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RESEARCH ARTICLE

Mycorrhizal effects on decomposition and soil CO_2 flux depend on changes in nitrogen availability during forest succession

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Abstract

1. Mycorrhizal fungi play a central role in plant nutrition and nutrient cycling, yet our understanding on their effects on free-living microbes, soil carbon (C) decomposition and soil CO_2 fluxes remains limited.
2. Here we used trenches lined with mesh screens of varying sizes to isolate mycorrhizal hyphal effects on soil C dynamics in subtropical successional forests.
3. We found that the presence of mycorrhizal hyphae suppressed soil CO_2 fluxes by 17% in early-successional forests, but enhanced CO_2 losses by 20% and 32% in mid- and late-successional forests respectively. The inhibitory effects of mycorrhizal fungi on soil CO_2 fluxes in the young stands were associated with changes in soil nitrogen (N) mineralization and microbial activities, suggesting that competition between mycorrhizae and saprotrophs for N likely suppressed soil C decomposition. In the mid- and late-successional stands, mycorrhizal enhancement of CO_2 release from soil likely resulted from both hyphal respiration and mycorrhizal-induced acceleration of organic matter decay.
4. *Synthesis*. Our results highlight the sensitivity of mycorrhizal fungi-saprotroph interactions to shifts in nutrient availability and demand, with important consequences for soil carbon dynamics particularly in ecosystems with low nutrient conditions. Incorporating such interactions into models should improve the simulations of forest biogeochemical cycles under global change.

KEYWORDS

enzyme activity, Gadgil effect, mycelial respiration, mycorrhizal types, mycorrhiza-saprotroph competition, soil C stabilization, soil nitrogen

1 | INTRODUCTION

Mycorrhizae are the most abundant symbiotic associations in terrestrial ecosystems, and play a central role in plant productivity (Klironomos et al., 2000), community structure (Jiang et al., 2017; Johnson et al., 2013) and ecosystem carbon (C) and nutrient cycling (Hughes et al., 2008; Lin et al., 2017). Through scavenging nutrients from soils and transferring a portion of these nutrients to host plants, mycorrhizal fungi can increase plant growth by up to 50% (Hoeksema et al., 2010). In return, these fungi get 3%–30% of photosynthetic C from plants to sustain their biomass (Zhu & Miller, 2003). As fungi die, hyphal necromass enters the soil food web, where it can become an energy source for free-living saprotrophs (Beidler et al., 2020) or become stabilized in slow-cycling soil pools (Fernandez & Kennedy, 2016). As such, mycorrhizal fungi are a key conduit for plant-derived C inputs to soil and in many ecosystems, a primary determinant of soil C sequestration (Clemmensen et al., 2013; Nottingham et al., 2010). While the effects of mycorrhizal fungi on soil organic matter (SOM) have been well-documented (Frey, 2019), we still have a limited understanding of the factors that govern mycorrhizal effects on soil CO_2 flux, as well as their interactions with other microbial guilds. This knowledge gap limits our ability to incorporate plant–microbe interactions into ecosystem and microbially explicit soil C models, which are needed to improve predictions of terrestrial ecosystem responses to global change (Orwin et al., 2011; Sulman et al., 2017).

Mycorrhizal fungi can influence soil CO_2 fluxes in myriad ways, with different consequences for soil C storage. First, external hyphae or bundles of hyphae (i.e. mycelium) respire C. These fluxes track photosynthesis (Johnson et al., 2002; Staddon et al., 2003), and can be large (15–26 g C m^{-2} $year^{-1}$ from soil or ~25% of soil respiration; Heinemeyer et al., 2007; Moyano et al., 2007), although they do not affect soil C storage. Second, mycorrhizal hyphae can directly accelerate decomposition (Bunn et al., 2019) by releasing extracellular enzymes and hydroxyl radicals to soil (Lindahl & Tunlid, 2015). Third, mycorrhizal hyphae can affect soil C storage via positive or negative interactions with free-living saprotrophic microbes (Verbruggen et al., 2016). Facilitative interactions occur when mycorrhizal hyphae release low molecular-weight organic compounds that desorb mineral-associated organic matter and stimulate SOM decomposition (Cheng et al., 2013; Keiluweit et al., 2015; Shahzad et al., 2015). In contrast, competitive interactions also occur when mycorrhizal hyphae compete with free-living saprotrophs for soil resources (water or nutrients), resulting in the depression of SOM decomposition, which is referred to as Gadgil effect (Fernandez & Kennedy, 2016; Gadgil & Gadgil, 1971). Given the complexity of mycorrhizal contributions to soil CO_2 fluxes, multiple approaches are often needed to partition these fluxes (Nottingham et al., 2010).

The degree to which mycorrhizal fungi accelerate versus decelerate decomposition has been hypothesized to relate to nitrogen (N) availability, though tests of this hypothesis are rare and the two competing processes—priming and microbial interguild competition—may be offsetting. Theory predicts that when N availability is low, competition between mycorrhizal fungi and saprotrophic microbes for N is most intense (Orwin et al., 2011). This has been referred to as mycorrhizal fungi-saprotroph nutrient competition hypothesis (Averill et al., 2014; Verbruggen et al., 2016). Likewise, priming effects and mycorrhizal-accelerated decomposition are believed to be greatest too when N availability is low (Sullivan & Hart, 2013; Sulman et al., 2017; Yin et al., 2018). Thus, there is need to investigate mycorrhizae-saprotroph interactions across a gradient of N availability to better understand the consequences of these interactions for soil C dynamics.

Mycorrhizal associations often reflect and affect nutrient availability in ecosystems owing to their difference in N acquisition capacity (Phillips et al., 2012; Read, 1991). Ectomycorrhizal (ECM) fungi can produce a variety of enzymes that release N from SOM, whereas arbuscular mycorrhizal (AM) fungi lack these enzyme systems (Read & Perez-Moreno, 2003; Tedersoo & Bahram, 2019). Accordingly, ECM fungi have been widely presumed to compete with saprophytic microbes for available N—a process that would suppress SOM decomposition (Averill & Hawkes, 2016). By contrast, AM fungal hyphae, which turn over more rapidly than ECM hyphae (Staddon et al., 2003), may stimulate saprophytic activity (Cheng et al., 2012; Wurzburger & Brookshire, 2017). However, other studies have reported positive effects of ECM fungi and negative effects of AM fungi on soil microbial activity and SOM decomposition (Lindahl & Tunlid, 2015; Verbruggen et al., 2016), casting doubt on the universality of these patterns. Moreover, there have been reports of both priming and mycorrhizal-saprotroph competition in AM- (Brzostek et al., 2015; Cheng et al., 2012) and ECM- (Averill & Hawkes, 2016; Brzostek et al., 2015) dominated ecosystems. Thus, there is little consensus on how and why mycorrhizal fungi may suppress versus stimulate decomposition (Frey, 2019).

Forest succession is a naturally occurring process of plant community replacement that alters soil structure (Erktan et al., 2016; Mardhiah et al., 2014), nutrient availability (Yang & Luo, 2011; Yang et al., 2011) and microbial community (Clemmensen et al., 2015). Mycorrhizal fungi can act as the vectors for forest succession, as well as key regulators in belowground C allocation (Clemmensen et al., 2013) and soil C decomposition (Averill & Hawkes, 2016). However, mycorrhizal effects on soil CO_2 fluxes during forest succession have not been examined.

Here, we took advantage of three AM-dominated successional subtropical forests in China to test the hypothesis that mycorrhizal effects on soil CO_2 fluxes depend on nutrient availability and biotic demand. We hypothesized that mycorrhizal fungi would suppress microbial activity and soil C decomposition in early-successional stands where N availability is relatively low and N demand by trees is relatively high. In contrast, we predicted that mycorrhizal fungi would enhance soil CO_2 losses via mycelial respiration and mycorrhizal-induced stimulation of saprophytic activities in the mid- and late-successional stands where N is abundant relative to demand. In addition, the constructive species changed from AM to ECM tree species along the forest succession (Table S1).

We also hypothesized that mycorrhizal fungi at late-successional stands would stimulate greater soil CO_2 losses than those at mid-successional stands.

