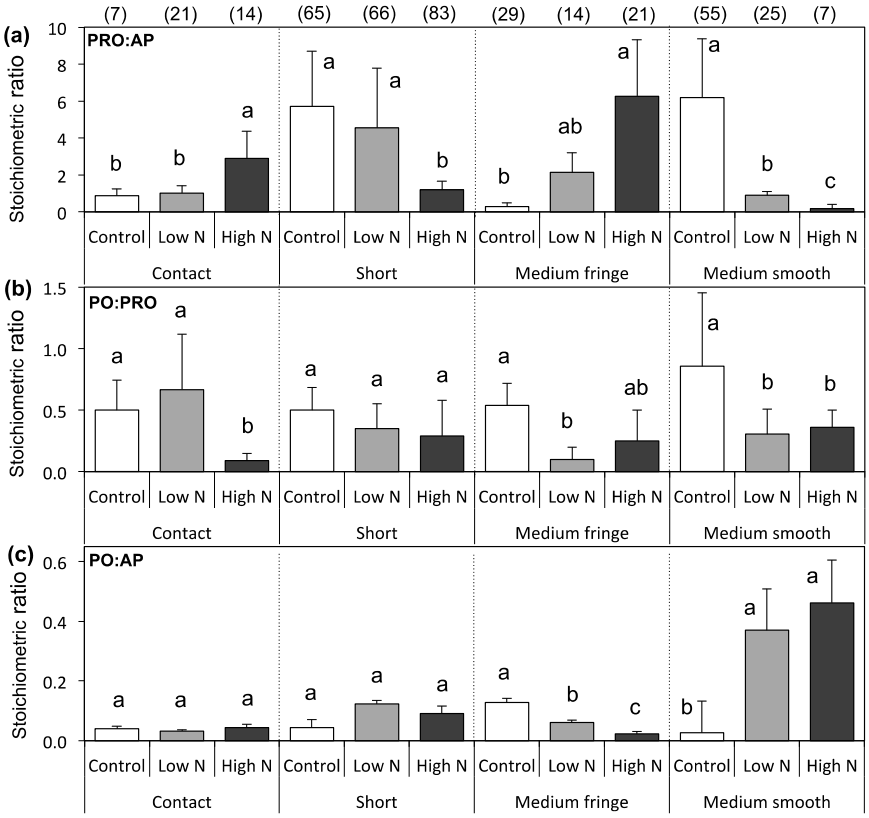

Figure 4. Mean (**a**) PRO:AP, (**b**) PO:PRO, and (**c**) PO:AP in ectomycorrhizal communities in control and N-fertilized plots based on hyphal exploration strategy. For each enzyme and hyphal strategy, columns with the same letter do not differ significantly at $p < 0.05$ based on Tukey's HSD test. Root tip number per exploration type is listed on the top panel.

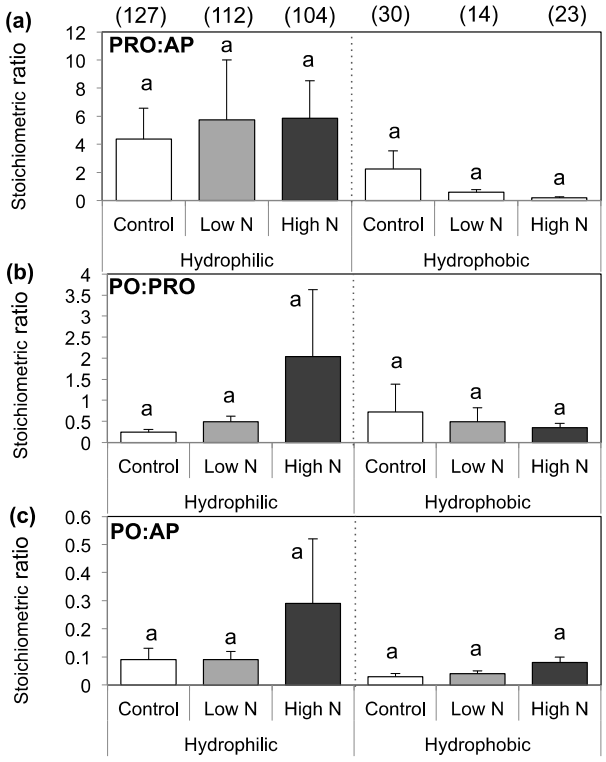

Figure 5. Mean (**a**) PRO:AP, (**b**) PO:PRO, and (**c**) PO:AP ratios based on mantle structure in control and N-fertilized plots. Mantle types with the same letter did not differ significantly at $p < 0.05$ based on Tukey's HSD test. Root tip number per hydrophobicity type is listed on the top panel.

