Stage-specific regulation of C-degrading genes and microbial metabolism activity during cultivation and incorporation of different leguminous green manure drives SOC pool accumulation in tropical soils

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Abstract

High temperatures and humidity in tropical climates hinder the effective accumulation of soil organic carbon (SOC) during fallow periods. Planting and returning leguminous green manure can promote the formation and long-term stability of SOC fractions through the microbial carbon pump (MCP) mechanism. However, the mechanisms of microbial carbon (C) sequestration at the functional gene level under different green manure crops and across management stages remain poorly understood. Here, we systematically analyzed the effects of *Crotalaria juncea* (CJ), *Crotalaria pallida* (CP), and *Sesbania cannabina* (SC) on SOC fractions, microbial C metabolic activity, and carbohydrate-active enzyme (CAZymes) gene abundance during cultivation and incorporation stages. Results indicate that compared to the fallow control (CK), both CP and SC significantly increased SOC and microbial biomass carbon (MBC) content during the planting and incorporation stages. Notably, the SC produced a greater increase during the incorporation stage, with SOC and MBC increasing by 51.3% and 49.1%. Both the cultivation and incorporation of green manure reduced the abundance of CAZyme genes involved in the degradation of plant-derived components (e.g., cellulose and hemicellulose). However, green manure selectively enriched chitin-degrading CAZyme genes during cultivation and peptidoglycan-degrading CAZyme genes during incorporation. Partial least squares path model revealed that changes in the functional gene profile significantly affected microbial C metabolic activity and SOC pools, and were regulated by microbial community reorganization. Moreover, CP and SC exhibited superior soil amelioration efficacy due to their higher nutrient accumulation and nitrogenase activity. Overall, variations in the abundance of CAZyme genes encoding the degradation of different C sources influence microbial C metabolic activity through the MCP mechanism, thereby promoting the accumulation of SOC pools under different leguminous green manures applications. These findings provide important theoretical and practical implications for improving soil fertility and C sequestration function in tropical farmland systems.

1. Introduction

Soil represents the largest organic carbon (C) reservoir in terrestrial ecosystems (Jia et al. 2024; Feng et al. 2024). Enhancing soil organic carbon (SOC) sequestration in croplands is therefore crucial for maintaining soil fertility and strengthening the resilience of agroecosystems (Hu et al. 2023; Zhu et al. 2025). SOC primarily originates from plant residues (e.g., litter and root exudates) and microbial necromass, with its accumulation and stability depending on complex biotransformation processes (Feng et al. 2024; Zhu et al. 2025). Plant biomass consists mainly of cellulose, hemicellulose and lignin, forming a structurally complex organic matrix characterized by marked differences in decomposability, with cellulose and hemicellulose being relatively labile and lignin more resistant to microbial degradation (Jing et al. 2021; Huang et al. 2024). As plant residues decompose, part of the plant-derived C is incorporated into microbial biomass and necromass, which are largely composed of peptidoglycan and chitin and constitute a major component of SOC (Huang et al. 2024). Microorganisms decompose and transform dead biomass through both “in vivo turnover” and “ex vivo modification”, sequestering its residues and metabolites through the subsequent “entombing effect” (Zhao et al. 2023; Zhu et al. 2025).

Notably, the microbial carbon pump (MCP) mechanism can convert some readily degradable plant-derived C into recalcitrant microbial-derived C (Huang et al. 2024; Feng et al. 2024), playing a crucial role in the formation and persistence of SOC components. Organic substrates of varying chemical properties exert selective pressure on microbial community composition and metabolic patterns, thereby influencing their C source utilization efficiency and metabolic strategies (Dong et al. 2024; Fu et al. 2025). Microorganisms secrete extracellular enzymes to degrade polysaccharides and aromatic compounds and accelerate organic matter mineralization (Chen and Sinsabaugh 2021). Furthermore, studies have shown a significant positive correlation between the abundance of C degradation-related genes and the activity of the corresponding extracellular enzymes (Chen and Sinsabaugh 2021; Huang et al. 2024). Therefore, exploring the coupled relationship between microbial community structure, functional genes, and metabolic activity is crucial for elucidating the mechanisms of SOC formation, particularly within tropical agroecosystems receiving different types of organic substrates.

Carbohydrate-active enzymes (CAZymes) are commonly used to study the functional responses of microorganisms in C cycling and play a crucial role in promoting SOC decomposition (Jing et al. 2021; Yu et al. 2024). According to the CAZyme families database (http://www.CAZy.org), glycoside hydrolases (GHs) and carbohydrate esterases (CEs) play a central role in the degradation of hemicellulose, cellulose, chitin, and glucans, while auxiliary activity enzymes (AAs) are crucial for lignin breakdown (Sime et al., 2024; Xiong et al., 2023). Furthermore, carbohydrate-binding modules (CBMs) facilitate substrate binding and recognition (Xiong et al. 2023). Recent studies have shown that the dynamics of SOC fractions are jointly influenced by C inputs and their degradation by different CAZyme families (Huang et al. 2024; Yu et al. 2024). Therefore, different C sources not only significantly alter soil physicochemical properties and nutrient cycling but also drive the reshaping of microbial community structure, leading to adjustments in C source utilization strategies and functional gene abundance, ultimately influencing SOC decomposition and C cycling (Huang et al. 2024; Dong et al. 2024; Fu et al. 2024). However, the response patterns of microbial CAZyme families to different types of SOC sources and their regulatory roles in SOC formation and turnover have yet to be systematically investigated.

The Nanfan National Seed Breeding Base leverages the mild winter climate of Hainan, China, to shorten breeding cycles, playing an irreplaceable strategic role in safeguarding food security. Locally, crop rotation or fallow periods are implemented from June to September each year to reduce pests and diseases and restore soil fertility. However, the high temperatures and humidity in tropical regions accelerate the mineralization of SOC (Wu et al. 2025). Cultivating and incorporating green manure crops during fallow periods increases ground cover, enhances soil fertility, and regulates microbial metabolic processes through the MCP mechanism to promote SOC sequestration (Zhou et al. 2025; Qiao et al. 2025). Green manures not only optimize soil microbial community structure and improve C source utilization efficiency, but also influence OM degradation by regulating extracellular enzyme activity (Zhou et al. 2020; Li et al. 2024). Owing to its generally low C:N ratio, high biomass, and rapid degradability, green manure sustains microbial activity and strengthens SOC sequestration potential (Xu et al. 2023; Li et al. 2024). During the green manure growth period, microorganisms utilize readily degradable C sources in root exudates and living biomass to proliferate and secrete extracellular enzymes, fixing some

of the plant-derived C as microbial necromass C, promoting the initial accumulation of SOC (Zhou et al., 2020). After green manure is returned to the field, microorganisms assimilate the green manure residue C and use extracellular enzymes to decompose complex organic matter, converting the microbial necromass into stable SOC by combining them with minerals, achieving long-term C sequestration (Oliveira et al. 2019; Yang et al. 2023). Although green manure application is known to enhance SOC accumulation through microbial metabolic processes and MCP, most existing studies have focused on a single stage (e.g., cultivation or incorporation phase) of the practice. Moreover, external C source interference not only changes the composition of microbial communities but also regulates carbohydrate metabolism (Xie et al. 2022; Xiong et al. 2023). Consequently, systematic insights into stage-specific microbial CAZyme responses and associated C stabilization pathways under green manure management remain lacking.

