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Enhanced abundance of generalist and litter saprotrophs explain increased tropical forest soil carbon with long-term nitrogen deposition

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Conflict of interest

Emma Sayer is a Senior Editor and Faming Wang is an Associate Editor of Functional Ecology, but both took no part in the peer review and decision-making processes for this paper.

Author contributions

Zhe Lu and Faming Wang conceived the ideas and designed methodology; Zhe Lu, Hui Li, Yao Chen, Guoming Qin, Jinge Zhou and Jingfan Zhang collected the data; Zhe Lu and Faming Wang analysed the data; Zhe Lu, Emma J. Sayer, and Faming Wang led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

Data supporting the results in this paper are available via the Supplementary file or archived for public access at https://doi.org/10.5281/zenodo.7927564 (Lu et al., 2023).

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Abstract

1. Nitrogen (N) deposition is a pervasive anthropogenic change that can alter the dynamics and storage of carbon (C) in tropical soils by altering the transformation of plant litter C to soil organic C (SOC). The response of fungi may be particularly important for explaining how N deposition affects SOC storage in tropical forests because they are the primary decomposers of plant litter. Fungi can be grouped into different ecological guilds, or functional groups, which often have contrasting responses to the same environmental change. However, little is known if shifts in fungal guilds and their functions explain increased tropical forest SOC storage under N deposition.
2. We addressed this knowledge gap by assessing the effects of long-term (six years) experimental N addition (+N) on fungal guilds, their functions, and SOC fractions in a tropical forest.
3. Total SOC, dissolved organic C (DOC), particulate organic C and fungal necromass C were all higher in +N soils. The fungal community in +N soils was dominated by generalist saprotrophs, and leaf saprotroph abundance increased nearly 93-fold compared to controls. These changes were accompanied by an increase in lignocellulolytic enzymes, indicating accelerated decomposition of recalcitrant litter C compounds. Chitosanase, which catalyzes synthesis of glucosamine, was 1.37× higher in +N soils than controls; correspondingly, fungal necromass C contributed 1.42× more to SOC. Greater abundance of saprotrophic enzymes involved in organic acid synthesis in +N soils was associated with higher concentration of Ca^{2+} and DOC.
4. We propose that N deposition increases SOC storage by enhancing saprotroph abundance and activity, whereby increased organic acid production supports fungal growth both directly and indirectly via the release of nutrients and DOC, resulting in greater inputs of necromass to the soil. Our findings highlight the importance of considering shifts in guild-level fungal abundance and function to investigate changes in SOC dynamics and storage.

Keywords

Fungal guilds, N deposition, plant litter, soil organic carbon, amino sugars, fungal community, tropical forest

Introduction

Increased nitrogen (N) deposition is a common human disturbance to natural ecosystems, especially in the tropics (Galloway et al. 2004, Liu et al. 2013). Nitrogen deposition in tropical forests influences plant growth (Lu et al. 2010), microbial activity (Cusack et al. 2011), and decomposition (Tian et al. 2019), with potentially far-reaching consequences for the turnover and storage of carbon (C) in biomass and soil. Globally, tropical forest soils store one-third of the C sequestered (almost 3000 Pg C), which is three times as much C as in Earth's atmosphere (Jackson et al., 2017). Small changes in such immense amounts of soil organic C (SOC) may accelerate climate change (Hicks Pries et al. 2017, Melillo et al. 2017). Interestingly, although elevated N inputs do not necessarily stimulate productivity in tropical forests (Cusack et al., 2011; Lu et al., 2018; Wright et al., 2018), N deposition has been linked to enhanced accumulation of SOC (Liu and Greaver 2010). The mechanisms by which N deposition influences SOC dynamics in tropical forests are therefore of broad relevance (Schulte-Uebbing and de Vries, 2018; Lu et al., 2021).

Soil fungi play a key role in forest soil C storage (Yang et al. 2022), but N deposition could substantially alter soil C sequestration by fungi in tropical forests. Fungi tend to have a high N requirement, because they dominate the decomposition of recalcitrant plant litter, which is composed primarily of cell wall materials such as carbohydrates (cellulose and hemicellulose) and aromatic C compounds (lignin; Berg and McClaugherty, 2020). These C-rich cell wall materials have a high C : N ratio, and decay slower than materials with a low C:N ratio (Baldrian 2006), suggesting that N limits the fungal decay of plant litter. Thus, N deposition could accelerate the litter decomposition rate by supplying decomposer fungi with additional N (Carreiro et al. 2000). Indeed, the transfer of plant litter C into soil organic matter by fungal decomposers (Sokol and Bradford 2019) could contribute to the increased SOC content that is often observed after experimental N addition (Liu and Greaver 2010). Moreover, fungal necromass is an important contributor to SOC, accounting for greater accumulation of SOC than bacterial necromass in many soils (Wang et al., 2021). The substantial contribution of fungi to SOC arises because they have greater living biomass than bacteria; and fungal cell compounds are more resistant to degradation (Liang and Balser, 2011; Liang et al., 2019). Increased fungal turnover with N deposition results in greater fungal necromass, which could further enhance SOC storage (Griepentrog et al. 2014). Thus, characterizing fungal-driven changes in SOC dynamics under N deposition is crucial for assessing the future C sequestration potential of tropical forests.

A better mechanistic understanding of fungal-driven changes in SOC dynamics might be gained by determining the responses of distinct fungal ecological guilds (also known as "functional groups'; Simberloff and Dayan 1991). These guilds coexist and often interact with each other (Devictor et al. 2008), but in many cases have contrasting responses to the same environmental change (Kubartová et al., 2012; Štursová et al., 2014; Clemmensen et al., 2015). Thus, analyses of the bulk fungal community could miss important ecological trends (Fierer et al. 2007, Moore et al. 2021), because they do not distinguish between ecological guilds and their distinct responses, which precludes identification of underlying mechanisms. The lack of information about fungal guild responses could explain why some studies measure increased total lignocellulolytic activity in soils in response to N deposition (Sinsabaugh and Moorhead, 1994; Carreiro et al., 2000; Knorr et al., 2005), whereas others observed reduced microbial activity and thus a slower decomposition rate of plant litter (Tian et al., 2019; Maaroufi et al., 2019; Lu et al., 2021). However, despite widespread changes in soil fungal community structure and functionality induced by N deposition, the potential impacts of N deposition on fungal guilds, and their linkages with soil C dynamics, have received limited research attention (Griepentrog et al. 2014, Moore et al. 2021). We propose that investigating changes in fungal guilds and guild-level functions could elucidate the mechanisms by which N deposition stimulates SOC storage in tropical forests.

Fungal guilds are generally divided into three trophic modes: saprotrophs, pathotrophs, and symbiotrophs, which can each be further subdivided into more specific trophic modes. Symbiotrophs comprise ectomycorrhizal, arbuscular mycorrhizal, endophyte, or lichenized fungi. Saprotrophs comprise dung, leaf, plant, soil, wood and undefined saprotrophs. Pathotrophs comprise animal and plant pathogens, parasites of fungi, lichens, and bryophytes, and Clavicipitaceous endophyte fungi (Nguyen et al. 2016). Although many fungi are specialists and therefore only belong to a single mode, some fungi can belong to two or all of these modes simultaneously and are thus considered generalists (Bebber and Chaloner 2022). Nearly half of all generalist fungi are saprotrophs with pathotrophic and symbiotrophic traits. Generalist fungi usually have greater capacity for resource uptake than specialists (Hu et al. 2022), because they can transiently occupy plant litter, sporulate rapidly in senescent and decomposed leaves, and infect more plant litters (Peršoh 2013). Saprotrophs usually increases in abundance following N fertilization (Moore et al., 2021), which could alleviate N deposition-induced C limitation (Treseder 2008) as they can incorporate litter C into soil. This mechanism could be especially important in the uppermost layers of tropical forests, where the litter of broadleaf tree species contains highly water-soluble C content, a valuable C resource to fungal decomposers (Berg and McClaugherty 2020). Consequently, we hypothesize that N deposition enhances accumulation of SOC in broadleaf tropical forest by increasing relative proportion of generalist saprotrophs in soil fungal communities. Hence, characterizing shifts in generalist *vs*. specialist fungal guilds could improve our understanding of changes in tropical forest SOC dynamics in response to N deposition.

