

Article

Regulatory Mechanisms of Medium-Term Crop Rotation on Soil Organic Carbon Storage in Red Soils at the Aggregate Level

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Abstract

Soil organic carbon (OC) storage in crop rotation systems benefits soil productivity and global climate change. However, the regulatory mechanisms and pathways by which soil OC storage is affected under medium-term crop rotation at the aggregate level are not fully understood. Herein, fifteen soil samples from five cropping systems (abandoned farmland, continuous cropping of tobacco, tobacco–pea rotation, continuous cropping of dasheen, and dasheen–ryegrass rotation for over 10 years) were collected from soil at 0 to 20 cm depths in Miyi County, Sichuan Province, China. The soil aggregates and aggregate-associated OC, enzyme activities, and microbial biomass were evaluated. The effects of medium-term crop rotation on soil aggregate-associated OC content and biochemical properties varied between crop types. Specifically, tobacco–pea rotation significantly decreased the proportion of macro-aggregates (0.25–2 mm); the contents of OC, Ca-OC, aliphatic C, alcohols, and phenols; enzyme activities; and fungal biomass in the aggregate fractions, compared with those associated with the continuous cropping of tobacco. In contrast, dasheen–ryegrass rotation significantly increased the recalcitrant OC content, β -glucosidase and polyphenol oxidase activities, microbial biomass in mega-aggregates (>2 mm) and macro-aggregates, and the recalcitrant OC content and enzyme activity in microaggregates (0.053–0.25 mm) and slit clay (<0.053 mm), relative to those in the continuous cropping of dasheen. Moreover, for the continuous-cropping soils, the OC contents were positively correlated with POD activity but negatively correlated with other enzymes. For the rotational soils, the OC content was positively related to the Fe/Al-OC, aromatic-C, aliphatic-C, and microbial biomass contents but negatively related to the carbohydrate content. The increased OC content was driven by the microbial biomass in the aggregate fractions, and medium-term crop rotation changed the negative effect of microorganisms on the OC content into a positive effect at the aggregate level. Overall, medium-term crop rotation enhances OC storage by improving soil structural stability and microbial community dynamics.

Keywords: crop rotation; soil aggregates; soil organic carbon; soil biochemical properties



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1. Introduction

Soil carbon (C) storage involves increasing the organic carbon (OC) content and sequestering OC in soil for long periods of time through agricultural management measures and ecological conservation practices [1,2]. Soils store a vast amount of OC globally, up to 3000 Pg C, which is essential for combating climate change [3]. The storage of OC

occurs predominantly within soil aggregates, where physical occlusion creates diffusion barriers that impede oxygen penetration, enzymatic catalysis, and microbial colonization, collectively stabilizing the protected C pool [4,5]. However, global soil OC stocks have declined by 20–69% in intensive agricultural ecosystems [6], potentially threatening crop production and the environment [7,8]. Various agricultural management practices (e.g., no-tillage, straw return, and crop rotation) have been shown to improve OC content, aggregate stability, and microbial communities [9–12]. Therefore, investigating how agricultural management practices influence soil aggregation and the associated OC is critical for understanding and predicting their potential benefits regarding crop productivity and environmental sustainability.

Crop rotation—where several crops are rotated sequentially in the same field—has a positive effect on soil OC storage by providing a rich organic matter source, maintaining microbial diversity and activity, and improving the soil structure [13,14]. Compared with crop rotation, continuous cropping significantly changes the structure and function of soil microorganisms, intensifies OC mineralization, and decreases soil OC storage [15]. Meanwhile, continuous cropping leads to soil acidification and nutrient imbalance, inhibits the activity of beneficial bacteria and mycorrhizal fungi, and limits OC sequestration processes that require specific nutrients, such as humification [16]. Many studies have investigated the roles of crop rotation on soil aggregates and the related OC but have yielded inconsistent results [14,17–19]. For example, through a 12-year field study conducted in Northwest China, Zhang et al. [20] demonstrated that a sustained wheat–maize cropping system significantly increased soil structural stability and OC storage in aggregates, while decreasing OC hydrophobicity in different aggregate size classes. Similarly, evidence from a 6-year field study demonstrated that crop rotation significantly elevated the topsoil (0–20 cm) OC concentration, the aggregate stability, the contents of macro-aggregate and associated OC, and the biochemical functionality through increased aryl-sulfatase, invertase, and other key enzyme activities [18]. Additionally, several meta-analyses have reported the positive effects of crop rotation on soil C-cycling enzymes and total, bacterial, and fungal PLFAs [21,22]. Contrary to expectations, studies by Guo et al. [23] and Yan et al. [19] did not observe significant improvements in OC stabilization within soil aggregates from crop rotation practices. The discrepancies in the findings likely emerge from the complex biotic and abiotic factors, such as soil properties [24]; regional climate patterns [25,26]; and cultivation strategies, including species selection and rotation duration [27,28]. This apparent context dependence generates significant challenges when modeling the medium-term impacts of rotational systems on OC storage within soil aggregates.