The quality and functional characteristics of green manure crops play a crucial role in determining the accumulation and stability of SOC pools. Studies have shown that legume green manures are more conducive to the accumulation of particulate organic carbon (POC) and mineral-associated organic carbon (MAOC) than non-legume green manures (Hu et al. 2024). This difference is mainly attributed to the fact that legume green manures improve substrate supply and nitrogen (N) availability due to their high-quality residues, thereby activating the microbial transformation pathway of SOC and promoting the accumulation and stabilization of bacterial and fungal necromass C (Hu et al. 2023, 2024). Given that *Crotalaria juncea* (*C*. *juncea*), *Crotalaria pallida* (*C*. *pallida*), and *Sesbania cannabina* (*S*. *cannabina*) exhibit excellent adaptability to hot and humid environments and are widely used leguminous green manures in tropical regions (Arone et al., 2024; Huan et al., 2014, 2017). Therefore, this study selected three species to systematically investigate their effects on soil chemical properties, microbial C metabolic activity, extracellular enzyme activities (EEAs), microbial functional gene abundance, SOC and its fractions during the cultivation and incorporation stages. Specific research objectives are to: 1) determine the abundance of microbial CAZyme genes involved in the decomposition of plant-, bacterial-, and fungal-derived C under different green manure types and management stages; 2) elucidate the relationships among microbial C metabolic activity, EEAs, and the abundance of CAZyme family genes; and 3) identify the key factors governing the formation of SOC fractions in tropical agricultural ecosystems. Based on these objectives, we hypothesize that the cultivation and incorporation of leguminous green manure modulate the abundance of C degradation genes and microbial metabolic activity by regulating the MCP function, thereby promoting the formation and accumulation of SOC in tropical regions.

2. Materials and Methods

2.1. Study site and soil characteristics

The experimental site was located at the Sanya Research Base of Nanjing Agricultural University in Batou Village, Yazhou District, Sanya City, Hainan Province (109.140°E, 18.384°N). The region has a tropical marine monsoon climate, with an average annual temperature of 26.5°C, an average annual sunshine duration of 2572.8 h, and an average annual precipitation of 1347.5 mm. The local cropping

season extends from October to April of the following year, while May to September is a fallow period during which green manure rotation or fallowing is commonly practiced to restore soil fertility and mitigate the disturbance of extreme weather events (e.g., typhoons). The soil type was identified as a Gleyed Paddy Soil (sandy loam) according to the Genetic Soil Classification of China (GSCC). The baseline soil properties were as follows: pH 5.71, SOC 9.38 g kg^{-1}, total nitrogen (TN) 0.52 g kg^{-1}, total phosphorus (TP) 1.51 g kg^{-1}, available phosphorus (AP) 81.03 mg kg^{-1}, and available potassium (AK) 323.81 mg kg^{-1}. Prior to the commencement of the experiment, the soil at the study site was amended with biochar and incorporated green manure in 2022.

2.2. Experimental design and soil sampling

A tropical leguminous green manure variety screening experiment conducted in October 2022 confirmed that *C. juncea* (CJ), *C. pallida* (CP), and *S. cannabina* (SC) exhibited superior performance due to their high biomass and nutrient accumulation (Table S8). Accordingly, these three species were selected as the green manure treatments in this study, together with a no green manure control (CK). A large-plot design was employed, with each treatment occupying 500 m^2. Green manure was sown on 15 June 2023 at rates of 45 kg ha^{-1} for CJ, 30 kg ha^{-1} for SC, and 30 kg ha^{-1} for CP. Prior to sowing, the soil was tilled and levelled to create a suitable microenvironment for seed germination, and sowing was timed following natural rainfall to enhance emergence. After sowing, seeds were lightly covered with soil using hand tools. The green manure was incorporated into the soil layer at 0–20 cm by rotary tillage at peak flowering on August 20, 2023. The aboveground biomass, nutrient content, and nodule nitrogenase activity of the three green manures were measured and are shown in Table S1. Throughout the experiment, no irrigation, weeding, or pest and disease control measures were applied to any treatment plots.

Surface soil samples (0–20 cm) were collected using a multi-point composite sampling method at the flowering stage of green manure (19 August) and one month after its incorporation (20 September). For each plot, seven to eight soil cores were taken with an auger and thoroughly mixed to form a composite sample of approximately 1 kg. Three composite samples were collected per treatment. All samples were collected in sterile plastic bags and immediately transported to the laboratory on dry ice. Each soil sample was divided into three subsamples according to analytical requirements: one was stored at 4°C for the determination of microbial biomass C (MBC) and N (MBN), microbial respiration (MR), and EEAs; another was stored at − 80°C for DNA high-throughput sequencing; and the remaining portion was air-dried at room temperature for the analysis of SOC fractions and other physicochemical properties (detailed descriptions are provided in the supplementary materials).

2.3. SOC fractions and microbial carbon metabolic activity

SOC was determined using a total organic carbon (TOC) analyzer (multi N/C 3100, Analytik Jena, Germany). Dissolved organic carbon (DOC) content was extracted using a 5:1 water-soil mixture, filtered, and quantified with the TOC analyzer. POC and MAOC fractions were separated following the method of particle-size fractionation (Huang et al. 2024). Briefly, 20 g of air-dried soil was dispersed in 60 mL of 5 g

L^{-1} sodium hexametaphosphate by shaking for 18 h, and the suspension was passed through a 0.053 mm sieve to obtain the POC (> 0.053 mm) and MAOC (< 0.053 mm) fractions. Both fractions were oven-dried at 65°C, ground to 0.15 mm, and analyzed for carbon content using the TOC analyzer.

MR was determined using the alkali absorption method, with respiration intensity expressed as the amount of CO_2 absorbed (Jia et al. 2025). MBC was measured by the chloroform fumigation extraction method and quantified using a TOC analyzer (Vance et al. 1987). The metabolic quotient (qCO_2) was calculated as the ratio of MR to MBC, while the microbial quotient (qMB) was obtained as the ratio of MBC to SOC (Huang et al. 2024).

The activities of C-hydrolytic enzymes [α-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CL), and β-xylosidase (XYL)] were determined using a 96-well microplate fluorometric assay (Bell et al. 2013). The activities of C-oxidative enzymes [peroxidase (POD) and polyphenol oxidase (PPO)] were assayed colorimetrically using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate, based on the formation of colored oxidation products (Huang et al. 2024).

3.4. Soil DNA extraction, metagenomic sequencing, and CAZyme annotation

Total genomic DNA was extracted from 0.2 g of fresh soil using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) following the manufacturer's instructions. The concentration and purity of the extracted DNA were determined using SynergyHTX and NanoDrop2000, respectively. DNA was fragmented using a Covaris M220 (Gene Corporation, China), and fragments of approximately 350 bp were selected. Paired-end (PE) libraries were constructed with the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, USA). Metagenomic sequencing was performed using the Illumina NovaSeq™ X Plus (Illumina Inc., San Diego, CA, USA) sequencing platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). High-quality reads were assembled into contigs ≥ 300 bp using MEGAHIT (Li et al. 2015), and open reading frames (ORFs) ≥ 100 bp were predicted and translated into amino acid sequences using Prodigal (Hyatt et al. 2010). All predicted genes were clustered at 90% identity and 90% coverage using CD-HIT to generate a non-redundant gene set (Fu et al. 2012). SOAPaligner was used to calculate gene abundance at a 95% sequence identity threshold (Li et al. 2008), and taxonomic annotation was performed by aligning sequences against the NR database using Diamond (Buchfink et al. 2015). A total of 7,694,730 bacterial genes and 3,505 fungal genes were obtained (Table S2).

The amino acid sequences of the non-redundant gene set were aligned against the CAZy database using HMMER to obtain CAZyme annotations (Lombard et al. 2014). The CAZy database classifies CAZymes into six families related to biomass degradation: glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), and auxiliary activities (AAs). Gene abundance for each CAZyme family was calculated by summing the abundances of corresponding genes, allowing comparison of soil C-cycling functional genes across different green manure incorporation treatments. A total of 9,817,688 and 10,370,136 CAZyme genes were identified in the metagenomes during the green manure cultivation and incorporation stages,

respectively, predominantly assigned to bacteria (97.2%), with fungi accounting for 0.01% and 2.8% unclassified (Table S3).