Guild-level functions likely determine the fungal-driven change in SOC sequestration with N deposition, because microbial decomposition of plant litter is considered a key determinant of long-term SOC sequestration (Makkonen et al. 2012), in which fungal necromass plays a dominant role (Yang et al. 2022). Fungal-driven SOC accumulation is tightly bound to fungal catabolism and anabolism (Bradford et al. 2016). Fungal catabolism mainly refers to the decomposition of the aboveground plant litter by saprotrophs in surface soils and fungal anabolism mainly refers to the conversion of the plant-derived C to fungal biomass, and subsequently necromass (Sokol et al. 2019). Saprotrophs secrete extracellular enzymes to degrade complex organic substrates in leaf litter on the soil surface (Baldrian 2006). Accordingly, in the uppermost (litter) layer of the forest floor, the fungal community is dominated by leaf saprotrophs (Voříšková and Baldrian, 2013; Tedersoo et al., 2014). Soil saprotrophs and other root-associated fungi are responsible for decaying the residues of plant litter (Rosling et al. 2011, U'Ren and Arnold 2016). Thus, it is conceivable that increased SOC in response to N deposition could be the result of up-regulated activities or abundances of fungal guild-level enzymes involved in litter decomposition, or the incorporation of organic C, and other growth elements (e.g. nutrients) into fungal biomass and necromass (Janssens et al. 2010, Zeng et al. 2016).

Here, we exploited a long-term field experiment with six years of N-addition treatments in a tropical forest in southern China to advance our understanding of the mechanisms by which fungal guilds regulate soil C turnover in response to atmospheric N deposition in tropical forests. We predicted that changes in fungal communities and activity would explain enhanced SOC storage in N-fertilized soils, but that the response of fungal guild-level functions to long-term N addition would differ from community-level functions. Specifically, we hypothesized that: (1) N addition enhances SOC sequestration by stimulating fungal turnover of plant-derived C to fungal necromass; (2) Generalist saprotroph abundance will be greater in soils with added N; and (3) Predicted saprotrophic enzymes abundances related to SOC turnover will be upregulated in soils with N-addition. To test these hypotheses, we analyzed the abundance of fungal ecological guilds and their enzyme activities, targeting functions associated with SOC sequestration. Furthermore, we linked fungal guild-level function with the dynamics of different soil C fractions to elucidate the mechanisms of fungal SOC sequestration.

2. Methods and Materials

2.1 Study site

We investigated fungal guilds and soil C fractions within a long-term N-addition experiment, which was performed in the Xiaoliang Research Station of Tropical Coastal Ecosystems, Chinese Academy of Sciences (21°270' N, 110°540' E), located in the southwest of Guangdong Province, China (Figure S1). The climate in this region is a typical tropical monsoon climate with an average annual temperature of 23 °C and an average annual precipitation of 1400 − 1700 mm and a dry season from November to March. The site was located in secondary broadleaf mixed forest on coastal land (c. 5 km from the coast) with a very small slope. The forest was originally planted with *Eucalyptus exserta* in 1959, which was clear-cut and planted with 312 tree species between 1964 and 1975. Thereafter, natural succession almost displaced all the planted species, creating a broadleaf evergreen secondary tropical forest with similar biodiversity and structure to natural forest (Mo et al., 2019). According to the Soil Taxonomic System of China, the soils at the site are classed as latosols developed from granite, with a pH of 4 and low phosphorus availability. Annual wet N deposition in the region is estimated as *c.* 40 kg N ha^{-1} $year^{-1}$ in 2012 (Mo et al. 2019, Wang et al. 2017).

2.2 Experimental design and soil sampling

The fertilization experiment was initiated in August 2009, and included four treatments (Figure S1, Zhang et al., 2022) but only N addition (+N) and control treatments are considered here. The treatments were randomly allocated to one 10 m × 10 m plot within each of five replicate blocks (Mo et al. 2019) and biodiversity and structure of the vegetation is similar across treatments. Fertilizer was applied to the +N plots from September 2009 by dissolving 476.6 g NH_4NO_3 (166.6 g N) in 30 L of groundwater and applying it every two months with a knapsack sprayer as close to the soil surface as possible, giving a total of 100 kg N ha^{-1} $year^{-1}$. The same volume of groundwater without N fertilizer was added to the control plots (Ctr). Soils were sampled in five replicate +N plots (one per block) and corresponding controls in May 2015. In each plot, five randomly located intact soil cores (0 – 5 cm) were collected and mixed thoroughly to form one composite sample per block. The soil samples were passed through a 2 mm sieve to remove loose debris, roots, and rocks. Each sample was then divided into three subsamples. The first subsample was air-dried for analyses of soil chemical properties and C fractions; and the second was fresh soil sample for determining dissolved organic C (DOC); the third was stored at -80 °C for analysis of fungal guilds and their functions.

2.3 Soil nutrient and carbon fraction analyses

To link the fungal guilds and their functions with SOC dynamics, five air-dried soil samples were used to analyze the SOC fractions and nutrient concentrations. We selected the nutrients which been previously reported to be associated with N deposition in tropical forests, including nutrient concentrations (Ca^{2+}, K^+, Na^+, Cl^-, SO_4^{2-}, Lu et al., 2014). SOC in the samples was divided into active and necromass fractions using 15 g air-dried soil, whereby the active C fraction was the sum of soil microbial biomass C (MBC), DOC, and particulate organic C (POC). In brief, MBC was determined by chloroform fumigation extraction (Vance et al. 1987). DOC was extracted with 0.5 M K_2SO_4 (henceforth DOC_{ex}) and measured on a TOC analyzer (TOC-VCPH Shimadzu Corp., Japan) and POC was determined by density fractionation using NaI solution according to Sollins et al. (2006), followed by high-temperature external thermal oxidation-ferrous iron titration following Walkley and Black (1934).

2.4 Fungal community analyses

Fungal community structure was determined using high-throughput sequencing techniques of

three randomly selected soil samples per treatment. Prior to DNA extraction, the soil samples were finely ground and total DNA was then extracted from the pulverized soil samples using a FastDNA SPIN Kit for Soil (MP Biochemicals, Solon, OH, USA) according to the manufacturer's instructions. The total DNA samples were concentrated, purified, and checked by a NanoDrop 2000 UV-visible spectrophotometer (Thermo Scientific, Wilminton, DE, USA; and used for the amplification of the 18S ribosomal DNA with fungal universal primer pairs 528F (5'-GCGGTAATTCCAGCTCCAA-3') and 706R (5'- AATCCRAGAATTTCACCTCT-3') spanning the V4-V5 hypervariable region (Degnan and Ochman 2012) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR reaction mixture, amplification cycling conditions and the sequencing process can be found in the Supplementary File 1. Sequencing was performed on an Illumina Nova 6000 platform (Illumina, San Diego, CA, USA). The acquired sequences were sequentially filtered, trimmed, and truncated for quality filtration by fastp version 0.19.6 to obtain clean tags (Chen et al. 2018, Martin 2011). The filtered sequences were then clustered into operational taxonomic units (OTUs) based on 97% similarity. Subsequently, the sequences representing each OTU were identified and classed into fungal phylotypes using the UNITE databases (Release 8.0, http://unite.ut.ee/index.php, Kõljalg et al., 2005).