4. Discussion

Despite the high levels of ambient N deposition, we found that three years of N fertilization produced shifts in ECM fungal community structure, and variously altered the abundances and potential enzyme activities of ECM fungal taxa as well as the stoichiometry of extracellular enzymes involved in P acquisition (AP) and lignin degradation (PO) (Question 1). Such shifts were related to some extent to changes in nutrient availability (P, K, OM) created by N fertilization (Question 2) and differences in enzymatic stoichiometry among ECM hyphal exploration types.

ECM fungal richness, diversity, and evenness were not significantly different across N fertilization treatments. These results reconcile with earlier studies in oak [30] and spruce forests [3,47–49], but seemingly contradict the majority of studies that show a rapid and substantial decline in ECM fungal diversity following N fertilization e.g., [6,9–11,30] and increasing soil N levels along natural gradients of productivity [50]. This result may reflect the short period (three years) over which the plots were fertilized [6,51] or (more likely) that high levels of ambient N deposition may have pre-empted any effects of the experimental N additions. Indicative of this condition, we found that even high N fertilization (300 kg N ha^{-1} $year^{1}$) did not significantly increase the levels of total and available N over those in non-fertilized plots, and the levels of available N across all plots (25–27 mg N kg^{-1} soil) exceeded those documented in an N-saturated pine forest in southern China [31]. Further, N additions are expected to alter the quantity and quality of resources (i.e., litter C:N) so as to influence soil C:N. However, we found that the soil C:N ratio did not differ significantly between N-fertilized and control (ambient N) plots. Taken together, these findings suggest the soil was N-saturated so that any new input of N (i.e., fertilizer) was likely leached from the system.

Even so, N fertilization was sufficient to alter ECM fungal community composition. The major genera found in our study (e.g., *Cenococcum*, *Thelephora*, *Tomentella*, *Sebacina*, *Inocybe*, *Russula*) are ubiquitous and dominant components in ECM communities. The most obvious indicators of

N-enrichment were the increased abundance of ECM taxa considered to be nitrophilic (*Russula*, *Tomentella*), the loss of N-sensitive fungi (*Cenococcum*), and the exclusion of protein-N-utilizing ECM species (*Suillus*, *Tricholoma*, *Cortinarius*, *Piloderma* [5,30,48,49]). These results also reflect the shift to an ECM fungal community comprising contact- (Russulaceae) and short-range exploration types (*Cenococcum*, Helotiales), and certain medium-distance types (Thelephoraceae, Atheliaceae). Our findings are in agreement with the well-documented patterns of ECM community change and species' abundances in response to N-deposition or fertilization noted elsewhere [8,11,30,49]. Unlike these former studies, soil K, P, and OM were the primary soil factors associated with changes in ECM fungal community structure, not N. This distinction could reflect the difference in major N input as NH_4-N versus NO_3-N in our systems. Nevertheless, our results show that imbalances of mineral nutrients will become increasingly important controls over ECM fungal community structure in ecosystems with chronic N deposition or saturation.

Forest soils can become increasingly deficient in K and P as a result of atmospheric N deposition or N fertilization, as occurred in our study plots [1]. However, ECM fungal communities respond differentially to deficiencies of K versus P. For instance, deficiencies of soil K reduce fine root growth and ECM colonization by impeding sucrose export from the leaves to the roots [52,53], but have no effect on ECM community composition [54]. Conversely, P-deficiency tends to increase C allocation belowground to stimulate root and ECM fungal growth and AP activity [55]. In our study, the decrease in ECM root colonization with N fertilization is consistent with K deficiency. In contrast, the decline in AP activity and changes in enzyme stoichiometry toward an increase in C-acquisition (PO) relative to P (AP) with N fertilization was a clear contradiction of previous studies [56]. The precise reason(s) for this response are beyond the scope of this study. However, it is possible that AP activity on ECM root tips may have been low relative to the activity in the foraging extraradical mycelium in other parts of the soil profile (i.e., functional compartmentalization [22,55]) or that there were changes in the physiological competence of ECM root tips owing to reductions in C-allocation from the host [52,53].

N fertilization also resulted in ECM communities with high PO activity. N-fertilized communities showed a net up-regulation in PO activity as individual ECM fungi showed an increased capacity to degrade SOM. This is in general agreement with the widespread capacity of ECM fungi to oxidize SOM [21,57,58] and the generally positive effect of soil N on litter degradation by ECM fungi [2,7]. Such increases in PO activity have been interpreted as a mechanism by which ECM acquire labile carbohydrates when host C allocation is low [22,58], or that ECM generally degrade polyphenolic-rich compounds as a consequence of mining for nutrients [20,22,59]. However, these mechanisms are not mutually exclusive, and may vary in their importance depending on the extent of N-enrichment and/or mineral nutrient deficits. Further studies are thus needed to determine the precise role(s) of each mechanism in this system. Even so, the positive relationship between PO and AP activity suggests that ECM fungi may have been similarly prospecting for P. Similarly, the significant correlations between PO and soil P, K, and Mg content are consistent with the concept that ECM use oxidative enzymes such as PO to mobilize mineral nutrients locked within organic matter-mineral complexes [13,17,20,24,57].