2.5. Data Analysis

One-way ANOVA followed by Duncan's post hoc test ($p < 0.05$) was performed using IBM SPSS Statistics 21 (SPSS, Chicago, IL, USA) to assess significant differences in soil chemical properties, SOC fractions, microbial C metabolic activity, EEAs, microbial community abundances, α-diversity indices, and CAZyme family gene abundances among different green manure treatments. Two-way ANOVA was conducted to evaluate the effects of different management stages of green manure (GS), green manure types (GT) and their interactions (GS×GT) on SOC and its fractions. Microbial community composition was visualized using principal coordinate analysis (PCoA) based on Bray–Curtis distances in R (v. 4.4.3). ANOSIM was used to test the significance of community differences among treatments. LEfSe analysis (microeco package, R) was used to identify significantly enriched taxa among all treatments based on the Kruskal–Wallis and Wilcoxon tests, with an LDA score threshold of > 3.0 ($p < 0.05$). Pearson correlation and Mantel tests were conducted in R using the "linkET" package to explore relationships between CAZyme family abundances involved in plant- and microbial-derived C degradation, microbial community composition, and microbial C metabolic activity. Linear regression (Origin 2024, v. 10.1.0.178) was employed to evaluate the relationships between C-degrading genes (based on the relative abundance of CAZyme families) and SOC fractions, as well as between specific EEAs and the corresponding CAZyme family gene abundances annotated in the CAZy database. The random forest model was used to calculate the percentage increase in mean squared error (%IncMSE) using the "randomForest" package in R to assess the importance of environmental variables. A partial least squares path model (PLS-PM) was developed using the "plspm" package to investigate the microbial sequestration pathways of SOC pools influenced by green manure cultivation and incorporation in tropical regions.

3. Results

3.1. Changes in SOC and its fractions

The management stage ($p < 0.01$) and type ($p < 0.001$) of green manures significantly influenced the content of SOC, POC, and MBC (Fig. 1). During the cultivation phase, CP and SC increased SOC and DOC compared to the CK, with the SC treatment showing the greatest increase, raising SOC and DOC by 11.7% and 42.0% respectively (Fig. 1A and D). Furthermore, the SC treatment elevated POC content by 26.0% relative to the CK treatment (Fig. 1B). Compared to the CK, all green manure treatments increased MBC, with respective increases of 23.5%, 31.4%, and 31.6% under the CJ, CP, and SC treatments (Fig. 1E). However, no significant effect was observed on MAOC across all treatments (Fig. 1C, $p > 0.05$). Green manure treatment exerts a greater influence on SOC and its fractions during the incorporation phase than during the cultivation phase, particularly with respect to SOC, POC and MBC. During the incorporation stage, all green manure treatments increased SOC content relative to the CK, with

increases of 25.3%, 39.8%, and 51.3% under the CJ, CP, and SC treatments respectively (Fig. 1A). POC and MBC content increased under CP and SC treatments relative to CK, with SC exhibiting the highest increase at 42.2% and 49.1% respectively (Fig. 1B and E). Furthermore, SC treatment significantly elevated MAOC and DOC content by 60.2% and 34.7% compared to CK (Fig. 1C and D). In all treatments, the proportion of mMAOC (61.1–67.6%) was higher than that of mPOC (32.4–39.0%) (Table S7), and the combined contribution of POC and MAOC to SOC exceeded 0.96 (Fig. 1F).

3.2. Changes in microbial community composition and diversity

PCoA revealed that the first axis explained 34.5% and 39.9% of the variation in bacterial and fungal communities, respectively, clearly distinguishing community structures across different management stages and types of green manure (Fig. 2A, $p < 0.001$). During the cultivation stage, the CJ treatment significantly increased the abundance of *Ascomycota* by 67.7% and the fungal Shannon index by 6.7% compared with the CK treatment (Fig. 2B and C). The CP treatment significantly increased the abundance of *Acidobacteria* by 11.0% relative to CK. Compared with CK, the SC treatment significantly increased the abundance of *Actinobacteria* by 5.1% and the bacterial ACE index by 2.9%, but significantly decreased the abundance of *Chloroflexi* by 19.6% and the bacterial Shannon index by 2.6%. During the incorporation stage, the CJ, CP, and SC treatments significantly increased the abundance of *Ascomycota*, *Basidiomycota*, and *Actinobacteria* by 23.7%, 33.6%, and 13.6%, respectively, compared with CK. In addition, relative to CK, the CP treatment significantly reduced both bacterial and fungal ACE indices, whereas the SC treatment significantly increased the bacterial Shannon index and the fungal ACE index.

LEfSe analysis further indicated that green manure treatments exhibited greater microbial biomarker species richness in soil compared to the CK (Fig. S1, $p < 0.05$, LDA > 3). Specifically, during the cultivation stage, bacterial taxon, *Acidobacteriia* was enriched in the SC treatment (Fig. S1A), while the fungal taxa, *Ascomycota* was preferentially enriched in the CJ treatment (Fig. S1B). Following green manure incorporation, the bacterial taxa, *Acidobacteria* and *Streptosporangiales* were enriched in the CJ and CP treatments, respectively (Fig. S1C). In addition, the fungal taxa, *Lecanoromycetes*, *Mucor*, and *Glomeromycetes* were enriched in the CJ, CP, and SC treatments, respectively (Fig. S1D).

3.3 Changes in the abundance of CAZyme genes encoding the degradation of plant- and microbial-derived components

NMDS analysis revealed distinct differences in CAZyme gene composition among the green manure treatments (Fig. S2, $p < 0.001$). A total of 73 CAZyme genes associated with the degradation of plant-derived (cellulose, hemicellulose, lignin), bacterial-derived (peptidoglycan), and fungal-derived (chitin and glucans) components were identified from the CAZy database (Table S4). The abundance of CAZymes

involved in the degradation of plant-derived substrates (> 90%) was higher than that of those associated with microbial-derived substrates (> 9%) (Table S5).

A total of 30 and 31 genes showing significant differences between the green manure treatments and the CK were identified during the cultivation and incorporation stages, respectively (Fig. 3A, $p < 0.05$). Specifically, during the cultivation stage, green manure treatments significantly increased the abundance of chitin-degrading CAZyme genes by 6.0–11.9% compared with CK (Table S5, $p < 0.05$), primarily due to the upregulation of GH19, GH20, and GH18, with the strongest response observed under the SC treatment (Table S6, $p < 0.05$). In addition, the CJ treatment significantly enhanced the abundance of CAZyme genes involved in glucans degradation (e.g., GH17 and GH55) by 7.8%. Relative to CK, the SC treatment further increased the abundance of CAZyme genes associated with peptidoglycan degradation (e.g., GH103, GH25, and GH73) by 3.4%, while reducing the abundance of lignin-degrading CAZyme genes (e.g., AA3 and AA7) by 2.5% ($p < 0.05$).

During the incorporation stage, the effects of green manure treatments on CAZyme gene abundance differed from those observed during the cultivation stage (Table S5 and S6). Compared with CK, green manure treatments significantly increased the abundance of CAZyme genes involved in glucans degradation by 5.8–9.6%, primarily driven by the concurrent upregulation of GH103 and GH108 across all three green manure treatments ($p < 0.05$). Among them, the SC treatment exhibited the largest increase, attributable to the significant upregulation of GH23 and GH25. Under the CP treatment, the abundances of CAZyme genes involved in hemicellulose degradation (e.g., CE1, GH16, GH26, GH36, and GH39) and chitin degradation (e.g., GH18 and GH20) were significantly increased by 1.1% and 7.3%, respectively. In contrast, the CJ treatment significantly reduced the abundance of CAZyme genes involved in cellulose degradation (e.g., GH1, GH5, GH8, and GH9) by 6.4%.

3.4 Changes in soil microbial C metabolism activity and chemical properties

During the cultivation stage, green manure application increased soil pH, AK, MBN, CL, and qMB by 1.1–3.2%, 27.7–112.6%, 16.5–20.2%, 22.5–31.0%, and 14.2–19.1%, respectively, compared with CK (Table S7, $p < 0.05$). BG activity increased by 4.7% and 6.0% under the CJ and CP treatments, respectively, and the CP treatment also increased AG activity by 19.5% ($p < 0.05$). In contrast, the SC treatment reduced PPO activity by 7.0% ($p < 0.05$). During the incorporation stage, green manure treatments increased soil pH, AK, DON, and PPO by 3.6–6.7%, 144.3–248.1%, 41.5–61.2%, and 8.3–10.1%, respectively, compared with CK ($p < 0.05$). In addition, the CP and SC treatments increased CL, POD, and MR ($p < 0.05$). No significant effects of green manure treatments were observed on PON or the C:N ratios at either the cultivation or incorporation stages ($p > 0.05$).