2 | MATERIALS AND METHODS

2.1 | Experimental sites and forest successional stages

The experiment was conducted at the Tiantong National Forest Ecosystem Observation and Research Station (29°48′N, 121°47′E), Zhejiang province, in China. Experimental blocks were established within a subtropical secondary forest succession, which was composed of three representative successional stages (early stage: 25 years young mixed woody community, middle stage: 55 years sub-climax *Schima superba* community and late stage: 120 years climax *Castanopsis fargesii* community; Table S1), based on both forest age and species composition (Yan et al., 2009; Zheng et al., 2019). In 2015, we established six replicate plots in each successional stage (18 plots in total) that was 50–300 m distance. Plots of three successional forests were >1,000 m apart from one another (Liu et al., 2019). Six replicate plots of each representative successional stage were located on similar slope positions and elevation, and at least 100 m apart from any forest edge. The soils, Acrisols and Cambisols in the FAO soil classification (Duchaufour, 1993), were developed from the same quartzitic parent material in each successional stage (Song et al., 2011; Yan et al., 2009). Details of edaphic conditions and community characteristics are shown in Table S1. More information about the experimental sites, such as soil nutrients and microbial traits, can be found from Liu et al. (2019) and Zheng et al. (2019).

2.2 | Experimental design

In August, 2016, four 0.7 m × 0.7 m subplots in the centre of tree interspaces were established in each plot for the measurements of soil respiration (*R*s), and its three components: heterotrophic respiration of free-living saprotrophs (R_H), autotrophic respiration of mycorrhizal fungi (R_{myc}) and autotrophic respiration of roots (R_{root}). Subplots were separated by 5–8 m buffer zone in each plot replicate. Three treatments were randomly assigned in each subplot (see Figure S1). Total *R*s, the sum of $R_{root} + R_{myc} + R_H$, was included in two subplots in each plot. The microbial respiration ($R_{myc} + R_H$), which is the sum of R_{myc} and R_H, was measured by quantifying CO_2 fluxes from trenched subplots (0.8 ~ 1.0 m depth) that were lined with 50 μm nylon mesh (length: 3 m; width: 1 m) in order to exclude root ingrowth. A third treatment, R_H, was achieved by lining trenches with 1 μm nylon mesh in order to exclude root and mycorrhizal hyphal ingrowth. We set 24 subplots (4 sub-plots × 6 plot replicates) at each successional stage. All the trenches were refilled according to their original soil profiles to minimize the disturbance of trenching.

2.3 | Soil respiration measurement

A PVC collar (20 cm in diameter and 10 cm in height) was installed 5 cm into the ground in each sub-plot, with 5 cm above the soil surface, after all the trenches were refilled. CO_2 flux was measured for all PVC collars 3 months after collar installation. R_{root} was measured as the difference in CO_2 flux between paired '$R_{root} + R_{myc} + R_H$' and '$R_{myc} + R_H$' treatments in each plot. R_H was measured as CO_2 flux in 'R_H' sub-plots. We defined the difference in CO_2 fluxes between '$R_{myc} + R_H$' and 'R_H' sub-plots as mycorrhizal-induced changes in respiratory C loss (or C decomposition). Such changes may arise from respiration by mycorrhizal mycelium, respiration from free-living microbes feeding on mycelial inputs and respiration from mycorrhizal associated organic matter decomposition.

In our study, we investigated soil CO_2 flux in each subplot from December, 2016 to February, 2018. Soil CO_2 fluxes and its components were calculated from March, 2017 to February, 2018. Mycorrhizal effects on soil C respiratory loss (or C decomposition) were estimated as:

$$\text{Mycorrhizal effect} = \left(R_{(R_{myc}+R_H)} - R_{(R_H)}\right)/R_{(R_H)} \times 100\%, \quad (1)$$

where $R_{(R_{myc}+R_H)}$ and $R_{(R_H)}$ is soil CO_2 fluxes in the $R_{myc} + R_H$ and R_H subplots respectively. If soil CO_2 fluxes in $R_{myc} + R_H$ subplot were lower than that in R_H subplot, we attributed this suppressed CO_2 flux to Gadgil effect (Fernandez & Kennedy, 2016; Gadgil & Gadgil, 1971) of mycorrhizal growth on soil C decomposition. On the contrary, if CO_2 flux in $R_{myc} + R_H$ subplot was higher than that in R_H subplot, we ascribed the increased CO_2 flux to stimulated soil C decomposition and/or mycorrhizal metabolic respiration (R_{myc}). To eliminate above-ground plant respiration, we clipped all small living plants (seedlings and herbs) inside the subplots at the soil surface one day ahead of the measurements. Because only few seedlings and herbs might grow in the subplots each month, the clippings would not stimulate *R*s. Soil CO_2 flux in each subplot was measured once a month from November 2016 to February 2018, between 8:00 and 12:00 (local time), using a LI-COR 8100 portable soil CO_2 flux system (LI-COR. Inc.). Over the course of the morning, soil CO_2 flux among different treatments at each successional stage was randomly measured.

2.4 | Soil sampling

Soils from 0 to 10 cm soil layer were seasonally sampled with a cylindrical core (diameter: 3.5 cm, length: 10 cm) at four random points in each subplot in February, May, August and December, 2017. Soils in the four cores were mixed as a composite sample and sieved through a 2-mm mesh. A part of subsamples were stored at −20°C for analysing microbial community composition, and then lyophilized prior to extraction of lipid. Soil mineral N was extracted seasonally from field moist samples by shaking with 1 M KCl at soil to solution ratio of 1:10 (w/v) for 30 min. The supernatant was filtered through Whatman 42 filter paper. Mineral N was determined in the supernatant using a Holland Skalar San~ (++) continuous flow analyser (Quik Chem from method 10–107-064-D for NH_4^+ and 10,107-04-1-H for NO_3^-, Germany).

2.5 | Microbial biomass and community structure

Microbial biomass C (MBC) and N (MBN) from 0 to 10 cm soil layer were determined in May, August and December, 2017, by subtracting the total dissolved C and N (DOC and DON) of non-fumigated subsamples from that of the fumigated subsamples with a conversion factor of 0.45 and 0.38 respectively (Brookes et al., 1985). Extracts were prepared by mixing 10 g field moist soil with 40 ml K_2SO_4 (0.5 M) distilled solution on a shaker for 30 min. The mixture was then centrifuged at 4,000 rpm for 10 min and filtered through Whatman 42 paper and then a 0.45 μm filter membrane. The total dissolved C concentrations in the K_2SO_4 extracts were determined using a SHIMADZU TOC-VCPH/CPN analyser (Germany), and the total dissolved N concentration in the K_2SO_4 extracts was determined using a continuous flow analyser (Holland, Skalar San++).

Soil microbial community structure in 0–10 cm layer was determined by analysing group-specific PLFAs from a representative subsample of 10 g fresh weight frozen soil in February, May and August, 2017, using the protocol described in Fanin et al. (2014). PLFAs i15:0, a15:0, i16:0, i17:0, a17:0 16:1ω7c, cy17:0, 18:1ω7c, and cy19:0 were used as biomarkers of soil bacteria, while the 18:1ω9 and 18:2ω6,9 PLFAs were used to characterize soil fungi and 16:1 w5c to quantify arbuscular mycorrhizal (AM) fungi (Frostegård et al., 2011; Swallow et al., 2009). Soil PLFAs were analysed using a gas chromatography analyser (Hewlett Packard 5890 GC; Agilent, USA).

Potential enzyme activities of four C-degrading enzymes (cellobiohydrolases, CBH; soil β-glycosidases, βG; peroxidase, PER and polyphenolic, PPO) were measured on soil subsamples, which were collected from the different treatments ($R_{root} + R_{myc} + R_H$, $R_{myc} + R_H$ and R_H) in May 2017. Enzyme measurements began within 48 hr after soil sample collections. Sample suspensions were prepared by mixing 2 g field moist soil with 125 ml of acetate buffer (pH 5.0) and homogenizing for 1 min with a hand blender. CBH and βG activities were assessed by the microplate fluorescence method with the substrate containing umbelliferone (MUB). PER and PPO activities were measured in microplates based on light absorption using L-3, 4-dihydroxyphenylalanine (L-DOPA) as the substrate. The CBH and βG reactions last for 4 hr at 25°C, then 0.2 mol/L NaOH was added to stop the reactions. The PER and PPO reactions last for 20 hr at 25°C in the dark. After the incubations, enzyme activities were measured using a multi-function microplate reader (SpectraMax M5, Molecular 210 Devices, USA) with 365 nm excitation and 450 nm emission filters. The enzymatic activities were expressed in units of μmol hr^{-1} dry weight soil g^{-1}. All enzyme assays followed the method of Saiya-Cork et al. (2002) and Su et al. (2020).