Studies have shown that physical protection, chemical bonding, and biological transformation play important roles in protecting soil OC [29,30]. Organic matter and minerals are bound in soil by binders (e.g., fine roots, polysaccharides, fungal hyphae, and glycoproteins) to form stable aggregates [31,32], which reduces the oxidative decomposition rate of organic matter, thereby prolonging its storage time in the soil [33]. Due to their larger pore spaces and permeability, macro-aggregates are more susceptible to external disturbances than micro-aggregates, thus resulting in OC loss [29,34]. Moreover, chemical reactions between OC and minerals, such as sorption, complexation, and co-precipitation, increase the stability of OC and reduce the probability of microbial decomposition [35,36]. However, the oxidative transformation of OC with minerals increases the surface oxygen-containing functional groups and is accompanied by bond cleavage to form small molecules, which may lead to decreased stability during interactions with soil minerals [37,38]. In addition, microorganisms incorporate soil C into their own biomass through a series of catabolic and anabolic reactions [39,40]. Following microbial death and decomposition, metabolites (e.g., extracellular enzymes, polymeric matrices, and quorum-sensing molecules) bind to

soil minerals, forming persistent organo-mineral complexes that evade microbial recycling and constitute the stabilized C fraction [40,41]. Although many studies have reported crop rotation's impacts on soil aggregates and related OC contents [14,17–19], the mechanistic insights into how medium-term crop rotation influences aggregate-specific OC storage by modulating these transition pathways have not been fully elucidated.

The current understanding of how multi-year crop rotation modulates the soil C pool remains incomplete, particularly in terms of quantifiable OC storage pathways at the aggregate level. Therefore, five field cropping systems (i.e., abandoned farmland, continuous cropping of tobacco, tobacco–pea rotation, continuous cropping of dasheen, and dasheen–ryegrass rotation) were used for 10 years in Southwest China to investigate the mechanisms of soil OC storage at the aggregate level. We hypothesized that (H1) medium-term crop rotation increases the OC and chemical-bound OC contents and the enzymes and microorganisms related to soil C-cycling in aggregate fractions relative to continuous monocultures and that (H2) the increased OC content is mainly driven by the microbial biomass in aggregate fractions, whose effect significantly differs between continuous cropping and rotation systems.

2. Materials and Methods

2.1. Study Site

This investigation focused on Miyi County ($26^{\circ}42'–27^{\circ}10' \text{ N}$, $101^{\circ}44'–102^{\circ}15' \text{ E}$) in the southwest of Sichuan Province, China, which covers 2153 km², with elevations ranging from 980 to 3447 m. This county exhibits climatic characteristics, including a mean annual temperature of 19.9 °C (ranging from 11.7 °C in January to 25.2 °C in July); 1101 mm of rainfall, concentrated in May–October (94%); and extended frost-free periods (307 days). The dominant agricultural soils are classified as Ultisols (US soil taxonomy) or red soils (China's soil classification system) [42,43], developed from clay-rich viscous alluvium.

2.2. Soil Sampling

This study consisted of five cropping systems: (1) abandoned farmland (control); (2) continuous cropping of tobacco; (3) tobacco–pea rotation; (4) continuous cropping of dasheen; and (5) dasheen–ryegrass rotation. In the rotational systems, tobacco and dasheen are ridge-cultivated in April annually. Following the September harvest, pea or ryegrass is sown, with harvest and residue removal occurring the subsequent year. Conversely, continuous cropping involves post-harvest fallowing of tobacco and dasheen fields, with residue removal after each cycle. These fields undergo spring plowing and are replanted with the same crops in April of the following year. The tillage depth during the tobacco and dasheen planting seasons is 30 and 40 cm, respectively, and no tillage occurs during the ryegrass and pea planting seasons. These cropping systems were converted from the traditional cropping system (continuous cropping of maize) for over 10 years. The fertilization rates and soil properties of each sampling site are listed in Table 1.