2.5 Fungal guild annotation by FUNGuild

To assign ecological classifications to our data, the table of UNITE-identified OTUs was parsed against the Python-based FUNGuild database (http://github.com/UMNFuN/FUNGuild). FUNGuild distils taxonomically complex sequencing data (OTUs) into more manageable ecological units such as guilds and trophic modes (Nguyen et al. 2016). Guild assignment relies heavily on accurate OTU taxonomic identification. In our study, 17.8% of orders, 34.2% of families, and 48.7% of genera were unidentified among the UNITE-based OTUs, respectively. Thus, most OTUs were assigned to fungal guilds at the genus level. However, when the genus is unidentified, some OTUs were assigned to fungal guilds based on family or order (see Supplementary File 2 for details). We retained most OTUs assigned to guilds with a confidence level of 'probable' or 'highly probable' at the genus level, and with a confidence level of 'possible' for most order/family level identification. We considered OTUs assigned to multiple trophic modes as generalists, as they are capable of switching between trophic modes depending on life stage and environmental conditions (Nguyen et al., 2016). Our FUNGuild-annotated soil fungal community comprised three major trophic modes (henceforth fungal guilds): symbiotrophs, saprotrophs, and pathotrophs (Figure 1), with 15 trophic subdivisions (Figure 2, Supplementary File 2). Trophic subdivisions containing OTUs represented by more than ten sequences were retained for sequential guild-level function annotation.

2.6 Guild-level function annotation

To predict fungal metabolic function, we assigned putative enzymatic profiles (E.C. number) to each fungal guild following Metcalf et al. (2016), using an updated PICRUSt2 (https://github.com/picrust/picrust2). PICRUSt2 is a software developed by Douglas et al. (2020), for predicting function (usually enzyme abundance) based only on the proportion of marker gene sequences (such as 18S ribosomal DNA gene in this study). After obtaining the enzyme abundance data, the KEGG database was applied to select the pathways related to SOC formation using basic local alignment search tools (Kanehisa et al. 2004). We selected 32 key enzymes associated with three pathways related to soil C dynamics: 1) amino sugar and nucleotide metabolism, 2) organic C degradation, and 3) organic acid formation. Then, we calculated the abundance of predicted enzymes involved in these three pathways for each fungal guild, whereby increased abundance of a predicted enzyme indicates up-regulation of enzyme production. Finally, to estimate the contribution of each ecological guild to the formation of SOC, the relative percentage of enzyme abundance were compared between control and +N soils for each trophic subdivision.

2.7 Verification of the function annotation results *via* glucosamine detection

To verify the accuracy of enzyme function predictions from the databases, we selected one of the three pathways to test whether trends in predicted-enzyme abundances were consistent with the changes in specific metabolites. We measured differences in glucosamine (GluN) as a key metabolite of amino sugar and nucleotide metabolism pathways. Amino sugar extraction was conducted following Indorf et al. (2011). Briefly, air-dried soil samples were hydrolyzed with 10 mL 6 M HCl at 105 ℃ for 6 h. The mixture was then cooled and filtered. The supernatant (0.5 mL) was evaporated by N_2 to remove HCl. The dried residues were resuspended with distilled water and dried again, then finally diluted in 2 mL distilled water for analysis. The concentrations of the GluN, Muramic acid (MurN) (mg kg^{-1} dry soil) were determined using a high-performance liquid chromatograph (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an octadecylsilyl silica gel column (Acclaim120 C18; 150 mm, 4.6 mm, 3 µm; Thermo Fisher Scientific, Waltham, MA, USA) after pre-column derivatization with ortho-phthaldialdehyde using standard solution. The concentrations of total GluN and fungal GluN (FGluN) were calculated using the equation as follows:

FGluN (mg kg^{-1}) = total GluN (mg kg^{-1}) – 2 × MurN (mg kg^{-1}) × (179.2/251.2)

Because GluN is derived from both fungal and bacterial cell walls, FGluN was calculated by subtracting bacteria-derived GluN from total GluN, assuming that MurN and GluN occur at a 1 to 2 molar ratio in bacterial cell walls, with molecular weights of 179.2 and 251.2 for GluN and MurN, respectively (Appuhn and Joergensen 2006). Accordingly, fungal necromass was calculated as:

Fungal necromass (mg kg^{-1}) = FGluN (mg kg^{-1}) × 9

where 9 is a conversion factor (Engelking et al., 2007; Zhou et al., 2022).

2.8 Data analysis and visualization

All data analyses and visualization were carried out in the *R* environment 4.2.0 (R Core Team 2022) unless otherwise stated. The abundances of OTUs and annotated enzymes were logarithmically transformed to satisfy assumptions of normality and variance homogeneity for analysis, but means and standard deviations are shown in all figures and tables. The differences in soil chemical properties and C fractions between the +N and control plots were tested using one-way analysis of variance (ANOVA, *aov* function). Statistical comparisons of fungal-associated parameters (OTU number for the three fungal guilds, log-transformed abundances of predicted enzymes, GluN and SOC fractions) between +N and control soils were assessed using Student's *t*-test with correction for false discovery rate (*t*.test and *p*.adjust functions).

To visualize intersecting sets of the three fungal guilds between +N and control soils, we used the number of OTUs in package *UpsetR* (Conway et al. 2017). To analyze taxa-function relationships, a Venn diagram was created in cytoscape version 3.6.0 (Gustavsen et al. 2019) using the number of OTUs in each fungal guild (symbiotrophs, saprotrophs, and pathotrophs) and key predicted enzymes involved in soil C-associated pathways. We calculated relative abundances of predicted enzymes for each guild and trophic subdivision as the proportion of a given enzyme in the whole fungal community. To visualize the relative abundance of predicted enzymes, a heatmap plot was generated with package *pheatmap* (Kolde and Kolde 2018), targeting enzymes most likely to be involved in organic C turnover. The abundances of predicted C-degrading enzymes in each trophic subdivision were illustrated by a bubble plot using packages *tidyverse* and *ggplot2* (Wickham, et al., 2016, 2019). To identify specific enzymes involved in organic acid formation and their response to N addition, we used a volcano plot to investigate significant differences in predicted enzymes between the +N treatments and controls using the package *DESeq2* (Love et al. 2014). Finally, to analyze the relationships between up-regulated predicted enzymes associated with organic acid synthesis (log2 Fold Change > 1 with $p < 0.05$) and soil chemical properties, we performed linear regression analysis

using the packages *tidyverse*, *vegan*, *grid*, and *gridExtra* (Oksanen 2015; Auguie, et al., 2017).