2.6 | Net N mineralization potential

In situ net N mineralization rate was measured in each subplot in May 2017, using a PVC core method (modified from Raison et al., 1987). Three round-bottomed PVC cores (2.5 cm diameter × 20 cm length) were inserted 10 cm into the ground in each plot. The PVC cores were fitted with plastic U-type tubes to prevent water penetration but allow the entry of the air. Cores were incubated in the field for ~30 days. When PVC cores were inserted, three similar soil cores were taken in adjacent locations. Soils in the three cores were mixed as a composite sample and sieved through a 2-mm mesh. Soil samples before and after incubation were extracted with 1 M KCl, and soil mineral nitrogen (N) was measured as the methods mentioned above. Soil net N mineralization rate was calculated as following:

$$A_{amm} = \left(c\left(NH_4^+\text{-}N\right)_{after} - c\left(NH_4^+\text{-}N\right)_{before}\right)/\Delta t, \quad (2)$$

$$A_{nit} = \left(c\left(NO_3^-\text{-}N\right)_{after} - c\left(NO_3^-\text{-}N\right)_{before}\right)/\Delta t, \quad (3)$$

where A_{amm} was soil ammonification rate; $c\left(NH_4^+\text{-}N\right)_{before}$ and $c\left(NH_4^+\text{-}N\right)_{after}$ were soil ammonium-N concentration in the initial and incubated samples respectively. A_{nit} was soil nitrification rate; $c\left(NO_3^-\text{-}N\right)_{before}$ and $c\left(NO_3^-\text{-}N\right)_{after}$ were soil nitrate-N concentrations in the initial and incubated samples respectively. Δt was incubation time.

2.7 | Data analysis

Linear mixed-effects models (LMMs) analysis was used to test for effects of forest succession and root-mycorrhizae exclusion treatments on annual soil CO_2 fluxes, net ammonification and nitrification rates, soil properties (e.g. the concentrations of available N, soil moisture and temperature) and soil enzyme activities, with plot replicate as the random factor, using the 'NLME' package in *R* software (Pinheiro et al., 2012). We also used LMMs to examine effects of forest succession, root-mycorrhizae exclusion treatments, and sampling time on soil microbial characteristics, including the abundance of soil fungi abundance, AM fungi concentration, bacteria: fungi ratio, MBC, MBN and MBC:MBN ratio. Pairwise comparisons were tested using the lsmeans function in 'LSMEANS' package with a Tukey's adjustment of *p*-values (Lenth, 2016).

Factors regulating mycorrhizal effects on soil C respiratory loss were explored by Spearman correlation analysis. These factors included thirteen soil properties and eighteen soil microbial characteristics (see Figure S6). Relative contributions of these variables to mycorrhizal effects were explored via a principal component analysis (PCA) and general linear models (GLMs). Prior to PCA, nonlinear correlations between variables were examined and data transformation was applied to linearize the interrelationships between these variables. Variables were considered non-independent or with collinearity and not included in PCA when correlation coefficient $\rho \geq 0.7$ and $p < 0.001$ using a Spearman correlation test (Figure S6; Demenois et al., 2018). Meanwhile, soil moisture, mycorrhizal growth-induced alteration in soil moisture (i.e. altered soil moisture), altered MBC:MBN ratio, altered ammonium-N concentration, soil nitrate-N concentration, altered nitrate-N concentration, altered soil nitrification, altered soil fungal and AM abundance, soil CBH, PPO and PER activities and altered CBH and PPO activities were not included in

the PCA due to non-significant correlation with mycorrhizal effects ($p > 0.1$). Mycorrhizal growth-induced alteration in soil properties and microbial characteristics were calculated as (variables ($R_{myc} + R_H$ subplots) − variables (R_H subplots))/variables (R_H subplots) × 100%). Following PCA, GLMs were used to quantify the best model to predict mycorrhizal effects. We used the PCA scores of the first and second axis (Dim 1 and/or 2) for linear modelling with mycorrhizal effects, which allowed us to deal with the limited number of observation (18 observations (6 plots × 3 successional stages) for 16 independent explanatory variables (Table 2, Grueber et al., 2011). The best model was quantified as the combination of variables (Dim 1 and/or 2) that produced the lowest corrected Akaike information criterion (AICc). Figures were drawn with Sigmaplot 10.0 and *R* software (R Core Team, 2019).

3 | RESULTS

3.1 | Microclimate, soil respiration and its components

Soil temperature did not significantly differ among different treatments across the successional gradients (Figure S2, Tables S2 and S3). Soil moisture was higher in mesh-ingrowth subplots ($R_{myc} + R_H$ and R_H treatments) than non-treated subplots ($R_{root} + R_{myc} + R_H$ treatment; $F_{2,40} = 32.3$, $p < 0.01$), but there were no difference between the two mesh-ingrowth treatments (Figure 1a–c; Tables S2 and S3). The temporal dynamics of soil respiration (*Rs*) and its components exhibited obvious seasonal variations, which was high during summer and low in winter (Figure 1d–f). The average *Rs* was 3.37,