3.5 Relationship between microbial C metabolism activity and CAZyme gene abundance

Mantel test analyses revealed that the abundances of CAZyme genes involved in the degradation of plant- and microbial-derived components were significantly positively correlated with extracellular enzyme activities (EEAs) (AG, CL, and POD), MR and microbial diversity (B-ACE, B-Shannon, and F-Shannon) (Fig. 3B, $p < 0.05$). Furthermore, linear regression further revealed significant positive relationships between soil EEAs and the abundances of their corresponding CAZyme genes (Fig. S3, $p < 0.001$). Specifically, a strong relationship was observed in the GH31 gene and AG activity ($R^2 = 0.55$), the GH3 gene and BG activity ($R^2 = 0.56$), the GH6 gene and CL activity ($R^2 = 0.57$), the AA2 gene and POD activity ($R^2 = 0.56$), the AA1 gene and PPO activity ($R^2 = 0.62$), and the GH10 gene and XYL activity ($R^2 = 0.75$), respectively.

3.6 Factors affecting changes in SOC and its fractions

Pearson correlation analysis indicated that soil pH, nutrient contents (AP, TN, AK, DON), and MR were significantly positively correlated with SOC and its fractions (Fig. 4A, $p < 0.05$). A random forest model assessed the importance of soil environmental factors (chemical and biochemical properties) on SOC ($R^2 = 0.64$), MAOC ($R^2 = 0.42$), and POC ($R^2 = 0.61$) (Fig. 4B). The results identified eight, five, and seven key factors, respectively ($p < 0.001$). Among them, MBC, pH, and MR were common key factors simultaneously regulating SOC, MAOC, and POC.

Linear regression analysis indicated that SOC and its fractions significantly decreased with increasing abundances of CAZyme genes associated with plant-derived components, and increased significantly with the increase of CAZyme gene abundance associated with bacteria-derived components (Fig. 4C, $p < 0.05$). Among them, SOC ($R^2 = 0.47, 0.46$), POC ($R^2 = 0.48, 0.43$) and MBC ($R^2 = 0.51, 0.47$) were more correlated with genes. In addition, SOC ($R^2 = 0.18$), POC ($R^2 = 0.18$), and MBC ($R^2 = 0.24$) significantly increased with the abundance of CAZyme genes linked to fungi-derived components ($p < 0.05$). PLS-PM was employed to investigate the effects of green manure input (GM), pH, nutrient supply capacity (NSC), microbial community (MC), C-degrading genes (CDG), and microbial C metabolic activity (CMA) on the SOC pool (Fig. 5). The results showed that CMA had a significant positive effect on the SOC pool (0.93, 0.38). In addition, soil pH (0.69) also exerted a significant positive influence on the SOC pool during the incorporation stage. GM had a significant positive impact on soil pH (0.73, 0.88) and MMA (0.62, 0.34). NSC was not only significantly promoted by soil pH (0.72, 0.82) but also had a notable effect on the MC (0.67, -0.78). The MC has a significant negative impact on CDG (-0.76, -0.65). Furthermore, CDG showed a significant negative effect on CMA during the planting stage (-0.43), but a significant positive effect during the incorporation stage (0.64).

4. Discussion

4.1. CAZyme functional genes are regulated by soil microbial community reorganization under different green manure treatments

In our study, different green manure species selectively shaped microbial community composition by altering substrate quality (Fig. 2A; Xiao et al., 2024). Specifically, *C. juncea* stimulated rapid turnover of labile C through the enrichment of *Ascomycota* (Zhang et al. 2021), while *S. cannabina* promoted *Actinobacteria* associated with the gradual transformation of structurally complex C and SOC stabilization (Fig. 2B; Yang et al., 2024; Zhang et al., 2021). In contrast, *C. pallida* favored *Acidobacteria* adapted to slow-metabolism conditions together with *Basidiomycota* capable of oxidative decomposition of complex plant residues (Kalam et al. 2020; Zhang et al. 2021). These distinct microbial regulatory effects among green manure treatments were primarily attributed to differences in plant chemical traits and residue decomposition characteristics (Table S1; Wang et al., 2025b; Zhou et al., 2023). Meanwhile, variations in soil pH regulation, N supply, and nutrient release induced by different green manures further drove the divergence in microbial community structure (Table S7; Yao et al., 2021). In contrast, under summer fallow management, the absence of fresh organic matter inputs led to a soil microbial community dominated by oligotrophic bacteria (e.g., *Chloroflexi*) and weakly decomposing fungi (e.g., *Olpidiomycota*) (Fig. S1), resulting in a limited potential for SOC degradation (Lori et al. 2023).

Changes in the composition of CAZyme functional genes were closely associated with shifts in microbial community structure (Fig. 5), indicating that community reorganization may directly drive variations in C degradation potential by regulating the utilization of dominant C substrates (Huang et al. 2024). This study reveals that the abundance of plant-derived CAZyme genes (> 90%) was higher than that of microbial-derived genes (> 9%) (Table S5), indicating that plant residues serve as the primary substrates driving soil C cycling (Feng et al. 2024). This finding aligns with Huang et al. (2024), who reported that the quantity and chemical composition of plant residue inputs directly determine the metabolic orientation of microbial communities (Huang et al. 2024). The PLS-PM analysis in this study revealed a significant and direct effect of green manure on microbial C metabolic activity (Fig. 5), further validating the pivotal role of plant-derived C inputs in regulating microbial functions. However, plant-derived C does not necessarily enter the atmosphere through direct mineralization pathways, but can be assimilated by microorganisms and transformed into more stable microbial residue C (Feng et al. 2024; Liu et al. 2025). This study found that green manure treatments reduced the abundance of CAZyme genes involved in the degradation of plant-derived components, while increasing the abundance of CAZyme functional genes involved in the degradation of microbial-derived components (Table S5). This indicates a shift in the SOC cycle pathway from direct plant residue mineralization to an SOC stabilization process driven by the MCP (Liu et al. 2025; Qiao et al. 2025). Specifically, during the green manure cultivation stage, soil microbial processes were primarily driven by root exudates, favoring copiotrophic microbial taxa (e.g., *Actinobacteria* and *Ascomycota*) characterized by rapid C turnover (Fig. S1A and B; Seitz et al., 2024; Wen et al., 2022). This shift was accompanied by an enhanced turnover of CAZyme genes involved in chitin degradation (e.g., GH18, GH19, and GH20) (Table S5 and S6; Drula et al., 2022; Ma et al., 2022; Seitz et al., 2024). In contrast, during the incorporation stage, microbial dynamics were dominated by inputs of plant residues, promoting slow-growing microbial groups (e.g., *Acidobacteria* and *Basidiomycota*) associated with the transformation of structurally complex C (Fig. S1C

and D; Wang et al., 2025a; Yang et al., 2021). Correspondingly, the abundance and transformation of CAZyme genes related to peptidoglycan degradation (e.g. GH103 and GH108) were increased (Table S5 and S6; Xiong et al., 2023; Yu et al., 2024). Interestingly, *S. cannabina* induced the greatest shifts in microbial community structure and functional gene profiles during both the cultivation and residue incorporation stages (Fig. 2, S1, 3A, and Table S5), which may be attributed to its high biomass input, favorable substrate quality, and pronounced dual regulation through root exudates and residue-derived C inputs (Table S1 and S7; Muhammad et al., 2021; Seitz et al., 2024). Overall, green manures mediate the functional reassembly of soil microbial communities by providing diverse substrates and modifying the soil microenvironment during plant growth, thereby facilitating the channeling of plant-derived C through distinct microbial pathways into the MCP and its subsequent stabilization as persistent SOC (Yao et al. 2021; Xiao et al. 2024).