3. Results

3.1 Effects of long-term N addition on soil carbon fractions

Total N and NO_3^- concentrations were significantly higher in the N-fertilized soils compared to controls (F = 38.9 and 12.25, p = 0.027 and 0.030, one-way ANOVA, Table S1) and SOC content was 1.15-fold greater in +N plots than controls (F = 11.88, p = 0.026, Table 1) in the surface (0 – 5 cm) soils. All measured SOC fractions except MBC and bacterial necromass C differed between +N and control soils. Although DOC contributed < 2% to total SOC in both treatments, K_2SO_4-extractable DOC (DOC_{ex}) concentrations were 14.5% higher in +N plots compared to controls (p = 0.023). POC accounted for > 90% of the active C pool in both the control and +N soils, but +N soils nonetheless contained 11.4% more POC than control soils (p = 0.002). Total necromass C accounted for over half of total SOC in both the +N (59.96%) and control (56.5%) soils but fungal necromass C was substantially 42.4% higher in the +N plots compared to controls (p = 0.011). Consequently, fungal necromass C contributed 89.1% to the total necromass C in the +N plots, compared to 76.6% in the controls.

3.2 Effect of long-term N addition on soil fungal ecological guilds

Based on FUNGuild annotations, we were able to assign most of the fungal OTUs (66.1%) to fungal guilds. The fungal community was dominated by saprotrophs in both control (55.5% of all OTUs) and +N soils (48.7% of all OTUs, Figure 1). The total number of saprotroph OTUs was significantly higher in +N soils (p < 0.05, corrected Student's t-test) with 109,664 OTUs compared to 79,885 in control soils. However, the proportion of OTUs representing saprotroph specialists was much lower in the +N soils (38.3%) compared to the controls (66.5% of all saprotrophs) (p = 0.018). The proportion of OTUs representing fungi capable of both saprotrophy and symbiotrophy did not differ between the +N plots (2.29%) and the controls (2.72%; p = 0.532). However, OTUs representing fungi capable of both saprotrophy and pathotrophy accounted for 27.1% of the total OTUs in the +N soils but only 3.05% in the controls. Finally, OTUs representing fungi capable of all three trophic modes accounted for 32.3% of all OTUs in the +N plots, which was significantly higher than the 28.4% in the control soils (p = 0.021; Figure 1). Thus, the fungal community was dominated by generalist saprotrophs in the +N plots but specialist saprotrophs in the controls.

Within the saprotroph subdivisions, the undefined saprotrophs dominated in both control and +N soils, with relative abundances exceeding 88% of all saprotrophs (Table S2). Nonetheless, there were significant shifts in other saprotroph subdivisions in response to long-term N addition. Importantly, the +N soils had a 93× higher abundance of leaf saprotrophs (p = 0.002) compared to control soils. In addition, the relative abundance of wood saprotrophs was 78.85% higher in +N soils compared to control (p = 0.018). Although the abundance of soil saprotrophs was 30.67% lower in +N soils than in the control soils, the difference was not significant (p = 0.39).

3.3 Effect of long-term N addition on fungal guild-level functions

Saprotroph-derived enzymes for organic C degradation

Saprotrophs contributed the most (nearly 50%) to enzymes associated with the degradation of organic C in both control and +N soils (Table S3). All subdivisions of saprotrophs were capable of degrading starch, cellulose, hemicellulose, pectin, chitin, and lignin, except for dung saprotrophs, which were not associated with laccase (E.C. 1.10.3.2) for lignin oxidation (Figure S2). The combined abundance of the enzymes responsible for starch and cellulose degradation (E.C. 3.2.1.1 and 1.1.99.18) was significantly higher in long-term +N soils compared to control (p = 0.031, Figure 2). In soil saprotrophs and undefined saprotrophs, the abundances of these enzymes did not differ between +N and control soils (all p-values > 0.05, Figure 2 and Table S5). However, in leaf saprotrophs,

the abundance of most C-degrading enzymes in +N soils was more than 3× higher than controls, and the highest (cellulose) was 15.84× higher (p = 0.004, Figure 2 and Table S4).

Enzymes related to organic acid synthesis

We identified 286 candidate enzymes related to organic acid synthesis of which 14 responded significantly to long-term N addition (Figure 3A and S3). Among these 14 enzymes, the relative abundances of 11 enzymes were significantly up-regulated whereas three enzymes were down-regulated in the +N soils compared to the control soils ($p < 0.05$, Figure 3A). The relative abundances of up-regulated enzymes involved in organic acid synthesis increased with soil concentrations of Ca^{2+}, SO_4^{2-}, and DOC_{ex} ($p < 0.05$, Figure 3B). The up-regulated enzymes were mainly contributed by saprotrophs, as > 57.0% of all up-regulated enzymes except pyruvate carboxylase (E.C. 6.4.1.1) were saprotroph-derived. Five of the up-regulated enzymes were responsible for catalyzing the synthesis of pyruvate (Figure 4), so pyruvate synthesis potential was predicted to be 2.91× higher in the +N soils compared to control (Table S6).

Fungal necromass formation

Apart from *N*-acetylglucosamine kinase (E.C. 2.7.1.59), all eight enzymes involved in fungal necromass formation were found in all three fungal guilds. However, their abundances varied among guilds (Figure 5). Glucosamine-related enzymes were mainly contributed by pathotrophs and saprotrophs, accounting for > 70% of the total abundance of each enzyme in both control and +N soils (Figure 5A and C). Notably, in the control soils, 71.67% of the total chitosanase (E.C. 3.2.1.132) was derived from saprotrophs and only 7.75% was derived from pathotrophs. By contrast, in the +N soils, 54.43% of the total chitosan was derived from saprotrophs and 30.2 % was derived from pathotrophs (Figure 5A). The absolute abundance of chitosanase was significantly 73.2% higher in +N soils compared to control ($p < 0.05$, Figure 5B). Consistently, GluN content tracked these changes in predicted enzyme abundances, whereby fungal GluN and total GluN were significantly 42.5% and 37.6% higher in +N soils compared to control soils ($p < 0.05$, Figure 5D). Thus, our results demonstrate that empirically measured GLuN and the putative enzyme abundances related to the relevant metabolic pathway responded similarly to N addition.

Discussion

Our study addresses gaps in our knowledge of how N deposition stimulates SOC storage by modifying the linkages between fungal guild functions and SOC dynamics. In support of our first hypothesis, fungal necromass contributed more to SOC in the +N soils, suggesting that N-addition stimulated fungal incorporation of plant-derived C into soil. In support of our second hypothesis, we observed a shift in fungal community composition, from saprotrophic-specialist-dominated to generalist-dominated, and a marked increase in leaf saprotrophs in response to added N. Finally, the upregulation of enzymes involved in SOC dynamics in +N soils lends support to our third hypothesis. Overall, the changes in saprotrophs and their putative enzyme abundances were linked to increased SOC and fungal necromass, as well as changes in DOC_{ex} and POC in +N soils. Thus, we propose that increased SOC storage in response to N deposition is underpinned by fungal community shifts towards generalist taxa and the up-regulation of enzymes involved in the degradation of leaf-derived C. Our findings also identify a potential pathway by which N-addition might enhance fungal SOC storage, whereby increased organic acid production promotes DOC release from leaf litter. Higher DOC concentrations then facilitate fungal growth and turnover, as well as the biosynthesis of GluN, Here, we discuss how these changes could explain the overall increase in fungal necromass and SOC in response to N addition.

4.1. Guild-level *vs*. community-level responses to N addition

This study combined FUNGuild and Picrust2 to predict the mechanisms of fungal-driven SOC sequestration. These amplicon-based tools have been widely used to estimate guild-level responses

of microbial communities (Metcalf et al., 2016; Douglas et al., 2020; Moore et al., 2021), but we verified their predictive power by measuring GluN content. The response patterns of putative GluN-producing enzyme abundances were consistent with measured GluN content in the soils, providing a direct link between annotated function and end-product. Thus, the annotated functions by FUNGuild and Picrust2 allow us to infer the mechanisms by which N deposition affects fungal guilds and their functions. Our findings represent a solid foundation for future studies using -omics approaches to investigate functional changes in fungal guilds in greater detail. For clarity and conciseness, we henceforth refer to annotated or putative enzyme abundance simply as 'enzyme abundance'.