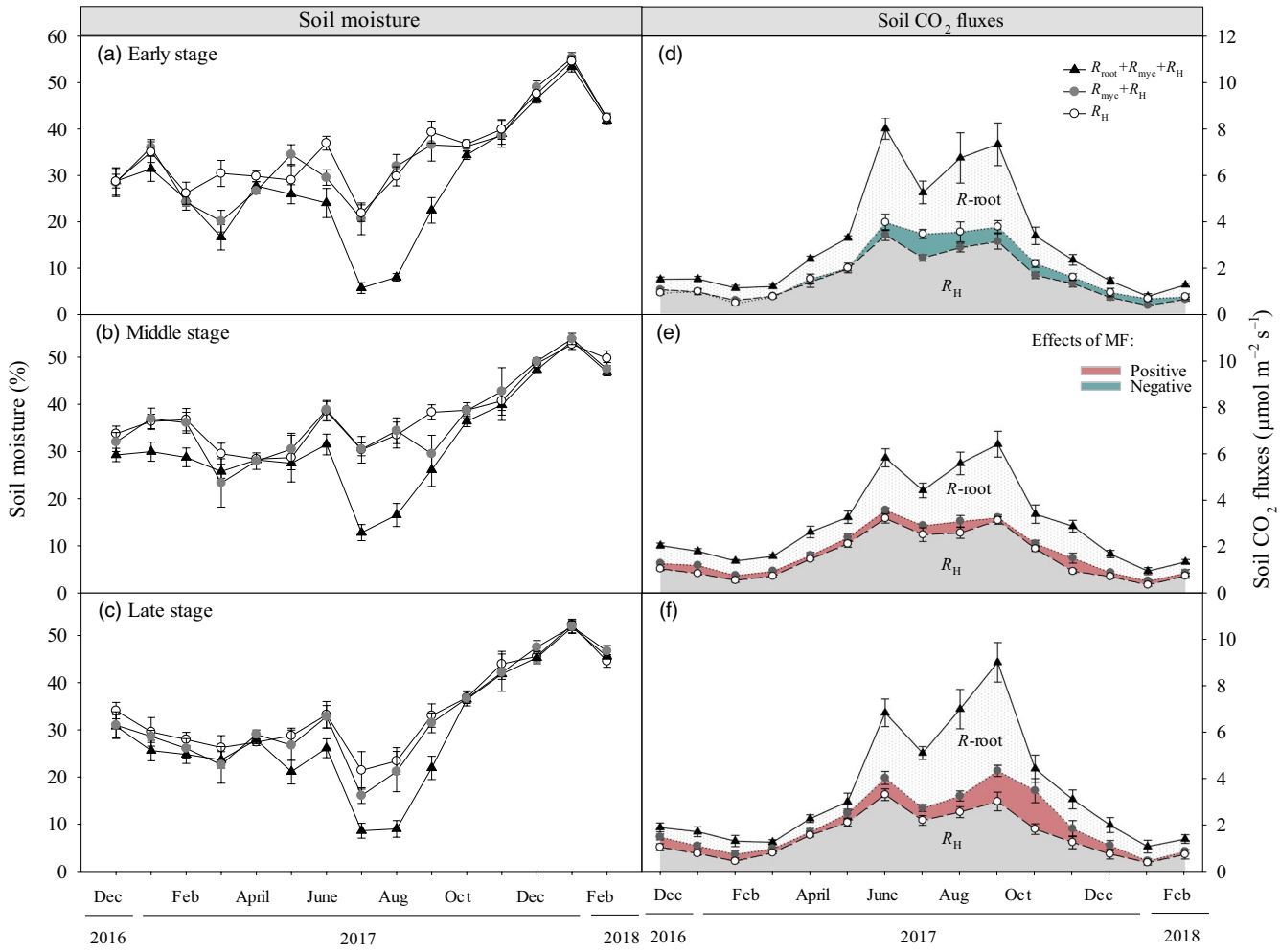

FIGURE 1 Temporal patterns of mean soil moisture (a, b, c) and mean CO_2 fluxes (d, e, f) at early-, mid- and late-successional stages of secondary forest succession respectively. Triangles and solid lines indicate soil moisture in non-treated plots (Showed by $R_{root} + R_{myc} + R_H$). Closed circles and hashed lines denote soil moisture in $R_H + R_{myc}$ plots. Open circles and dashed lines indicate soil moisture in R_H plots. The area in blue represents mycorrhizal growth-induced decrease in soil C mineralization (i.e. suppression of mineralization), whereas the area in red represents mycorrhizal growth-induced increase in respiration (i.e. enhancement of CO_2 flux). Mycorrhizal effects (%) were calculated as: $(R_{(R_{myc}+R_H)} - R_{(R_H)})/R_{(R_H)} \times 100\%$, $R_{(R_{myc}+R_H)}$ and $R_{(R_H)}$ is annual CO_2 fluxes in the $R_{myc} + R_H$ and R_H subplots respectively. The inset in panel (f) shows the net effects of mycorrhizal fungi on soil C mineralization at each stage of succession, where E = early-successional, M = mid-successional and L = late-successional stage. Vertical bars represent the standard error. Notes: R_{root}: root respiration; R_{myc}: mycorrhizal respiration and R_H: soil heterotrophic respiration

3.1 and 3.69 μmol m^{-2} s^{-1} in the early, middle and late stages of forest succession, respectively, with no significant difference among the successional stages (Table S2; $F_{2,15} = 2.4$, $p = 0.12$). However, succession altered the relative contribution of heterotrophic respiration (R_H) to *R*s. The average R_H accounted for 54.5% of *R*s at the early successional stages, which was higher than those at the middle (46.9%) and late stages (42.2%) of forest succession (Table S4; $F_{1,15} = 7.5$, $p < 0.05$).

Mycorrhizae-induced changes in soil respiratory C loss (or C decomposition) were calculated by subtracting respiration rates in the $R_{myc} + R_H$ treatment from those in the R_H treatment, which differed among three successional stages. Mycorrhizal growth suppressed soil C decomposition by 17% at the early-successional stage (Figure 1d; Table S5; $p < 0.05$), but accelerated soil respiratory C loss (by 20% and 32% respectively) at the mid- and late-successional stages (Figure 1e–f; $F_{1,15} = 7.4$, $p = 0.02$). Mycorrhizal growth increased soil CO_2 release by an average of 0.45 μmol CO_2 m^{-2} s^{-1} at the late-successional stage, which was higher than that of mid-successional stage (0.23 μmol CO_2 m^{-2} s^{-1}, Table S4; $t = -2.33$, $df = 10$, $p = 0.04$).

3.2 | Microbial biomass and nitrogen availability

Root and mycorrhizal growth ($R_{root} + R_{myc} + R_H$ and $R_{myc} + R_H$ vs. R_H treatments) increased soil fungal abundance, and decreased soil bacteria:fungi ratio (Figure 2a–f; Table S6). Soil arbuscular mycorrhizal (AM) fungi abundance in $R_{root} + R_{myc} + R_H$ and $R_{myc} + R_H$ treatments was higher due to root and mycorrhizal growth compared to R_H treatment, but only during the growing season (Figure 2e–f; Table S6; $F_{2,125.9} = 29.9$, $p < 0.05$). Soil microbial biomass carbon (MBC) based on the seasonal measurement in the non-treated control ($R_{root} + R_{myc} + R_H$) subplots was 921, 792 and 878 mg/kg on average in the early, middle and late stages of forest succession,

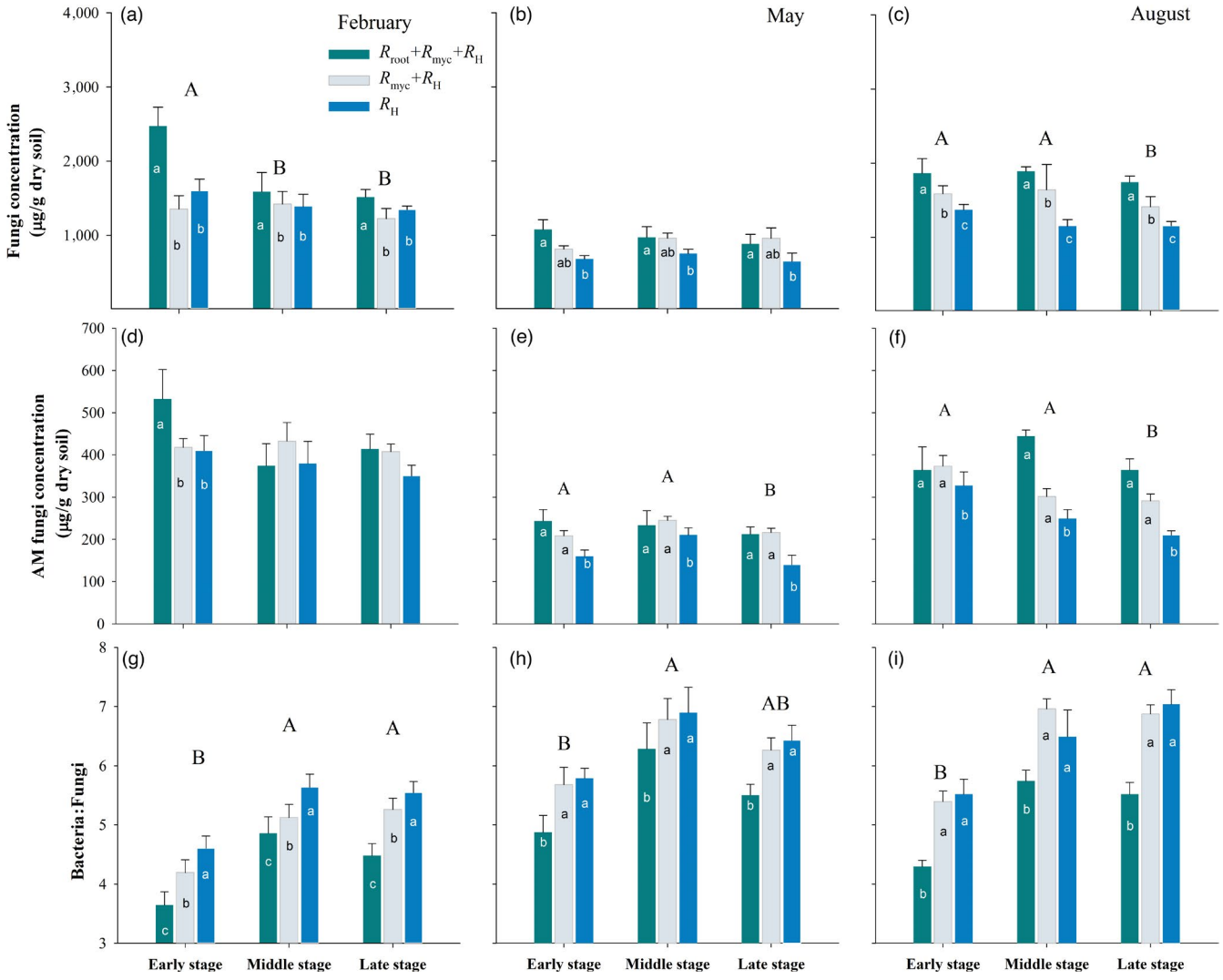

FIGURE 2 Mean fungi content (a, b, c), arbuscular mycorrhizal (AM) fungi content (d, e, f) and bacteria: fungi ratio (g, h, i) among different treatments at early-, mid- and late-successional stages of secondary forest succession in February, May and August, 2017 respectively. Different lowercase letters above bars show significant effects of treatments, and capital letters indicate significant differences among different successional stages, $p < 0.05$