4.2. Carbon degradation genes affect microbial C metabolism activity under different green manure treatments

The abundance of C-degrading genes increased in parallel with the corresponding enzyme activities (Fig. S3), indicating that enzyme activity is regulated by functional genes (Chen and Sinsabaugh, 2021; Huang et al., 2024). However, different types of green manure elicited distinct responses in soil C-hydrolyzing and C-oxidizing enzymes (Table S7). Application of *C. juncea* significantly enhanced BG, CL, and PPO activities, suggesting a simultaneous stimulation of cellulose and hemicellulose hydrolysis pathways and a modulation of microbial allocation to oxidative enzymes (Valášková and Baldrian 2006; Xu et al. 2020). Its higher proportion of labile C input and suitable C:N ratio may promoted the rapid activation of key CAZyme genes and accelerated metabolic turnover (Table S1 and S7; Prommer et al., 2020). In contrast, *C. pallida* induced the most comprehensive metabolic response, significantly increasing MR and qMB as well as multiple enzyme activities (AG, BG, CL, PPO and POD) (Table S7). This is primarily attributed to its strong N-fixing capacity (Table S1), which alleviates microbial N limitation, allowing more energy to be directed towards C degradation (Castellano-Hinojosa and Strauss 2020). Furthermore, its high biomass and substantial residue return provide abundant SOC and N substrates, further stimulating microbial growth, respiration, and synchronous activation of C degradation pathways (Table S1 and S7; Hu et al., 2024). *S. cannabina* mainly enhanced CL, PPO and POD activities (Table S7), indicating a preferential effect on cellulose degradation and the oxidative enzyme system, with relatively limited stimulation of hemicellulose-hydrolyzing enzymes (Valášková and Baldrian 2006; Xu et al. 2020). This is likely due to its residues being rich in labile C and having a relatively low C:N ratio (Table S1 and Fig. 1D), which leads microbes to prioritize readily degradable substrates to sustain rapid metabolism (Table S7), while simultaneously activating cellulose degradation and oxidative enzyme activities (Trivedi et al. 2016).

Moreover, different green manure management practices significantly altered the activity patterns of soil C metabolism-related enzymes and the expression profiles of their corresponding functional genes (Table S6 and S7). Microbial community can rapidly respond to C source availability through gene

functional expression under substrate presence or resource limitation conditions (Wang et al., 2024). During the planting phase, green manure input enriched soluble SOC substrates (Xu et al. 2023), markedly enhancing enzyme activities associated with carbohydrate degradation (AG, BG, and CL) while simultaneously increasing microbial metabolic intensity (Table S7). This indicates that green manure provides the microbial community with readily degradable C sources and energy support, stimulating the initiation of polysaccharide (e.g., chitin) degradation pathways (Fu et al., 2024; Mason-Jones et al., 2023; Zhou et al., 2025). The incorporation of large amounts of green manure residues into the soil increased substrate complexity, continuously reshaping the allocation of genes involved in the degradation of plant- and bacteria-derived components, thereby directly regulating the intensity of microbial carbon metabolic activity (Fig. 5; Jia et al., 2025; Xiao et al., 2024). Specifically, POD, CL, and MR activities were significantly increased and positively correlated with plant- and bacteria-derived functional genes (Table S7 and Fig. 3B), indicating a tightly coupled response between enzyme-mediated substrate decomposition and microbial functional gene allocation, which collectively enhanced microbial C turnover and SOC transformation (Chen and Sinsabaugh 2021). The AA1 and AA2 genes showed significant correlations with PPO and POD activities respectively (Fig. S3D and E), indicating enhanced oxidative degradation of lignin and aromatic compounds during green manure residue decomposition (Thirunavukkarasu et al. 2025). This process not only influences the mineralization rate of recalcitrant C but also governs the formation and transformation of stable components within the SOC pools. Collectively, green manure-mediated C degradation genes affect the metabolic performance of microbial communities and the formation of SOC pools (Fig. 5; Dong et al., 2024).

4.3. The formation of SOC and its fractions is regulated by microbial functional genes and C metabolic activity

This study found that both green manure cultivation and incorporation enhanced the contents of SOC and its fractions to varying degrees (Fig. 1). When considering the management stages, *C. juncea*, *C. pallida*, and *S. cannabina* all significantly increased SOC primarily during the incorporation phase (Fig. 1A). Comparing green manure types, *C. pallida* and *S. cannabina* contributed more to the accumulation of SOC and MBC (Fig. 1A and E). This may stem from their high biomass production and active N fixation capacity in root nodules (Table S1), which enhances microbial growth and the formation of microbial residues, thus promoting the simultaneous accumulation of SOC and MBC (Table S7; Hu et al., 2024; Wang et al., 2025b). In addition, both species markedly increased DOC during the planting phase and POC during the incorporation phase (Fig. 1B and D), indicating that they not only increased labile C inputs via the rhizosphere but also facilitated the formation of POC during residue decomposition (Gentsch et al. 2024). In contrast, *C. juncea* increased MBC during the planting stage and SOC during the incorporation stage (Fig. 1A and E), suggesting that its active rhizosphere C inputs first stimulate microbial activity, while residue return subsequently drives the accumulation of more stabilized forms of SOC (Arone et al., 2024). Notably, MAOC accounted for a consistently larger proportion of SOC than POC (Table S7). This indicates that regardless of the differences in C input pathways of green manure, a considerable proportion of C ultimately enters the mineral-bound state through microbial transformation or residue decomposition, achieving long-term sequestration (Sokol et al., 2019b).

Concurrently, soil chemical properties and microbial C metabolic activity exhibit significant synergistic regulatory effects during the formation of SOC fractions (Sokol et al. 2019; Huang et al. 2024). Soil pH, AP, TN, AK, DON, and MR showed significant positive correlations with SOC and its various fractions (Fig. 4A). This indicates that appropriate nutrient supply and a relatively stable acid-base environment provide a favorable ecological foundation for microbial C assimilation and SOC accumulation (Tao et al. 2023). The correlation patterns of MBC were largely consistent with those of SOC (Fig. 4A), indicating that microbial biomass serves as a sensitive indicator and driving factor for SOC variation (Cotrufo et al. 2013; Tao et al. 2023). Furthermore, the highly significant positive correlations between SOC, MAOC, and MBC with the C:N ratio (Fig. 4A) suggest that a higher C:N ratio favors C retention during microbial assimilation and residue stabilization processes (Tao et al., 2023). Notably, the significant positive correlations between C-related enzymes (AG, BG, CL, POD) and qMB with POC and DOC (Fig. 4A) align with findings from Zhang et al. (2020). These hydrolytic and oxidative enzymes collectively drive the formation and transformation of the labile C pool, reflecting the close coupling between microbial C metabolic activity and SOC fraction accumulation (Fig. 5; Xu et al., 2020). Random forest analysis further emphasized the co-regulatory role of factors such as MBC, pH, and MR in the accumulation processes of SOC, MAOC, and POC (Fig. 4B). Microbial biomass provides the potential for C transformation, whilst microbial respiration drives C decomposition and cycling (Cotrufo et al. 2013; Tao et al. 2023). The pH indirectly influences C stability by regulating substrate solubility, nutrient availability, and enzyme activity (Zhong et al., 2023).

This dynamic change in the soil C pool is influenced not only by environmental factors such as exogenous C inputs and soil properties (Fig. 4A; Jia et al., 2024; Li et al., 2024), but more profoundly governed by the decomposition and transformation processes regulated by microbial functional genes (Fig. 4C; Huang et al., 2024; Jing et al., 2021). This is consistent with our research hypothesis. As the abundance of CAZymes genes associated with plant-derived components (hemicellulose, cellulose, lignin) increased, SOC and its fraction contents decreased significantly (Fig. 4C). This indicates that enhanced degradation potential of plant polymers may accelerate SOC mineralization, thereby reducing SOC accumulation (Thirunavukkarasu et al., 2025). This finding suggests that within green manure utilization systems, accelerated decomposition of plant-derived substrates may lead to C release as CO_2 rather than conversion into stable C pools (Liu et al. 2022). Conversely, bacterial-derived components (peptidoglycan) showed a significant positive correlation between CAZymes gene abundance and SOC, POC, and MBC (Fig. 4C), indicating bacteria play a pivotal role in microbial residue turnover and C source recycling. Peptidoglycan degradation not only facilitates C cycling from microbial residues but also promotes its binding with mineral particles to form MAOC, representing a crucial pathway for long-term SOC sequestration (Zhao et al. 2025). Furthermore, fungal-derived components (chitin, glucans) associated with CAZymes genes exhibited a highly significant positive correlation with DOC (Fig. 4C). This indicates that fungi, through the secretion of extracellular enzymes during the primary decomposition of complex OM, release soluble C compounds, thereby driving the formation of the dissolved C pool (Fu et al. 2024).