As predicted, the responses of several guild-level enzymes to N addition differed from total enzyme abundances. Previous results have reported limited, no, or positive effects of N fertilization on the fungal-driven hydrolysis of plant-derived C (Waldrop et al., 2004; Morrison et al., 2016; Moore et al., 2021), which indicates variable effects of N addition on enzyme activities associated with litter decomposition. Our findings present a way to reconcile these discrepancies, because we demonstrate the SOC dynamics are linked to differences in guild-level enzyme synthesis, rather than changes in total enzyme abundance. For instance, the total abundance of hemicellulose-degrading α-L-arabinofuranosidase (Lundell et al. 2014) did not differ between +N plots and controls. Instead, we observed increases in the analogous enzymes α-L-arabinofuranosidase and α-*N*-acetylglucosaminidase, which are derived from leaf saprotrophs, reflecting the greater abundance of leaf saprotrophs in the +N plots. Similarly, we observed no overall increase in the total abundance of lignin-oxidizing enzyme laccase (Hatakka, 1994) in the +N soils, but leaf saprotroph-derived laccase increased substantially (Figure 3). Although leaf saprotrophs account for only a small proportion of saprotrophs, they are important in driving aboveground plant C into forest soils (Kaiser and Kalbitz 2012, Kramer et al. 2012). Leaf saprotrophs dominate the litter layer (Voříšková and Baldrian, 2013), and their greater abundance could explain higher litter decay rates in the long-term N-fertilized soils (U'Ren and Arnold 2016, Zhang et al. 2022). Thus, such guild-level responses to N addition are more likely to reveal changes in fungal metabolism of C and the role of fungi in SOC than the response of the whole fungal community.

The observed shifts in fungal community composition, from saprotrophic-specialist-dominated to generalist-dominated, after N addition expands our second hypothesis to demonstrate changes in guild-level functions. Many saprotrophs in the +N soils are considered generalists because they also exhibited functions associated with pathotrophy (in which they damage host cells to obtain nutrients) and symbiotrophy (Koide et al., 2005). These generalists are capable of quickly infecting, sporulating and decomposing plant litter (Peršoh 2013) and can accelerate the decomposition of dead plant tissues (Lindahl et al. 2007, Osono and Hirose 2011) by boosting lignocellulolytic activity in litter (Korkama-Rajala et al. 2008). We therefore propose that the changes in fungal community, including the increase of leaf saprotrophs and generalist saprotrophs, collectively explain the increased enzyme abundances for litter decomposition in N-fertilized soils. The up-regulation of lignocellulolytic enzymes suggests that N addition alleviated nutrient limitation to decomposers caused by the high lignin:N ratio in plant litters (Carreiro et al. 2000). Such up-regulation of enzymes for litter decomposition also potentially contributed to greater incorporation of plant C into soils, supporting our third hypothesis. Accelerated degradation of recalcitrant plant C by generalist saprotrophs could account for the greater relative contribution of plant C inputs to SOC with N addition observed in other studies (Xu et al. 2021) and would also explain why N-addition enhances SOC storage without stimulating litter production (Cusack et al. 2011, Lu et al. 2018). Hence, we propose that greater fungal turnover of plant litter is a likely mechanism contributing to increased SOC accumulation with N deposition.

4.2. Increased fungal necromass as a key source of SOC

Greater abundance of generalist saprotrophs likely also underpins the higher concentrations of fungal necromass (and therefore SOC storage) with N addition. A recent meta-analysis demonstrated that fungi contribute a much greater proportion to total necromass than bacteria (Wang et al. 2021). In our study, necromass C contributed >50% to total SOC in both +N and control soils, but a much greater amount of necromass was derived from fungi (> 80%) than bacteria. The amino sugar GluN is generally considered a time-integrated biomarker of fungal necromass (Ma et al. 2018) and many studies estimate fungal necromass from the GluN content in soils (Griepentrog et al. 2014, Hu et al. 2022). However, measurements of total GluN content cannot elucidate the contribution of different guilds to fungal necromass. By analyzing the contribution of three guilds to fungal necromass at the molecular level via the abundance of chitosanase, a key enzyme for GluN biosynthesis, we revealed that much of the chitosanase was derived from saprotrophs in +N soils (54.4%). Thus, enhanced GluN biosynthesis, and therefore higher soil GluN content, in +N soils is most likely the result of greater abundance of saprotrophs. Given the shift towards generalist saprotrophs in the +N soils, we propose that N-addition results in C-limitation fungal growth by reducing the litter C:N ratio. Multitrophic saprotrophs (i.e., generalists) then become dominant because of their ability to quickly acquire C from plant litter, especially in broadleaf tropical forests where litter water-soluble C content is relatively high (Berg and McClaugherty 2020).

4.3. DOC as a pathway for increased fungal C turnover

The observed increases in both DOC_{ex} and enzymes related to organic acid formation are a further indication of upregulated fungal activity as a key mechanism contributing to greater SOC stocks in the +N plots. Litter-derived C is the primary source of SOC (Cotrufo et al. 2015), but the main rate-limiting step in the turnover of litter C to SOC is the conversion of plant-derived C to DOC by free-living decomposers (Allison et al. 2010, Miltner et al. 2012). DOC is mainly derived from recent litter and humus (Berg and McClaugherty 2020), and the decomposition of litter, humus, and microbial necromass directly contribute to the amount of DOC in soils (Kaiser and Kalbitz 2012, Tu et al. 2011). Most DOC in soils is composed of low-molecular-weight organic acids (Griffiths et al. 1994), which are important C source for many soil microorganisms (Van Hees et al. 2005). Accordingly, in our +N soils, we observed concomitant increases in i) the abundances of enzymes for litter decomposition, ii) DOC_{ex} concentrations and iii) abundances of enzymes involved in the synthesis of organic acids, which were mostly contributed by saprotrophs. The up-regulation of enzymes involved in organic acid synthesis potentially enhanced the production of low-molecular-weight acids, such as pyruvate, which had a 2.91-fold greater synthesis potential in +N soils in our study. Furthermore, the relative abundances of enzymes linked to organic acid synthesis were related to concentrations of DOC and soil nutrients (Ca^{2+}, SO_4^{2-}), suggesting that increased organic acid production by saprotrophs not only contributed to higher DOC concentrations in +N soils, but also helped mobilize nutrients (Ström et al. 2005). Thus, increased abundance and activity of saprotrophs with N-addition likely enhances SOC storage by releasing DOC and nutrients to fuel rapid growth and turnover, resulting in greater inputs of fungal necromass to the soil.

4.4 Conceptual model of fungal-driven SOC storage with N-deposition

Enhanced SOC storage in response to N fertilization is a long-term ecological process, because the overall average soil C turnover time is years to decades (Carvalhais et al. 2014). Our study, using soils collected after six years of N addition, provides valuable insights into the role of fungal guilds in SOC storage in a tropical forest. Based on our findings, we propose a conceptual model of the mechanisms and pathways by which N deposition stimulates increased C turnover and SOC storage in tropical forests (Figure 6): 1) Changes in fungal community structure are characterized by a shift from a community dominated by specialist saprotrophs to a community dominated by generalist

saprotrophs and a marked increase in the abundance of leaf saprotrophs. 2) These shifts facilitate the fungal decomposition of plant litter, accelerating the transformation of plant-derived C compounds to SOC. 3) During decomposition, upregulated synthesis of organic acids not only supports microbial growth directly, but also indirectly by releasing nutrients and DOC. 4) Finally, increased saprotroph abundance is associated with greater abundance of enzymes involved in the synthesis of GluN, the predominant contributor to increased SOC under N addition.