respectively, with no significant difference among successional stages (Figure 3a–c, Table S7; $F_{2,15} = 0.5$, $p > 0.05$). MBC in the root-mycorrhizae exclusion plots (R_H treatment) was higher than that in the $R_{myc} + R_H$ plots at the early-successional stage, but similar to those in the $R_{myc} + R_H$ plots at the mid- and late-successional stages (Figure 3a–c). Microbial biomass nitrogen (MBN) and the ratio of microbial C to N (MBC:MBN ratio) were unaffected by mycorrhizal exclusion treatment ($R_{myc} + R_H$ vs. R_H treatment, MBN: $t = 1.52$, $df = 116$, $p = 0.28$; MBC:MBN ratio: $t = -0.62$, $p = 0.81$) and forest succession (Figure S4a–f, Table S7; MBN: $F_{2,15} = 2.7$, $p = 0.1$; MBC:MBN ratio: $F_{2,15} = 1.4$, $p = 0.28$).

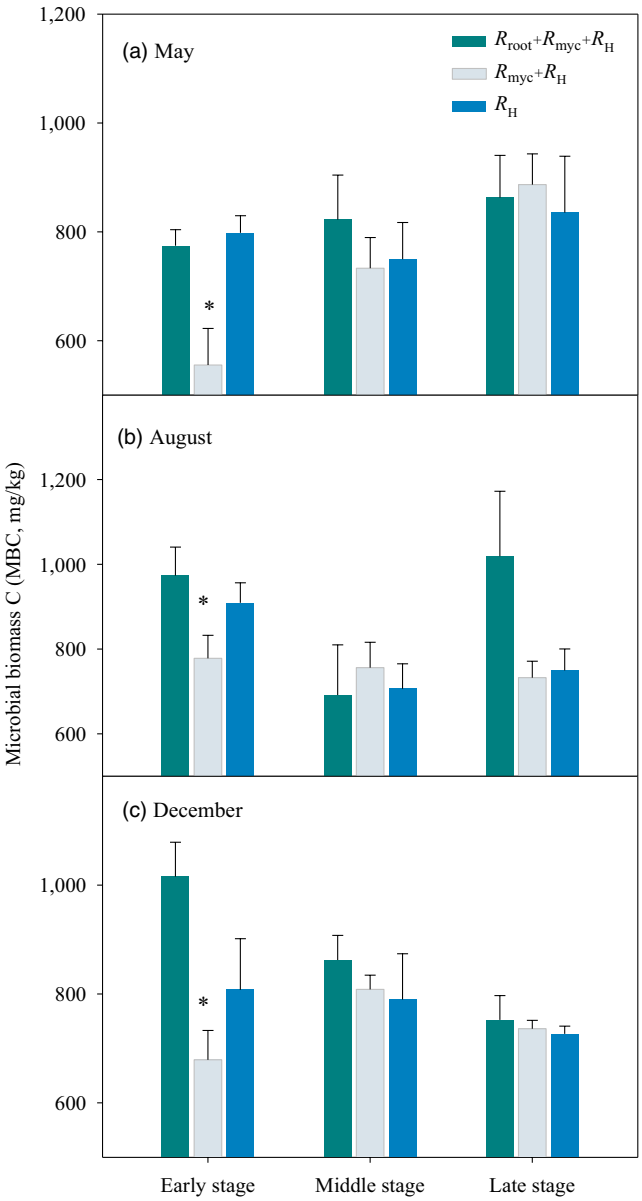

FIGURE 3 Microbial biomass carbon (C) among three treatments at different stages of secondary forest succession in May (a), August (b) and December (c), 2017. * shows significant effects of treatments when $p < 0.05$. Vertical bars represent the standard error

3.3 | Extracellular enzyme activities

Responses of soil C-degrading enzyme activities to root-mycorrhizae growth varied with different stages of secondary forest succession (Table 1). The presence of roots and mycorrhizal fungi ($R_{root} + R_{myc} + R_H$ and $R_{myc} + R_H$ vs. R_H treatments) largely decreased soil β-glycosidases (βG) and peroxidase (PER) activities (βG: $F_{2,10} = 7.5$, $p = 0.01$; PER: $F_{2,10} = 5.8$, $p = 0.02$), but did not affect soil cellobiohydrolases (CBH) and polyphenolic oxidase (PPO) activities at the early-successional stage (Table S8; CBH: $F_{2,10} = 1.8$, $p = 2.2$; PPO: $F_{2,10} = 0.01$, $p = 0.99$). However, these treatments did not impact the activities of soil C-degrading enzyme at the mid- and late-successional stages (Table 1; CBH: $F_{2,24} = 0.31$, $p = 0.73$; βG: $F_{2,24} = 0.11$, $p = 0.90$; PPO: $F_{2,34} = 2.9$, $p = 0.07$; PER: $F_{2,24} = 2.4$, $p = 0.11$). Mycorrhizal growth-induced difference in soil βG and PER activity was tightly correlated with altered soil respiratory C loss throughout forest succession (Figure S6, βG: $\rho = 0.57$, $p < 0.05$; PER: $\rho = 0.54$, $p < 0.05$).

3.4 | Net nitrification and ammonification

Soil net ammonification rate in the non-treated control ($R_{root} + R_{myc} + R_H$) subplots had an average of 20.2 mg kg^{-1} $month^{-1}$ at the early stage of forest succession, which was higher than that at the mid-successional stage (10.0 mg kg^{-1} $month^{-1}$) but lower than that at the late-successional stage (30.9 mg kg^{-1} $month^{-1}$; Table 1 and Table S5; $F_{2,15} = 19.9$, $p < 0.001$). Soil net ammonification rates in $R_{root} + R_{myc} + R_H$ and $R_{myc} + R_H$ treatments were higher than that in R_H treatment at the early-successional stage ($F_{2,10} = 7.0$, $p = 0.01$), but lower than that in R_H treatment at the late-successional stage ($F_{2,10} = 4.7$, $p = 0.04$). Soil net nitrification rate in the $R_{root} + R_{myc} + R_H$ plots was 13.9 mg kg^{-1} $month^{-1}$ at the early stage of forest succession, and increased along forest successions (Table 1 and Table S5; $F_{2,15} = 70.2$, $p < 0.001$). $R_{root} + R_{myc} + R_H$ treatment had higher soil net nitrification rate compared to R_H treatment at the late-successional stage ($F_{2,10} = 5.8$, $p = 0.02$), but there was no difference among treatments at the early- and mid-successional stages (Table 1, $p > 0.05$).

3.5 | Mycorrhizal contributions to soil C efflux

Principal component analysis (PCA) showed that the first principal component (Dim 1) and its combination with Dim 2 had significant impacts on mycorrhizal effects (Table S9). The best model (AICc = 145) on mycorrhizal effects was that one which only included Dim 1 (Table S9), explaining 74% of the variation in mycorrhizal effects on soil C respiratory loss (Figure 4b). Variables related to soil N supply (e.g. soil ammonium concentration, soil ammonification rate, soil TN and altered ammonification rate) had the largest loading weight in Dim 1 (Table 2).