Collectively, green manure influences microbial metabolic activity and alters CAZymes gene expression encoding degradation pathways for diverse C sources through the MCP regulatory mechanism, thereby promoting the formation of SOC and its fractions (Fig. 5). These findings not only reveal the multifaceted mechanisms by which green manure regulates soil C dynamics but also hold significant theoretical and practical implications for enhancing soil fertility and C sink functions in tropical regions. Nevertheless, this study retains certain limitations: Although the degradation potential of C has been inferred from metagenomic data, continuous experiments are still needed to validate the effects of long-term gene expression and metabolic processes. Future work could integrate transcriptomic analysis and isotope tracing techniques to conduct long-term, site-specific studies, thereby elucidating the sustained mechanisms by which green manure influences soil C sequestration.

5. Conclusion

This study investigated the effects of different green manures on SOC accumulation and its microbial driving mechanisms during both the cultivation and incorporation phases in tropical regions, focusing on cascading responses from microbial community structure and functional genes to metabolic activity. Our study suggests that green manure inputs optimized the soil microenvironment via root activity and plant residue inputs, driving the succession of functional microbial communities represented by *Acidobacteria*, *Actinobacteria*, and *Ascomycota*. This succession enhanced the abundance of CAZyme genes involved in the degradation of plant- and microbial-derived components and increased microbial metabolic activity, thereby accelerating C accumulation by regulating the decomposition and transformation of labile and recalcitrant C in the soil. This study particularly emphasized the crucial role of green manure-driven decomposition of dead microbial biomass (peptidoglycan and chitin) in regulating metabolic activity. *Crotalaria pallida* and *Sesbania cannabina* exhibited superior soil amelioration efficacy among the three legume green manures due to their higher nutrient accumulation and nitrogenase activity. These findings underscore the pivotal role of microbial functional traits in driving C sequestration under green manure management, providing crucial theoretical underpinnings for enhancing C sinks and promoting sustainable management in tropical agricultural systems.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

Long Jia: data curation, formal analysis, investigation, methodology, writing - original draft, writing - review and editing. Peng Li: writing - review and editing. Tong Su: formal analysis, writing - review and

editing. Zhuochen Lou: software, formal analysis. Jiale Ye: methodology, writing -review and editing. Ruiyun Yang: software, formal analysis. Jiaguo Jiao: project administration and funding, writing -review and editing. Huixin Li: funding acquisition. Feng Hu: funding acquisition.

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Figures

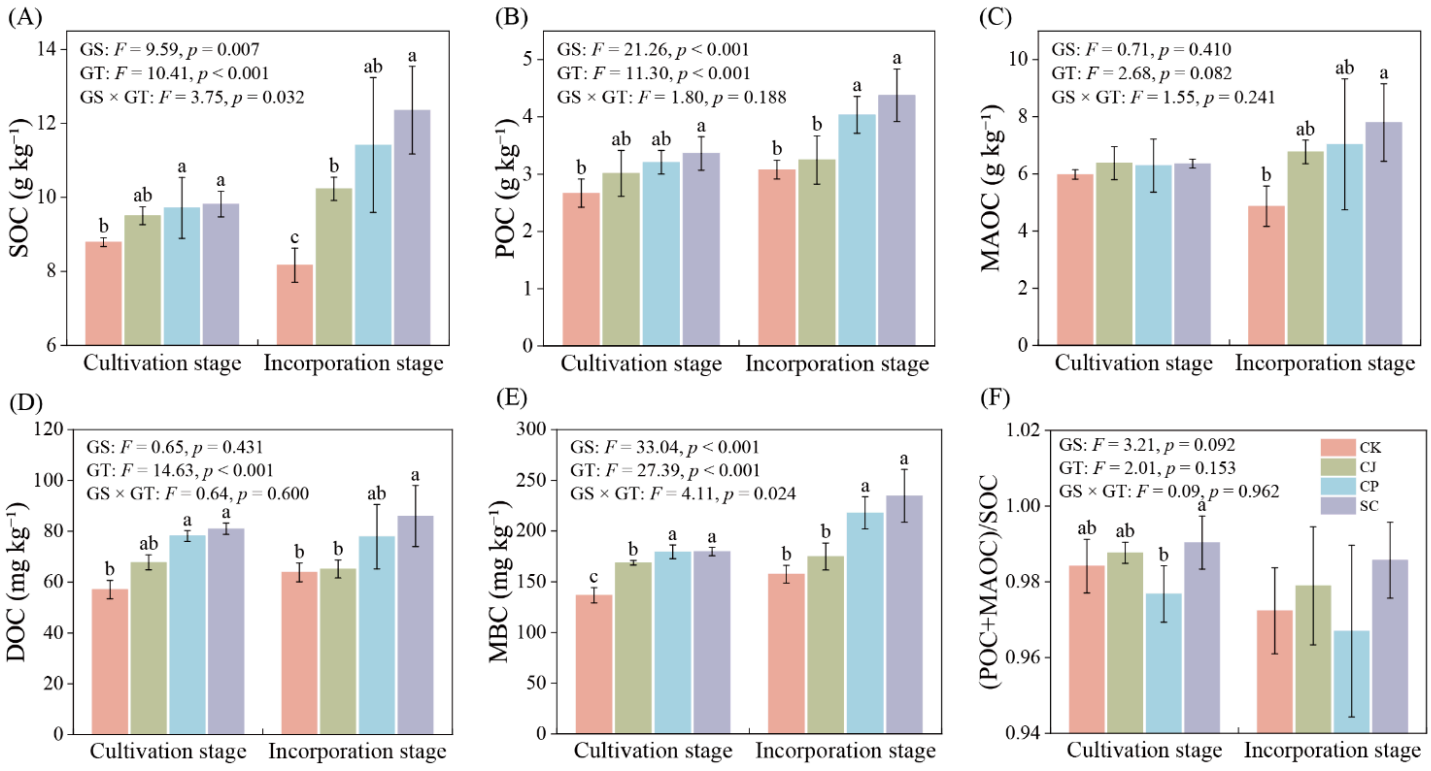

Figure 1

Characteristics of soil organic carbon (SOC) and its fractions under different green manure treatments. Different lowercase letters indicate significant differences among green manure types within the same management stage ($p < 0.05$). The effects of green manure management stage (GS), green manure type (GT), and their interaction (GS × GT) are also presented. CK: no green manure; CJ: *Crotalaria juncea*; CP: *Crotalaria pallida*; SC: *Sesbania cannabina*;POC: particulate organic carbon; MAOC: mineral-associated organic carbon; DOC, dissolved organic carbon; MBC, microbial biomass carbon.