Collectively, our findings advance research into soil C dynamics in tropical forests by demonstrating distinct functional responses of fungal guilds to N deposition. Our work lays solid foundations for future studies using -omics approaches to investigate changes in the synthesis of guild-level fungal enzymes, which will further elucidate the key role of fungi in global biogeochemical cycling.

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Figures and Tables with captions

Table 1. Effect of long-term nitrogen fertilization (+N) on soil carbon (C) fractions at 0 – 5 cm depth in a tropical forest, showing means ± SE for *n* = 5. The abbreviation Ctr refers to the control soils.

Carbon fraction (g Kg^{-1})	Ctr	+N	*F*-value	*p*-value
Soil organic C	22.7 ± 3.48	26.2 ± 4.50	11.9	**0.026**
✧ Active C				
Microbial biomass C	0.26 ± 0.05	0.29 ± 0.12	0.102	0.766
Dissolved organic C (K_2SO_4-extractable)	0.41 ± 0.02	0.48 ± 0.02	12.6	**0.023**
Particle organic C	5.10 ± 0.02	5.68 ± 0.09	49.2	**0.02**
✧ Necromass C				
Bacterial	2.49 ± 1.23	1.71 ± 0.87	1.41	0.266
Fungal	9.83 ± 0.08	14.0 ± 0.13	20.5	**0.011**

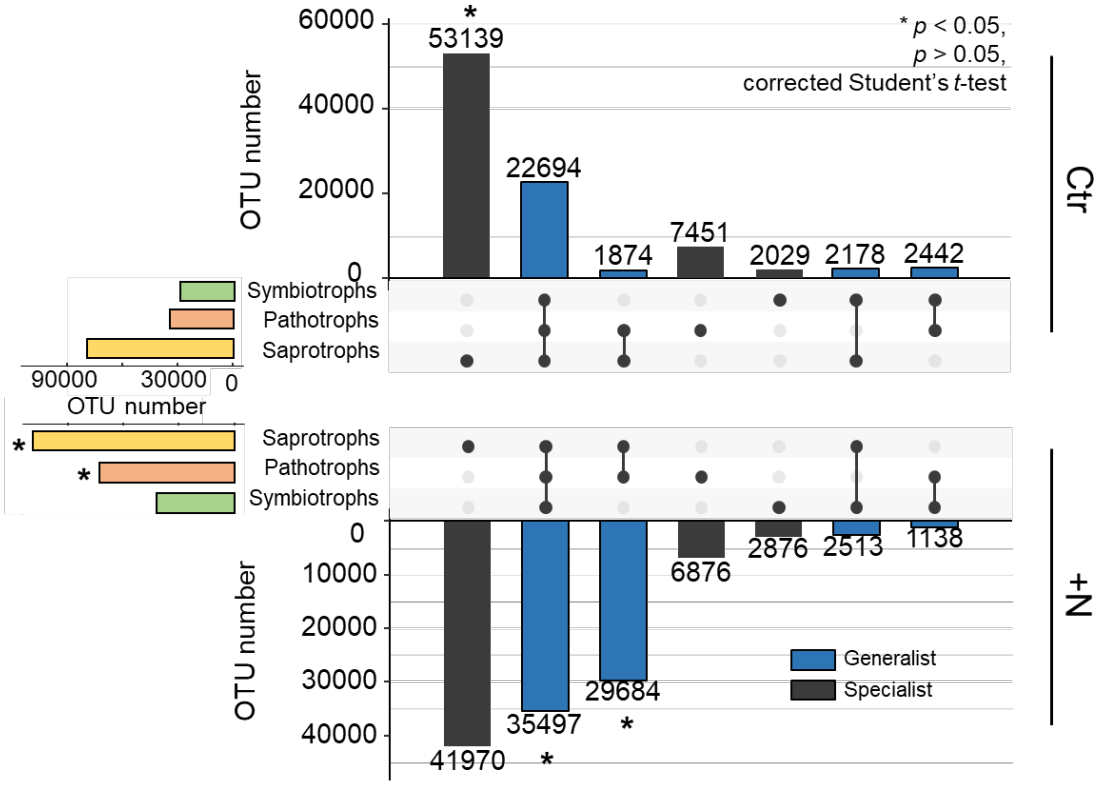

Figure 1. Effect of long-term nitrogen (+N) fertilizer on soil fungal communities at 0 – 5 cm depth in a tropical forest, showing three ecological fungal guilds (trophic modes): saprotrophs, pathotrophs, and symbiotrophs. Asterisks indicates significant changes in fungal trophic modes by long-term N fertilization at $p < 0.05$, based on the number of operational taxonomic units (OTU); the location of the asterisks indicates the treatment with greater abundance.

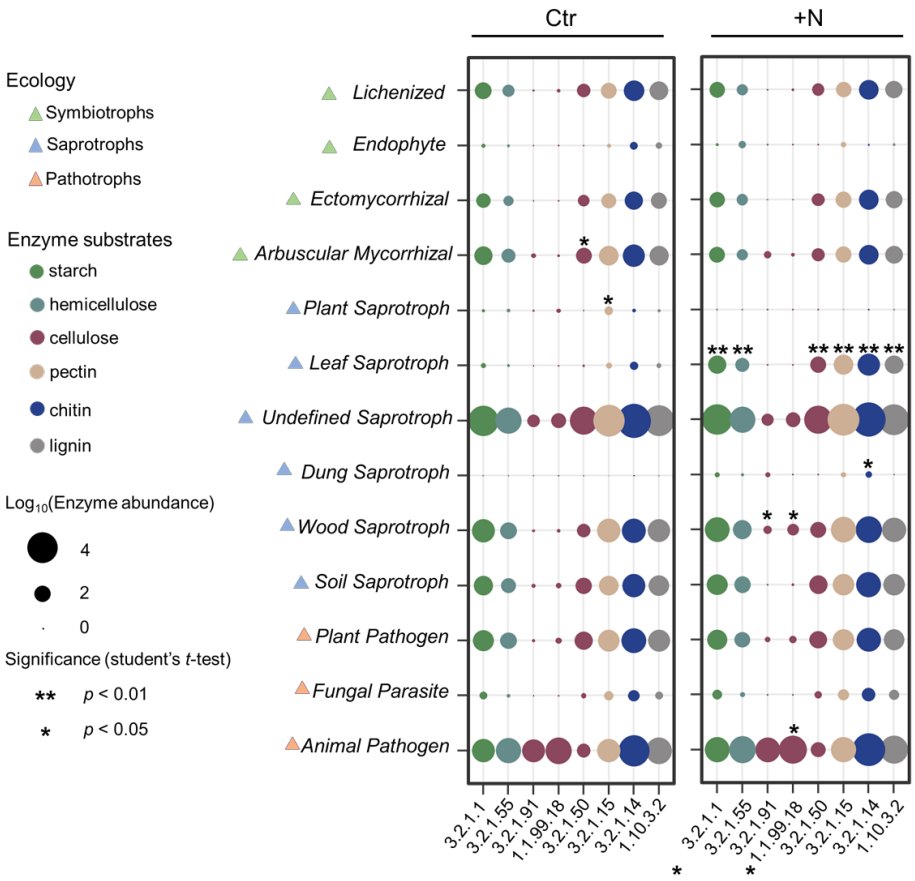

Figure 2. Abundances of enzymes involved in the degradation of organic C associated with three fungal guilds in control (Ctr) and long-term nitrogen-fertilized (+N) soils. The soil fungal community was classed into three ecological guilds representing trophic modes: symbiotrophs (green), saprotrophs (orange), and pathotrophs (blue) by FUNGuid annotation. The three guilds were further split into trophic subdivisions. Enzymes were grouped into six categories according to the substrates they degrade, and Enzyme Catalogue (E.C.) numbers are shown. * and ** indicate significant changes in potential fungal organic carbon degradation in +N soils at $p < 0.05$ and 0.01, respectively; the location of asterisks indicates which treatment had the higher enzyme abundance.