TABLE 1 The mean soil C degrading enzyme activities, soil ammonification and nitrification rates under different treatments at three stages of forest succession (Data shown by mean (standard error))

Treatments	Early stage	Middle stage	Late stage
CBH (μmol g^{-1} hr^{-1})			
$R_{root} + R_{myc} + R_H$	1.7 (0.9)	2.8 (0.6)	2.5 (0.9)
$R_{myc} + R_H$	1.3 (0.4)	3.2 (1.3)	2.3 (0.6)
R_H	2.5 (0.4)	3.5 (1.4)	3.8 (1.1)
βG (μmol g^{-1} hr^{-1})			
$R_{root} + R_{myc} + R_H$	27.6 (6.9)**b**	34.6 (4.5)	48.8 (7.8)
$R_{myc} + R_H$	26.9 (2.6)**b**	29.2 (6.7)	44.2 (7.3)
R_H	60.8 (14.2)**a**	32.7 (6.6)	41.4 (9.1)
PER (μmol g^{-1} hr^{-1})			
$R_{root} + R_{myc} + R_H$	110.6 (14.8)**b**	144.4 (30.2)**b**	222.4 (48.1)
$R_{myc} + R_H$	133.2 (9.0)**b**	345.1 (106)**a**	276.6 (71)
R_H	219.6 (37.7)**a**	347.2 (42.2)**a**	279.8 (98)
PPO (μmol g^{-1} hr^{-1})			
$R_{root} + R_{myc} + R_H$	24.6 (8.6)	57.6 (12.5)	33.5 (8.3)
$R_{myc} + R_H$	25.8 (9.7)	56.0 (14.0)	38.4 (8.9)
R_H	24.8 (4.1)	28.7 (10.0)	23.7 (13.4)
Soil ammonification rate (mg kg^{-1} $month^{-1}$)			
$R_{root} + R_{myc} + R_H$	20.1 (1.5)**Bb**	10.0 (2.1)**C**	30.9 (4.0)**Aa**
$R_{myc} + R_H$	29.9 (5.1)**Ab**	10.8 (2.8)**B**	18.6 (6.9)**ABab**
R_H	44.8 (6.7)**Aa**	6.9 (1.5)**B**	10.6 (1.9)**Bb**
Soil nitrification rate (mg kg^{-1} $month^{-1}$)			
$R_{root} + R_{myc} + R_H$	13.9 (2.7)**Bab**	20.8 (6.3)**B**	101 (14.6)**Ab**
$R_{myc} + R_H$	37.7 (7.6)**Ba**	27.8 (3.8)**B**	146 (11.8)**Aab**
R_H	5.1 (1.5)**Bb**	27.3 (5.4)**B**	165.1 (23.0)**Aa**

Note: Different lower-case letters indicate significant differences among the three respiration partitioning treatments. Different capital letters indicate significant differences among the three successional stages. Significance was determined for $p < 0.05$. $R_{root} + R_{myc} + R_H$ represents non-treated control, $R_{myc} + R_H$ represents root exclusion but allowing mycelial growth treatment, R_H represents root and mycelial exclusion treatment. Bg, β-glycosidases; CBH, cellobiohydrolases; PER, peroxidase; PPO, polyphenolic.

4 | DISCUSSION

4.1 | Mycorrhizal fungi-saprotroph interactions on soil C efflux

Understanding microbial interguild interactions and their effects on soil C is crucial for predicting ecosystem responses to global change (Alberton et al., 2005; Talbot et al., 2008). In this study, we hypothesized that mycorrhizal fungi would suppress saprotrophic activity in early-successional stands due to their strong competition for N, and stimulate saprotrophic activity in older stands due to reduced competition for N. Overall, our hypotheses were partially supported. We found that changes in N availability during succession can influence mycorrhizae-saprotroph interactions, with dramatic consequences for soil C cycling (Table 2; Figures 4 and 5). In the early-successional forests with low soil N (Table S1), soil C decomposition was suppressed by ~17% by mycorrhizal growth, which supports the 'mycorrhizal fungi-saprotroph nutrient competition' hypothesis (Averill & Hawkes, 2016; Koide, 2019; Verbruggen et al., 2016). By contrast, in the relatively N-enriched mid- and late-successional forests, we observed the accelerated C respiratory losses. The factors responsible for the elevated CO_2 fluxes are not clear, likely resulting from both greater hyphal respiration and mycorrhizal-induced priming. Regardless of the mechanism, our results suggest that mycorrhizal fungi play a central role in soil C cycling in forests and as such, mycorrhizal effects should be accounted for in future investigations.

It is well known that early-successional forests often accumulate and sequester C in soils, whereas late-successional forests sustain a state of C input/output equilibrium (Shao et al., 2019; Yang et al., 2011). However, the degree to which interguild microbial interactions (e.g. between mycorrhizal fungi and free-living saprotrophs) affect inputs and outputs of soil C remains equivocal. Competition between mycorrhizal fungi and saprotrophs has been reported to accelerate soil C accrual by reducing SOC decomposition (Clemmensen et al., 2015) but the resources being competed for have rarely been identified. Based on the distinct contributions of different variables from Dim 1, we found that variables related to soil N supply (e.g. soil ammonium concentration, soil ammonification rate, soil TN and

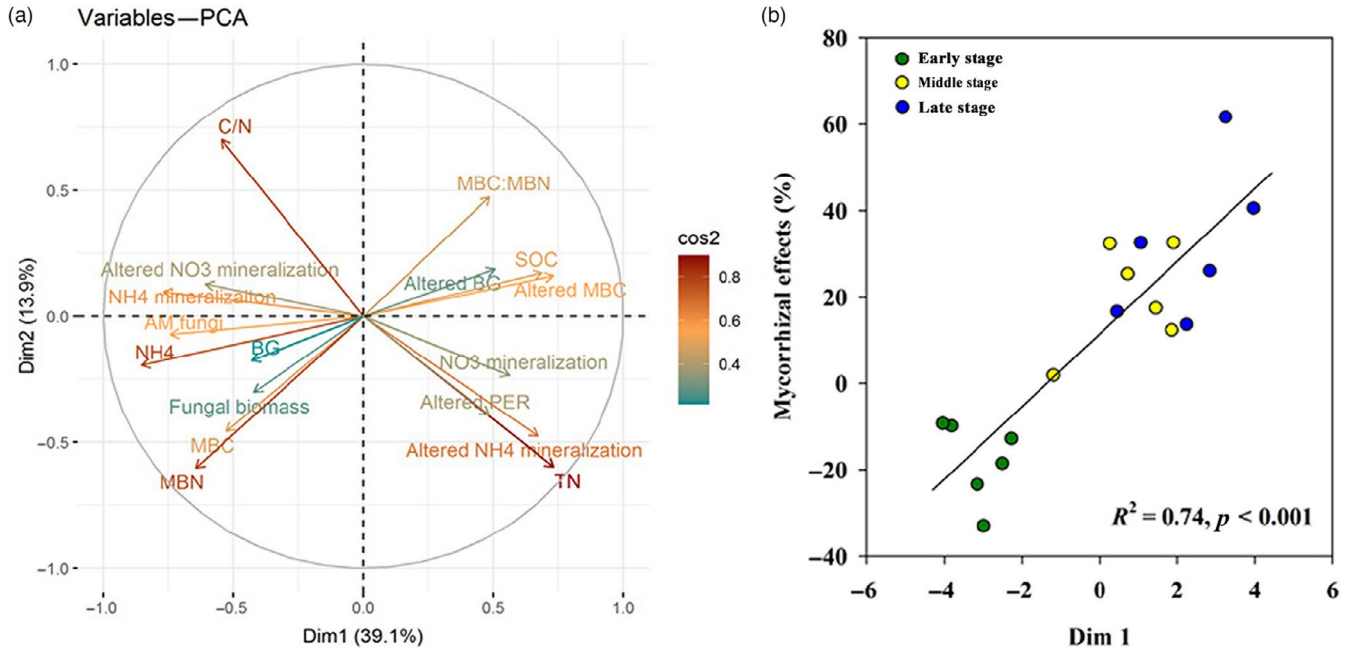

FIGURE 4 Principle component analysis of soil and microbial characteristics (a) and best general linear model for predicting mycorrhizal effects on soil C respiratory loss (b). The best model (AICc = 145) on mycorrhizal effects was that one that only included Dim 1. AICc of the model = 145, levels of significance: intercept < 0.01; Dim 1 < 0.01. PCA values of the first axis were used as a predictor for soil CO_2 efflux effects. Coloured dots correspond to each plot. Plant communities are indicated by different colours: dark green is the early-successional community; yellow is the mid-successional community; blue is the late-successional community