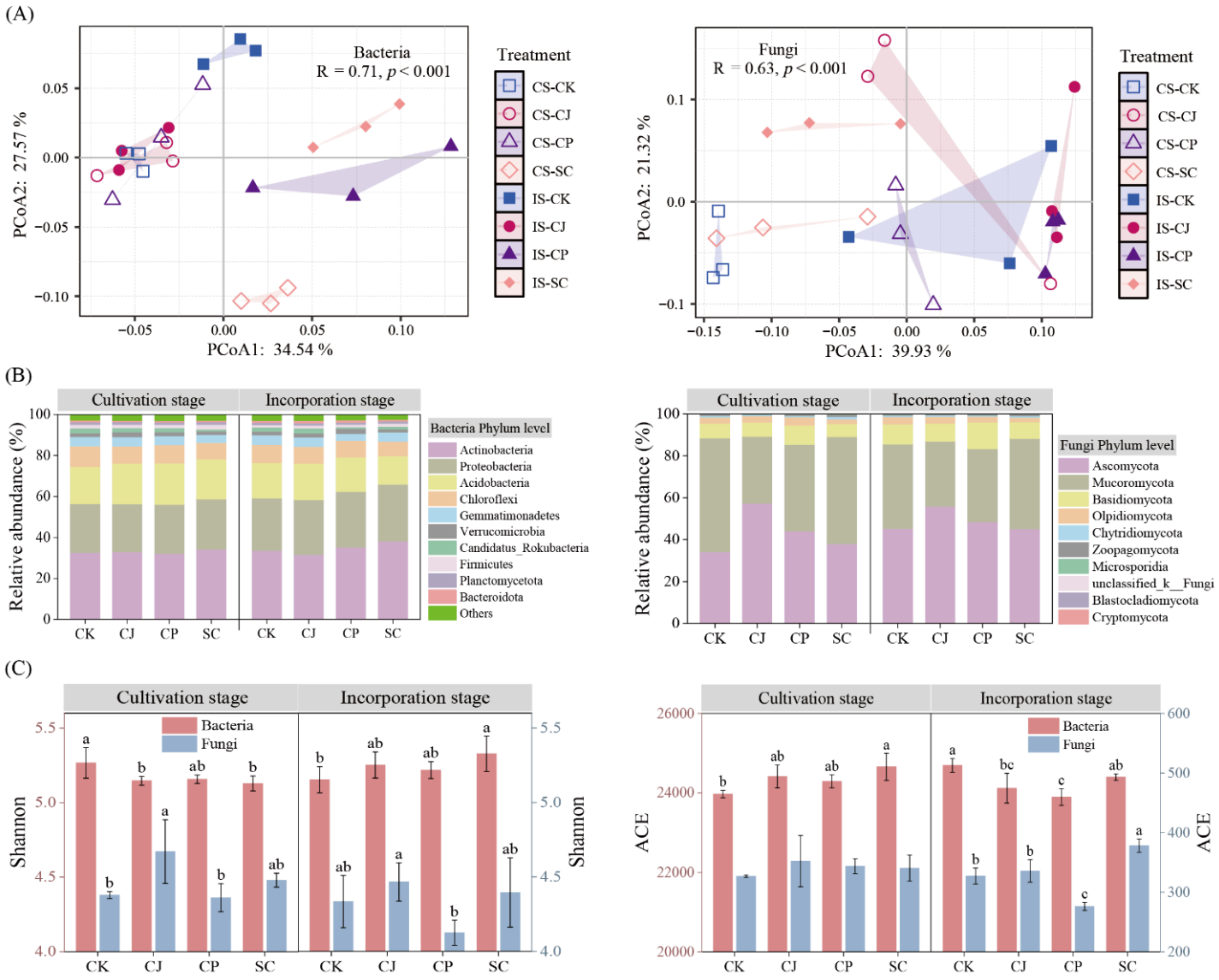

Figure 2

Differences in microbial community structure (A) under different green manure treatments based on principal coordinates analysis (PCoA), relative abundance (B), and diversity indices (C). Different lowercase letters indicate significant differences among green manure types within the same management stage ($p < 0.05$). Abbreviations are same as described in Fig. 1. CS: cultivation stage; IS: incorporation stage.

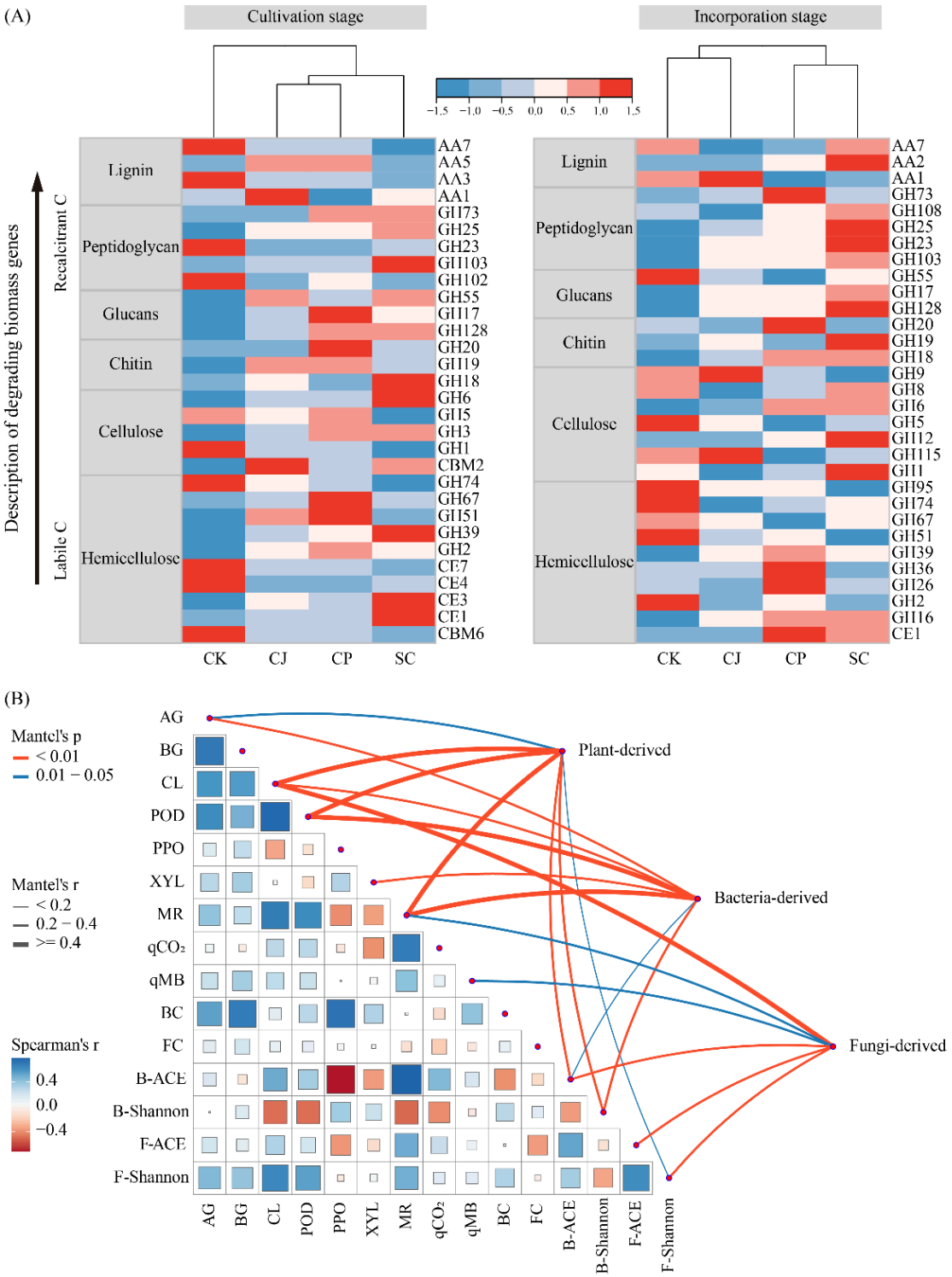

Figure 3

Clustering analysis (A) of the relative abundance of CAZyme genes involved in the degradation of plant- and microbial-derived components, and their associations with microbial carbon metabolic activity based on Mantel tests (B). AG: α-glucosidase; BG: β-glucosidase; CL: cellobiohydrolase; XYL: β-xylosidase; POD: peroxidase; PPO: polyphenol oxidase; MR: microbial respiration; qCO_2: metabolic

quotient; qMB: microbial quotient; B-: bacteria-; F-: fungi-; BC: bacterial community; FC: fungal community.