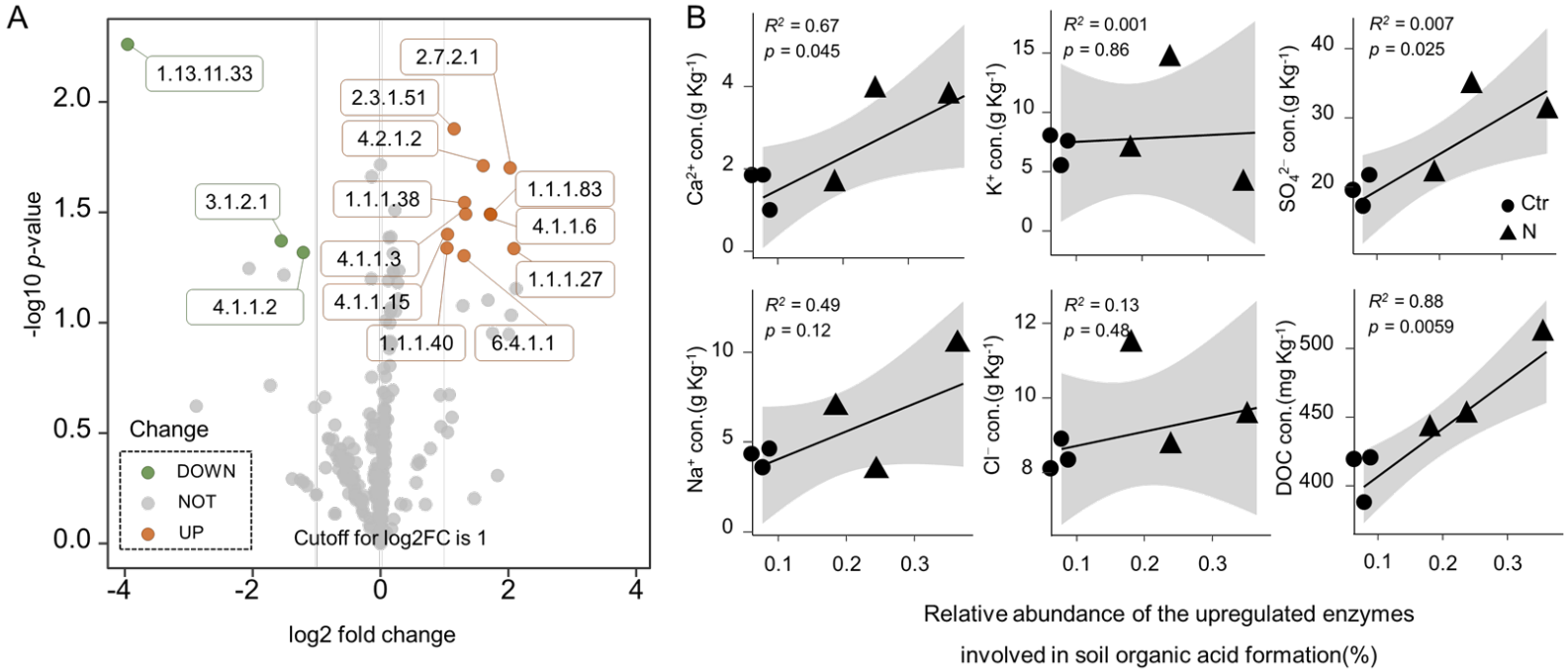

Figure 3. Changes in Picust2-annotated enzymes involved in organic acid synthesis under long-term N fertilization (A), and the relationships between the relative abundances of up-regulated enzymes and concentrations of calcium (Ca^{2+}), potassium (K^{+}), sulphur (SO_4^{2-}), sodium (Na^{+}), chlorine (Cl^{-}) and dissolved organic carbon (DOC) in the soil (B). Significantly down-regulated (green) or upregulated (orange) enzymes (> 2-fold change and $p < 0.05$) were identified *via* difference analysis, and Enzyme Catalogue (E.C.) numbers are shown.

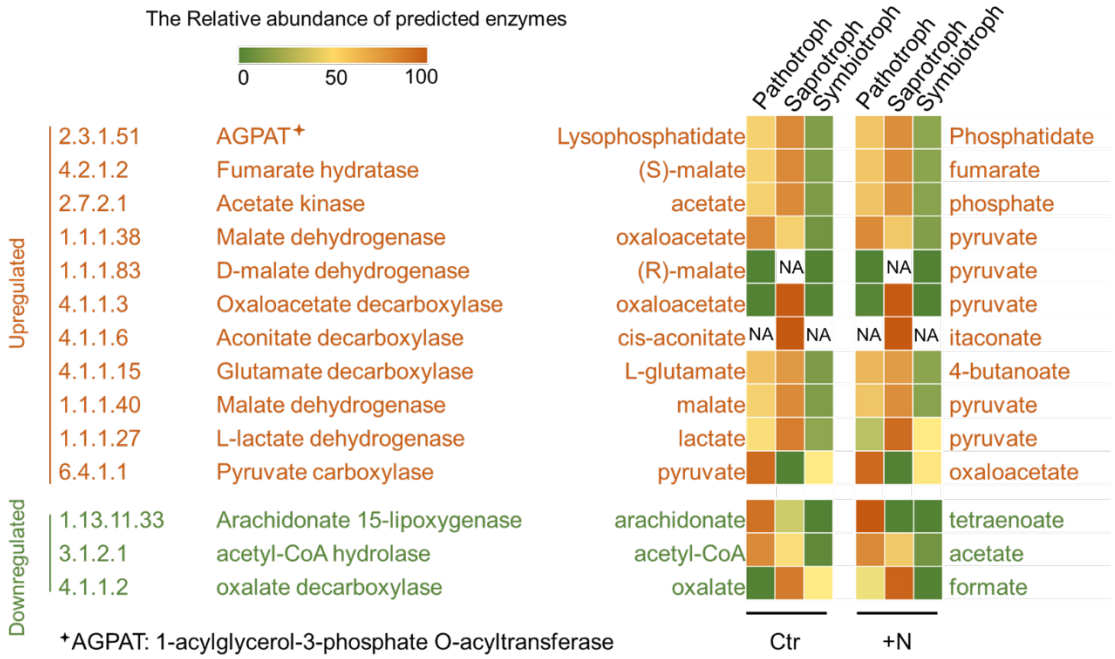

Figure 4. Heatmap showing the relative abundances of each Picust2-annotated enzyme (E.C. number) associated with three fungal guilds (trophic modes) in control (Ctr) or long-term N-fertilized (+N) soils. Up-regulated and down-regulated enzymes are identified in orange and green, respectively, and Enzyme Catalogue numbers and enzyme names are given. The left- and right-hand sides of the heatmap represent the substrates and products of the reactions catalyzed by each enzyme, respectively.