Without examining the relationship between ECM enzyme levels and chemical modifications of SOM, we cannot exclude the possibility that ECM fungi also acquired labile C from degraded substrates. In our analyses of ECM enzyme stoichiometry and potential energetic trade-offs, only ECM fungi with the medium-distance smooth exploration type (Thelephoraceae) showed an increase in SOM-degrading potential relative to P acquisition. This is not surprising, since the Thelephoraceae tend to dominate in organic-rich soil horizons [18,60] and show some of the strongest potential activities of degradative enzymes [4,6,61,62]. In addition, studies have shown that when host plants allocate less C to their ECM fungi, either due to dormancy or defoliation, the relative activity of C-degrading enzymes increases significantly [17,55,63].

ECM fungi may also experience trade-offs between the energetic demands associated with root tip colonization versus the metabolically expensive production of enzymes. Under ambient N conditions (control), ECM species with lower levels of root colonization showed disproportionately high levels of

potential enzyme activity—an outcome that is consistent with energetic trade-offs [46]. Communities in N-fertilized plots displayed a different pattern, whereby root tip abundance was positively associated with enzyme activity (Figure 3). Such results are more typical of competitive interactions; that is, where certain ECM taxa pre-emptively colonized roots and utilized resources [64,65] owing to competition for spatial co-existence (a limited availability of root tips) or overlap in resource utilization (limited C availability). Alternatively, the abiotic conditions created by N fertilization may have selected for more closely-related taxa than expected by chance (environmental filtering [62]; e.g., members of the Russulaceae and Thelephoraceae dominated N-fertilized plots). However, additional studies are needed to distinguish between these explanations.

There is now substantial evidence that labile-C compounds derived from roots and fungal tissues are the dominant inputs into stable SOC stores [66]. Up to 30% of the total C assimilated by plants may be transferred to their ECM partner, meaning that any change in the symbiosis will have profound effects on SOC storage. Our results address two sets of dynamics relating SOC to N availability. The first focuses on the dynamics associated with increasing potential enzyme activity for SOM degradation and the release of labile C compounds. Such elevated inputs of labile C may concomitantly stimulate microbial activity and SOC decomposition (the "priming effect" hypothesis). These changes would be expected to be associated with a more rapid turnover of the SOC pool and changes in litter chemistry. However, more rapid decomposition is not synonymous with reductions in total SOC stocks if coupled with similar increases in litter inputs and soil C stabilization. Conversely, soil microbes may preferentially metabolize these pools of labile C over the mining of complex polymers (the "preferential substrate utilization" hypothesis). This effect, combined with the observations that N fertilization can inhibit SOC decomposition [1,2], may lead to increases in SOC following N fertilization.

The second focuses on ECM community dynamics. Carbon transferred to ECM fungi can also contribute to SOC storage if fungal tissues decompose more slowly than non-mycorrhizal roots [67] or if the fungal residues persist in long-term SOC stores. As a corollary, the tissue quality (C:N, melanin content) of fungal necromass can influence degradation dynamics [68]. Thus, shifts in ECM community composition and root colonization—and especially the differences in the extent of root tip colonization between different ECM fungal species and their tissue chemistry—could feedback to influence the quantity and quality of fungal residues entering the long-term C pool [69].

In the near future, this region in China is predicted to experience increasing N deposition. Based on our results, we can hypothesize that increasing inputs of N are likely to exacerbate soil P and K deficiencies, and compromise the capacity of ECM fungal communities to acquire mineral nutrients. In addition, variations in ECM community composition and species' functional plasticity could undermine the contributions of fungal residues to long-term SOC stores. These findings add to the accumulating evidence that increasing inputs of N—either from atmospheric deposition or fertilization—will continue to impact forest health and productivity by altering soil mineral resources and ECM community structure and functioning. Such prospects point to the need for a better understanding of the role(s) of ECM functional traits, their interactions with host plant growth and nutrient status, and their link to the relative soil nutrient availabilities. This multi-faceted approach is urgently needed to improve forest health and productivity in this region, as well as patterns of C stabilization and loss.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/9/3/99/s1; Supplementary S1: details of nutrient analyses. Figure S1: potential AP, PO, and PRO enzyme activity in ECM hyphal exploration types in response to N fertilization. Figure S2: potential AP, PO, and PRO enzyme activity in ECM hyphal hydrophobicity types in response to N fertilization.

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