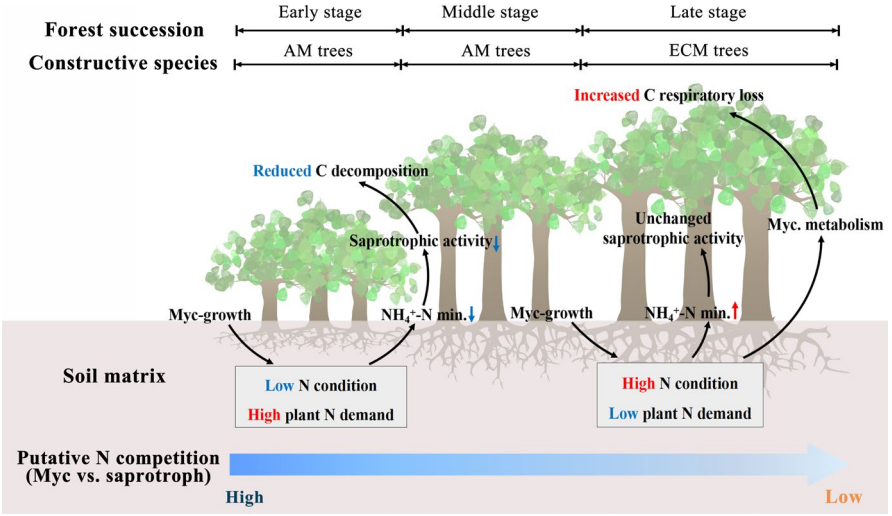

FIGURE 5 A conceptual framework to explain how mycorrhizae-saprotroph competition for soil N supply regulates soil CO_2 efflux in successional forests. Myc is mycorrhizal fungi, and NH_4^+-N min. is the ammonification rate. Small arrows represent the positive (red ones) and negative (blue ones) effects of mycorrhizal growth in the figure. Mycorrhizal fungi strongly compete with soil saprotrophs for limited N supply to reduce saprotrophic activity, leading to the suppression in microbial C decomposition and ammonification rate in the early-successional forests. Mycorrhizal growth did not affect soil saprotrophic activities in the mid- and late-successional forests with high soil N content, but largely increase soil CO_2 flux through mycorrhizal metabolism

altered ammonification rate) were the primary abiotic factors governing mycorrhizal effects (Table 2). Overall, N cycling was slowed in the youngest stands owing to reduced ammonification rates (which controls N supply) and lower TN (Figure S5; Table S1). This was exacerbated by mycorrhizal growth, which decreased ammonification rates in the young forests but not in the older forests (Figure S5). We did not quantify the effects of other nutrients, although some nutrients, especially soil phosphorus (P), might influence mycorrhizae-saprotroph interaction to some degree. However, previous studies conducted in our sites suggested that the early-successional forests were in a relatively N-limited condition (Yan et al., 2006). Thus, it seems reasonable to conclude that competition for N between

mycorrhizal fungi and soil saprotrophs was greatest in the young stands (Table 1), which explains the greater SOC accumulation rate (Table S1) and suppression of soil CO_2 fluxes compared to those in the mid- and late-successional forests.

What role do the mycorrhizal fungi play in this process? Early successional stands were dominated by plants associating with AM fungi, and these fungi can compete with free-living micro-organisms for N (Hodge & Storer, 2015), although the degree to which such competition affects soil C decomposition is debated (Verbruggen et al., 2016). We found that the presence of mycorrhizal fungi significantly decreased soil MBC and enzyme activity (e.g. βG and PER) in the young AM-dominated forests (Figure 3; Table 1). AM fungi can reduce soil N availability via mycelial uptake, which can suppress microbial activity (Veresoglou et al., 2012) in ways consistent with our results (Figure 3; Table 1). Although plant growth in subtropical and tropical forests is often presumed to be P limited (Vitousek et al., 2010; Wright, 2019), N limitation has been observed in young subtropical forests (Yan et al., 2006) and in aggrading tropical forests recovering from agricultural abandonment (Davidson et al., 2007). Thus, competition for soil N would be stronger in the early-successional forests, potentially leading to suppressed soil C decomposition. Second, AM roots and fungi can release exudates that directly suppress soil saprotrophic activity (Einhellig et al., 1993; Perry et al., 2005). Antimicrobial compounds have been reported in soils of AM-associating tree species such as *Cunninghamia Lanceolata* and *Schima Superba* in subtropical forests (Huang et al., 2000). However, this mechanism seems less likely to be driving soil C suppression in our system since tree community composition was relatively similar in the young versus mid-successional stands. Microbial biomass carbon (MBC) values in the non-treated control plots were similar to that in the R_H plots at the early-successional stages, which may be attributed to rhizosphere effects. It is well known that rhizosphere C input can increase microbial growth and enhance microbial activities (Finzi et al., 2015; He et al., 2020). We did not find significant mycorrhizal effects on soil microbial biomass and C-degrading enzyme activities in the mid- and late-successional forests (Table 1). This might be attributed to relatively high N availability in the mid- and late-successional forests, which may alleviate the N competition between mycorrhizae and saprotrophs (Lilleskov et al., 2002) and offset the negative effects of mycorrhizal fungi on soil microbial activities.

TABLE 2 Contributions of soil and microbial characteristics to Dim 1 of the principal component analysis

Variables	*Eigen vectors*	Contribution (%)	Cos^2
NH_4^+-N concentration	−0.332	11.66	0.74
AM fungi	−0.288	8.88	0.56
NH_4^+-N mineralization	−0.299	8.76	0.55
Soil TN	0.284	8.72	0.53
Altered MBC	0.284	8.44	0.55
SOC	0.267	7.74	0.49
Altered NH_4^+-N mineralization	0.26	7.30	0.46
MBN	−0.25	6.79	0.43
NO_3^--N mineralization	0.218	4.83	0.31
C:N ratio	−0.213	4.67	0.28
MBC	−0.205	4.46	0.30
Altered βG activity	0.196	4.18	0.26
MBC/MBN	0.188	3.78	0.22
Altered PER activity	0.187	3.52	0.24
βG activity	−0.167	3.15	0.20
Fungal biomass	−0.164	3.13	0.20

Note: SOC: soil organic carbon; Soil TN: soil nitrogen concentration; M: arbuscular fungi; Cos^2 is calculated as the squared coordinates for variables on the factor map of principal component analysis: cos^2 = coord × coord. Altered soil properties and microbial characteristics were calculated as: (variable(R_{myc} + R_H) − variable(R_H))/variable(R_H) × 100%.

There are two possible explanations for the elevated CO_2 fluxes in the older stands. First, mycorrhizal hyphae could have directly or indirectly enhanced enzyme and hydroxyl radical activities, both of which would reflect accelerated soil C decomposition. It is well-established that roots and mycorrhizal hyphae promote zones of intense microbial activity (referred to as the rhizosphere or mycorrhizosphere), where nutrient transformations and microbial activities are typically enhanced (Meier et al., 2015; Phillips & Fahey, 2008). So why did not mycorrhizal inputs enhance microbial biomass and enzyme activities in the mid- and late-successional stands (Figure 3; Table 1)? It is possible that our sampling frequency (monthly) and approach (i.e. not collecting soil adjacent to hyphae) limited out ability to detect the dynamic changes that likely occurred. We observed positive correlations of the mycorrhizal effect (on soil C respiratory loss) with the change in microbial biomass, and the change in the extracellular enzymes βG and Perox (Figure S6). Moreover, in August, when mycorrhizal effects on CO_2 fluxes were greatest in the late-successional stands (Figure 1), mycorrhizal ingrowth increased bacteria and fungi (Figure 2), being consistent with how mycorrhizal priming is believed to occur (Zhang et al., 2018). Thus, while we have limited direct evidence of mycorrhizal-induced priming, it seems reasonable to conclude that it contributed to the elevated CO_2 fluxes in the older stands. Future studies that use ^{13}C and ^{14}C isotope tracer techniques for the released CO_2 and SOC fractionation may be able to determine the relative importance of mycorrhizal hyphae versus mycorrhizal-accelerated decomposition (Staddon et al., 2003; Verbruggen et al., 2016). The other possible factor that likely contributed to the elevated CO_2 losses in the older stands is stimulation of hyphal respiration. In our study, changes in microbial biomass in the mycorrhizal subplots of the older stands suggest that increases in mycorrhizal biomass (and hence respiration) likely occurred.