The soil was sampled from the five cropping systems prior to sowing in the spring of 2021. All sampling plots had similar physical–geographical conditions. For each cropping system, three adjacent plots (20 m × 100 m; $n = 15$) were established 30 m apart. Within each plot, six soil cores (0–20 cm depth) were systematically collected from non-fertilized and non-ridge areas. Field-moist subsamples from each plot were homogenized into composite samples, with visible plant debris manually removed prior to any laboratory analysis. The subsamples were subjected to different preservation protocols: (i) air drying at ambient conditions for wet aggregate separation and physicochemical analysis, (ii) cryopreservation at -80°C for subsequent phospholipid fatty acid (PLFA) profiling, and (iii) short-term storage at 4°C to test the enzymatic activity related to C metabolism.

Table 1. Fertilization amounts and soil properties for five cropping systems.

Sample ID	Seasonal Gap	Preceding Gap	Average Annual Fertilizer Application (kg/ha/year)				pH	OC (g/kg)	TN (g/kg)	Particle Composition (%)		
			N	P ₂ O ₅	K ₂ O	Manure				Sand	Clay	Silt
CK	Weed	Weed	0	0	0	0	5.7	5.9	0.4	25.2	58.5	16.3
CT	Fallow	Tobacco	212.5	109.8	563	0	5.4	21.6	1.6	42.3	52.5	5.2
RPT	Pea	Tobacco	210	99.8	504.8	0	4.7	16.5	1.1	12.5	59.8	27.7
CD	Fallow	Dasheen	180	180	180	400	4.1	28.5	2.1	21.5	67.7	10.8
RDR	Ryegrass	Dasheen	180	180	180	400	4.7	32.0	2.0	18.3	55.5	26.5

Note: CK, abandoned farmland; CT, tobacco continuous cropping; RPT, tobacco–pea rotation; CD, dasheen continuous cropping; RDR, dasheen–ryegrass rotation; N, nitrogen; P₂O₅, phosphorus pentoxide; K₂O, potassium oxide; OC, organic carbon; TN, total nitrogen.

2.3. Laboratory Analysis

The wet-sieving process, according to Cambardella and Elliott [44], was used to fractionate the soil samples into different water-stable aggregate size classes. The soil aggregates were fractionated using a soil aggregate analyzer (Model SAA 8052, Shanghai, China) with sieve sizes in the following order: 2, 0.25, and 0.053 mm. Briefly, naturally dried soil samples (50 g) were first immersed in deionized water on top of a 2 mm sieve for 5 min under ambient conditions (20 °C). Vibrational separation was then performed at 3 cm amplitude with 30 times per minute for 30 min. Sieve-retained fractions were quantitatively collected in a beaker: >2 mm (mega-aggregates, ME), 0.25–2 mm (macro-aggregates, MA), and 0.053–0.25 mm (micro-aggregates, MI). The <0.053 mm fraction (silt–clay complexes, SC) was isolated via gravitational sedimentation for 24 h, followed by removal of the supernatant. Following stabilization through oven-drying at 40 °C, the aggregate samples were weighed. The subsamples were homogenized using a 0.25 mm sieve for OC quantification. The total organic carbon (TOC), active organic carbon (AOC), and recalcitrant organic carbon (ROC) contents in the aggregates were analyzed via the Walkley–Black oxidation procedure [45].

Following the procedure described by Xu et al. [46], sodium sulfate and sodium hydroxide–sodium pyrophosphate solutions (Suzhou, China) were used to extract calcium-bound OC (Ca-OC) and iron/aluminum-bound OC (Fe/Al-OC), respectively, from the aggregate fractions. The OC content in the extract was determined using a TOC analyzer (Atmoslytic, San Francisco, CA, USA). The functional groups of soil C were identified using Fourier-transform infrared spectrometry [47,48]. Specifically, the soil samples were dried at 60 °C for 8 h, and the sample and potassium bromide (spectrally pure) (Suzhou, China) were completely ground in an agate mortar at a ratio of 1:100. The tablet was pressed at 75 kPa, and the spectrum was recorded. A combined scan of 64 repetitions was used with a resolution of 4 cm^{−1}, and the spectral measuring range was 400–4000 cm^{−1}. The relative content of each functional group was determined by calculating the ratio of each absorption peak to the total peak area.