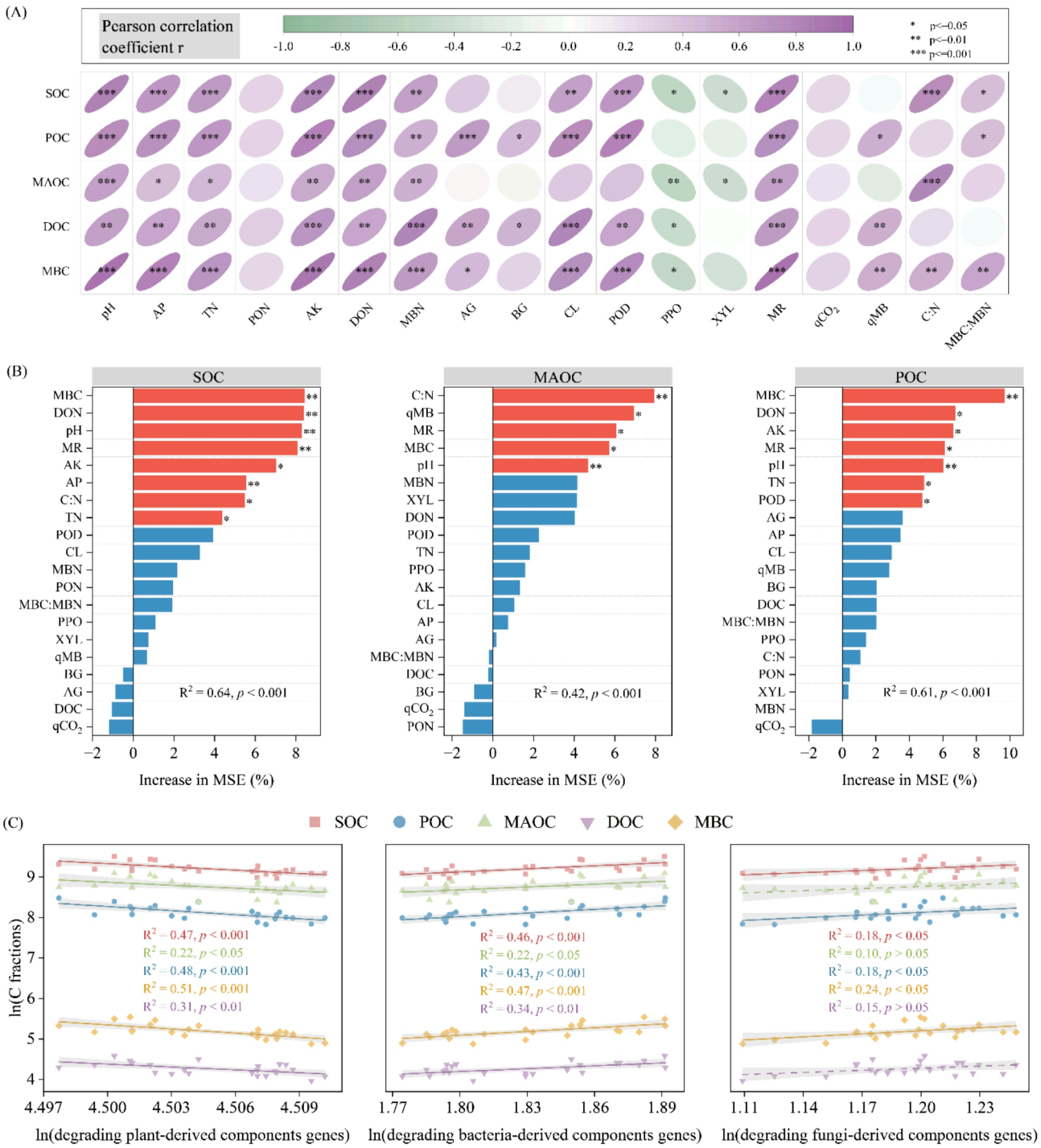

Figure 4

Relationship between soil biochemical properties, carbon degradation genes and SOC fractions. Pearson correlation between soil chemical properties, microbial C metabolic activity, and SOC fractions (A).

Relative importance of independent variables controlling SOC, POC, and MAOC as indicated by the percentage increase in mean squared error (MSE%) using the random forest model (B). Ln-Ln linear regression analysis between C fractions and CAZyme genes encoding plant- and microbial-derived components (C). Abbreviations are same as described in Fig. 1 and 3. TN: total nitrogen; AK: available potassium; AP: available phosphorus; DON: dissolved organic nitrogen; PON: particulate organic nitrogen; MBN: microbial biomass nitrogen. *p < 0.05, **p < 0.01, ***p < 0.001.

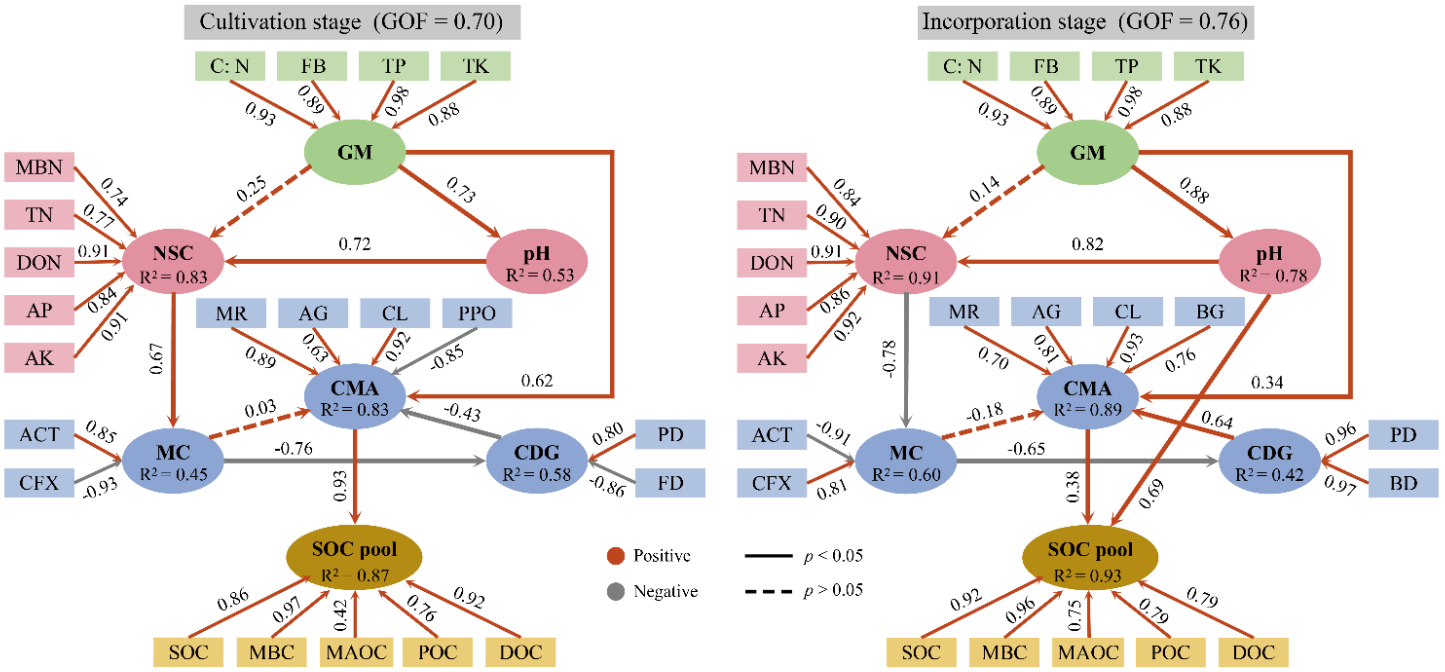

Figure 5

Partial least squares path model (PLS-PM) illustrating the effects of green manure cultivation and incorporation on SOC pools. Abbreviations are same as described in Fig. 1 and 3. GM: green manure; NSC: nutrient supply capacity; MC: microbial community; CDG: carbon degradation gene; CMA: microbial carbon metabolic activity; FB: fresh biomass; ACT: Actinobacteria; CFX: Chloroflexi; PD, BD, and FD: CAZyme genes involved in the degradation of plant- (PD), bacterial- (BD), and fungi-derived (FD) components. The boxes represent observed variables, and the circles represent latent variables. Red lines indicate positive correlations; gray lines indicate negative correlations. Solid or dashed lines indicate significant (p< 0.05) or insignificant (p > 0.05) relationships, respectively.

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