Mycorrhizal respiration is rarely quantified in ecosystems, especially in forests at lower latitudes (Clark et al., 2001). In this study, we estimated mycelial respiration at the mid- and late-successional stage was 12 and 19 mg C m^{-2} hr^{-1}, which represents 16% and 21% of autotrophic respiration (R_A) respectively (Table S4). To date, few studies have estimated mycelial respiration in natural ecosystems (Nottingham et al., 2010), and our research is the first to our

knowledge in subtropical forests. Mycelial respiration was estimated as 15 mg C m^{-2} hr^{-1} in a temperate agro-ecosystem (Moyano et al., 2007) and 26.6 mg C m^{-2} hr^{-1} in a tropical AM-dominated secondary forest (Nottingham et al., 2010), which accounted for 25% and 26% of R_A respectively. Our estimation of mycelial respiration was close to that of Moyano et al. (2007) but lower than that of Nottingham et al. (2010), implying an important pathway of C fluxes from subtropical trees to the atmosphere. Mycelial respiration is higher in the late- relative to the mid-successional forests (Table S4), which may be attributed to the difference in plant photosynthesis (Heinemeyer et al., 2010), soil nutrient availability (Hughes et al., 2008) and mycorrhizal type (Clemmensen et al., 2015). Plant photosynthesis in the late-successional forests has been reported to be higher compared to those in the early-successional ones (Yang, 2001). Higher nutrient availability in the late-successional forests might also induce greater mycelial C use efficiency (Hagenbo et al., 2019), stimulating the respiration of external hyphae. Meanwhile, ecomycorrhizal fungi (ECM) associated with the constructive species in the late-successional forests might respire greater CO_2 than those arbuscular mycorrhizal (AM) fungi in the mid-successional forests, due to the greater mycelial network in ECM than AM fungi (Nottingham et al., 2010).

It is important to note that the methods for isolating mycorrhizal effects may affect soil moisture, and hence the estimates of mycorrhizal respiration (Averill & Hawkes, 2016). However, we found no difference in soil moisture and temperature between two mesh ingrowth treatments (Figure 1 and Figure S2; Table S2). As such, we attribute the main treatment effect to root-mycorrhizae exclusion rather than to potential environmental disturbance. Likewise, we anticipate that other disturbances related to the trenching would be insignificant several months after experimental instalments, which was similar to previous studies (Averill & Hawkes, 2016). Mycelial length density generally decreases with the distance from the plant roots (Papp et al., 2018), probably resulting in the higher fungal PLFA biomass in non-treated control relative to the mycorrhizal-growth (R_{myc} + R_H) mesocosms (Figure 2a,b). Thus, mycorrhizal effects on R_S may be underestimated in this study by using mesh ingrowth mesocosms. Meanwhile, a nylon mesh with 1 micron opening size likely allowed some mycorrhizal fungi with small diameter hyphae to enter. Despite this limitation, this method is generally accepted to explore the effects of mycelial growth on soil C and nutrient cycling (Heinemeyer et al., 2010; Nottingham et al., 2010). Although the limitations of experimental methods were not fully overcome, our experimental design was still suitable to explore the underlying mechanism by providing different mycorrhizae-growth gradients.

4.2 | Implications for future experiments and model development

Mycorrhizal fungi play key roles in soil C cycle, serving as conduits for plant-derived C (Heinemeyer et al., 2007; Moyano et al., 2007; Nottingham et al., 2010) but also mediators of SOC decomposition (Averill & Hawkes, 2016; Nottingham et al., 2013). Our results provide some insight to explain how and why mycorrhizal fungi affect soil C dynamics. First, inhibitive effects of mycorrhizal growth on soil C decomposition suggest that traditional root-mycorrhizae exclusion methods might overestimate R_H and underestimate R_A in nutrient-limited forest ecosystems. Hence, future experimental designs should pay attention to the importance of mycorrhizal C decomposition and its contribution to R_s. On the other hand, the respiration of mycelial hyphae accounted for a large part of R_s (9%~12%, Table S4) in the mid- and late-successional forests, indicating an important pathway of C flux from subtropical forest trees to the atmosphere. Given our limited understanding of what factors (e.g. forest types, mycorrhizal status and soil nutrient conditions) control mycelial respiration, more experiments are needed to elucidate the governing mechanism, especially in the context of climate change.

Second, the changes in mycorrhizal taxa along forest succession might considerably influence soil N cycling. Our results indicate that fungi in the late-successional forests are better at mining organic N than those in the early- and mid-successional forest, but suppress soil nitrification (Table 1). Unfortunately, the governing mechanism was not explored in this study since we did not measure the successional changes in mycorrhizal taxa. Considering that the constructive species in the late-successional forests are ectomycorrhizal trees, ectomycorrhizal fungi might help plants to forage N more efficiently (Liu et al., 2019). Which ectomycorrhizal taxa do this function of N acquisition is still unclear. Some ectomycorrhizal taxa like *Cortinarius* are known to mine organic matter for N, whereas others may be less able to do this (Lindahl et al., 2021; Tedersoo & Bahram, 2019). Hence, it is important to quantify the changes in ectomycorrhizal taxa and how these dynamics influence soil N cycling in future experimental designs.

Third, mediation of mycorrhizae-saprotroph interactions on soil C decomposition suggests these dynamics should be considered for inclusion in ecosystem models. As an important symbiosis that transport soil available nutrient to host plants, the activities of mycorrhizal fungi usually reflect the interactions among plants, soil and saprotrophic micro-organisms (Zhu & Miller, 2003). A few terrestrial biogeochemical models have considered these interactions (Oleson et al., 2010; Zaehle & Dalmonech, 2011), but the magnitude of interactive effects is difficult to quantify (Du et al., 2018; Thomas et al., 2013, 2015). Our results showed that the magnitude and direction of mycorrhizae–saprotroph interactions depended on nutrient availability gradients across successional stages, which were likely controlled by the balance between plant and mycorrhizal N demand and plant and fungal-induced changes in N supply. Incorporating this mechanism into C–N coupled models might be beneficial to quantify plant–saprotroph interaction and improve the simulation accuracy.

5 | CONCLUSIONS

Our study reveals that mycorrhizal fungi-saprotroph competition for soil available N likely regulates soil C efflux in subtropical successional forests. Our data suggest that mycorrhizal fungi compete

with soil saprotrophs for limited N supply and suppress microbial C decomposition in the early-successional forests, while it accelerates soil C respiratory loss in the mid- and late-successional forests. Thus, the magnitude and direction of mycorrhizal effects on microbial utilization of C during organic matter decomposition may be regulated by the balance between plant N demands and N supply along forest successional gradients. In addition, mycelial respiration accounted for more than 16% of belowground autotrophic respiration in the mid- and late-successional forests, indicating the critical role of mycorrhizal fungi in driving belowground C processes.

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AUTHORS' CONTRIBUTIONS

R.L. designed the experiment and wrote the manuscript; X.Z. conceived, designed and oversaw the experiment and revised the manuscript; Y.H., G.Z., H.Z., N.L., B.S., C.L., E.Y., X.C. and X.W. participated in the experimental design; J.S. and M.W. took part in statistical analysis; L.Z., S.H.B. and R.P.P. discussed and revised the manuscript together.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/1365-2745.13770.

DATA AVAILABILITY STATEMENT

Data of soil CO_2 fluxes, soil properties and microbial characteristics are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.8gtht76q3 (Liu et al., 2021).

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SUPPORTING INFORMATION

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