The activities of soil C-cycling enzymes, namely β -glucosidase (β -GC), peroxidase (POD), polyphenol oxidase (PPO), and invertase (IVT), were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's instructions. The basics behind enzyme analysis are illustrated in Table 2. The C-cycling enzyme activities were expressed in nanomoles per hour per gram of soil. Moreover, the soil microbial community structure was assessed using the PLFA method, as described by Bossio and Scow [49]. Briefly, lipid extraction was performed on 6 g of lyophilized soil using a chloroform–methanol–citrate buffer mixture (1:2:0.8) (Suzhou, China). A sequential elution with chloroform, acetone, and methanol through solid-phase extraction columns (Supelco Inc., Bellefonte, PA, USA) separated the phospholipids from

the neutral and glycolipid fractions. Following methylation to fatty acid methyl esters, microbial community profiling was conducted using an Agilent 7890A gas chromatograph system (Agilent Technologies, Santa Clara, CA, USA) equipped with MIDI software (v4.5, MIDI Inc., Newark, DE, USA). PLFA abundances (nmol/g dry soil) were normalized to represent community composition, while total PLFA content served as a proxy for total microbial biomass. The biomarker assignments included bacteria (14:0, 15:0, a15:0, i15:0, i16:0, 16:0, 16:1 ω 7c, 16:1 ω 9c, a17:0, i17:0, cy17:0, 18:0, 18:1 ω 7c, and cy19:0) [14,50], fungi (16:1 ω 5c, 18:2 ω 6c, and 18:1 ω 9c) [51,52], and actinomycetes (10Me16:0, 10Me17:0, and 10Me18:0) [52]. PLFAs below 0.5% relative abundance were excluded [53].

Table 2. Soil C-cycling enzyme assay principles.

Enzyme	EC Number	Substrate	Reaction Principle	Measured Product	Detection Wavelength
β -glucosidase	3.2.1.21	p-Nitrophenyl- β -D-glucopyranoside	Hydrolysis: pNPG $\rightarrow$ p-Nitrophenol + Glucose	p-Nitrophenol	405
Peroxidase	1.11.1.7	Pyrogallol + H ₂ O ₂	H ₂ O ₂ -dependent oxidation: Pyrogallol $\rightarrow$ Purpurogallin	Purpurogallin chromophore	470
Polyphenol oxidase	1.10.3.1	Pyrogallol/L-DOPA	Oxidation: Pyrogallol $\rightarrow$ Purpurogallin (O ₂ -dependent)	Purpurogallin chromophore	430
Invertase	3.2.1.26	Sucrose (8% w/v)	Hydrolysis: Sucrose $\rightarrow$ Glucose + Fructose	Reducing sugars	540

2.4. Data Analysis

To quantify the impact of crop rotation on soil aggregate stability, the mean weight diameter (MWD) and the geometric mean diameter (GMD) were calculated using the following formulas [54]:

$$MWD = \sum_{i=1}^n X_i \times W_i \quad (1)$$

$$GMD = \exp \left[\frac{\sum_{i=1}^n W_i \times \ln X_i}{\sum_{i=1}^n W_i} \right] \quad (2)$$

where W_i is the weight fraction (%) of the i th aggregate fraction, and X_i is the mean diameter of each class (mm).

Statistical processing was conducted with SPSS 25.0 (IBM SPSS, Somers, NY, USA) and R 4.2.2 “<http://www.r-project.org/>” (accessed on 31 October 2024). Graphs were generated using Origin 9.0 (OriginLab Corp., Northampton, MA, USA). For inter-group comparisons, the datasets were analyzed via one-way ANOVA followed by Tukey’s post hoc test ($p < 0.05$). The relationships between soil aggregate size distribution, biochemical properties, and OC content at the aggregate level were assessed using Spearman’s correlation analysis. Subsequently, partial least squares–path modeling (PLS-PM) was used to evaluate the effects of soil aggregate size distribution and biochemical properties on the OC content at the aggregate level using the R package “plsrm” [55].