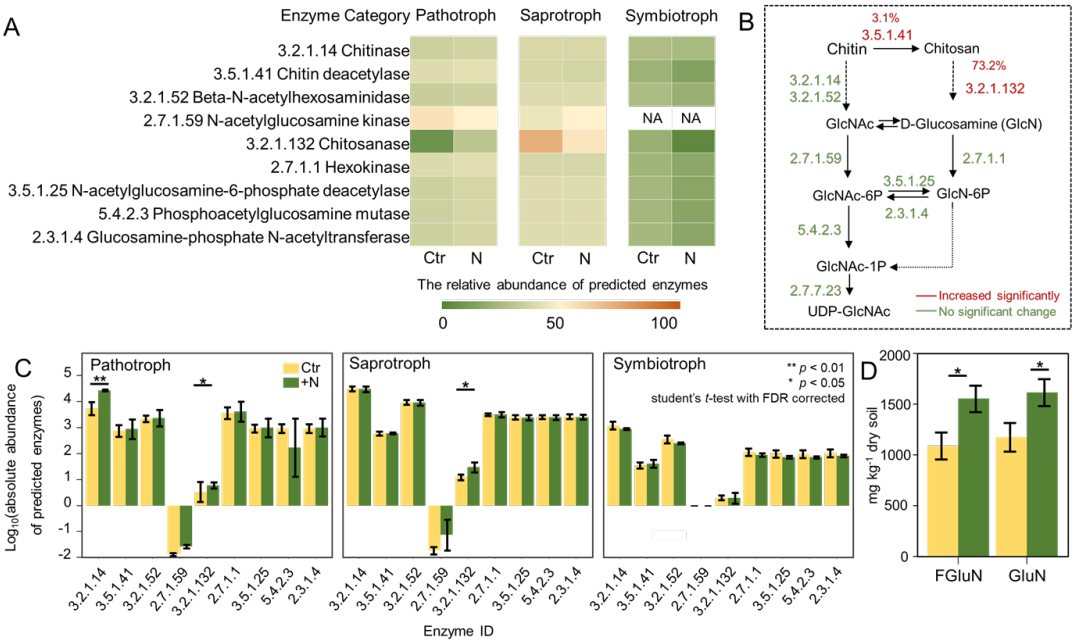

Figure 5. Abundances of the Picust2-annotated D-glucosamine (GluN)-associated enzymes in soils, showing (A) The relative abundance of each enzyme associated with three fungal guilds (trophic modes) in the control (Ctr) and long-term N-fertilized (+N) soils; (B) A conceptual model showing fungal-driven GluN biosynthesis pathways, with up-regulated enzyme shown in red and non-significant changes in green; (C) Log-transformed abundances of enzymes associated with each fungal guild in Ctr and +N soils; (D) Total and fungal glucosamine (GluN and FGluN, respectively) contents in Ctr and +N soils. In panels C and D, values are presented as means ± SE for $n = 3$, and * indicates a significant increase in fungal GluN biosynthesis pathway in +N soils at $p < 0.05$. Enzyme Catalogue (E.C.) numbers are shown in panels A – C.

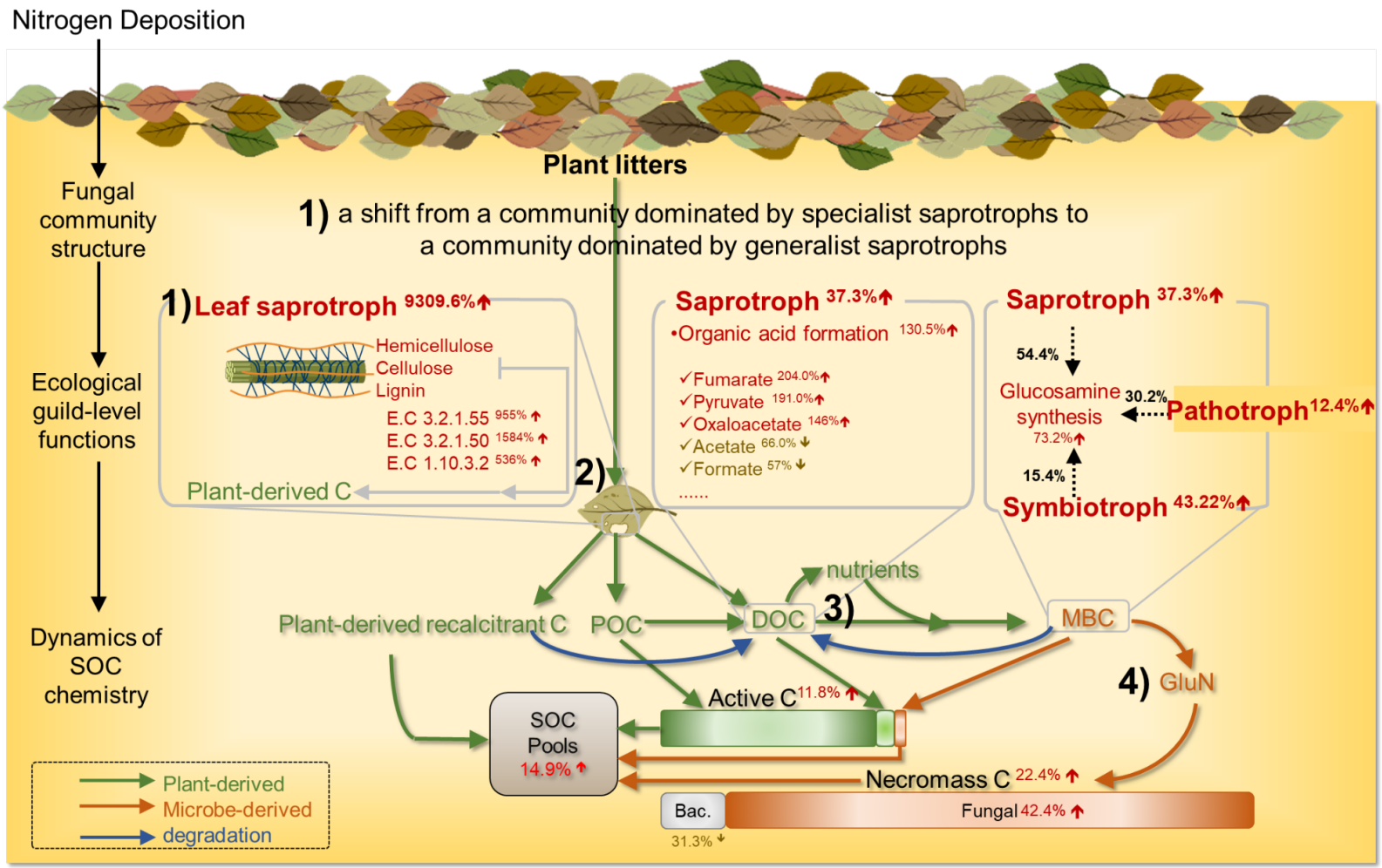

Figure 6. A conceptual model showing how guild-level functions of the soil fungal community influence the dynamics of soil organic carbon (SOC) under nitrogen (N) deposition. Up- and down-regulated ecological guilds and enzymes are shown in red and yellow, respectively, where up and down arrows represent up- and down-regulation, and percentages indicate the associated increase or decrease in long-term N-fertilized soils compared to controls. Green, orange, and blue arrows represent plant-derived C pathways, microbial pathways, and carbon (C) degradation, respectively. Black dotted arrows represent the contribution of the three fungal guilds to the abundance of chitosanase (E.C. 3.2.1.132), catalyzing the biosynthesis of glucosamine.