3. Results

3.1. Soil Aggregate Size Distribution and Stability

Averaged across all treatments, MA was the predominant soil mass, with a proportion of 55.4% (48.9–60.6%), while ME, MI, and SC accounted for 18.7% (9.8–31.0%), 19.3% (10.8–31.5%), and 6.6% (3.2–10.0%) of the soil mass, respectively (Figure 1). Compared with the abandoned farmland, the two cropping systems based on dasheen significantly increased the proportion of ME and the values of MWD and GMD but decreased the proportion of MI ($p < 0.05$; Figures 1 and 2). Moreover, tobacco–pea rotation significantly

decreased the proportion of MA (−19.8%) but increased the proportion of MI (57.7%) compared with the continuous cropping of tobacco ($p < 0.05$; Figure 1). Medium-term crop rotation had no effect on the MWD and GMD (Figure 2).

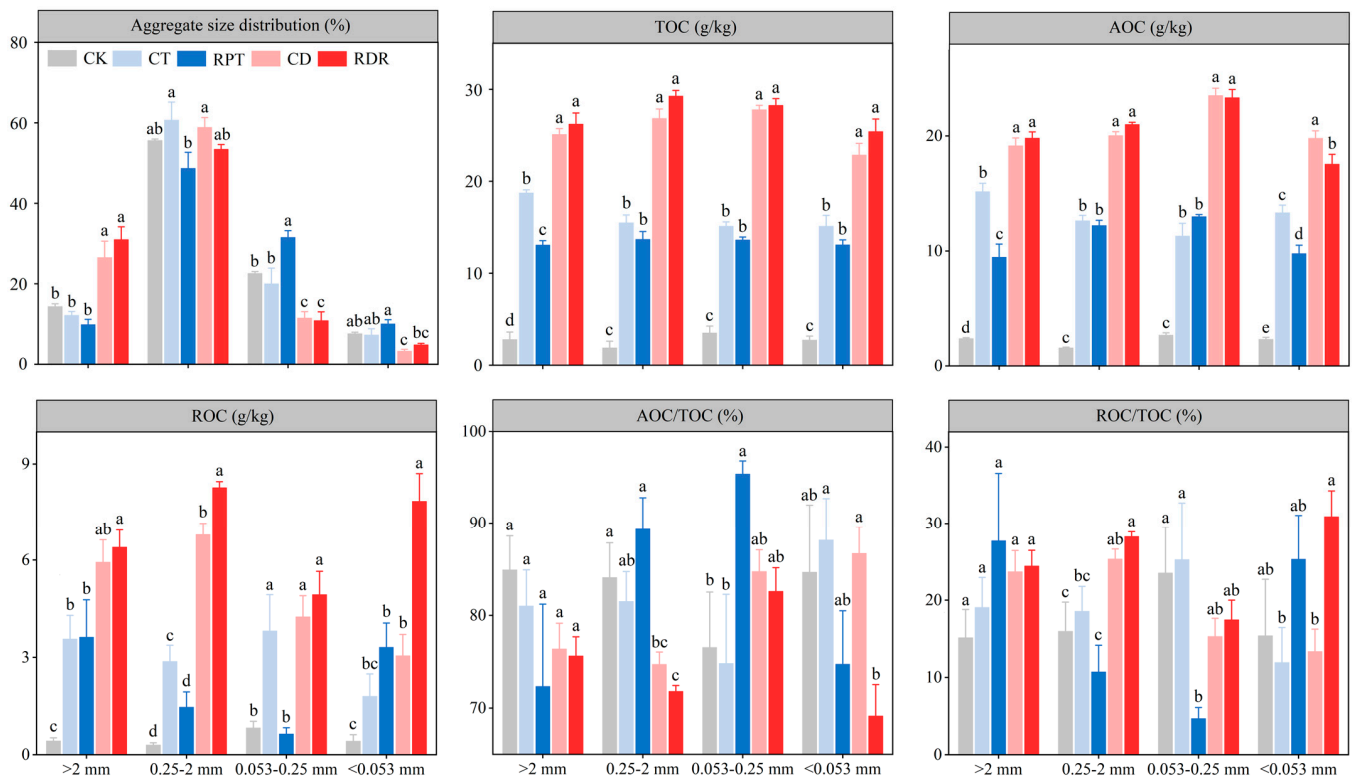


Figure 1. Aggregate size distribution, total organic carbon (TOC), active organic carbon (AOC), recalcitrant organic carbon (ROC), AOC/TOC, and ROC/TOC in the soil aggregate fractions under different cropping systems. Error bars represent the standard deviation of three independent replicates ($n = 3$). Different lowercase letters indicate significant differences among cropping systems based on Tukey's test ($p < 0.05$). CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation.

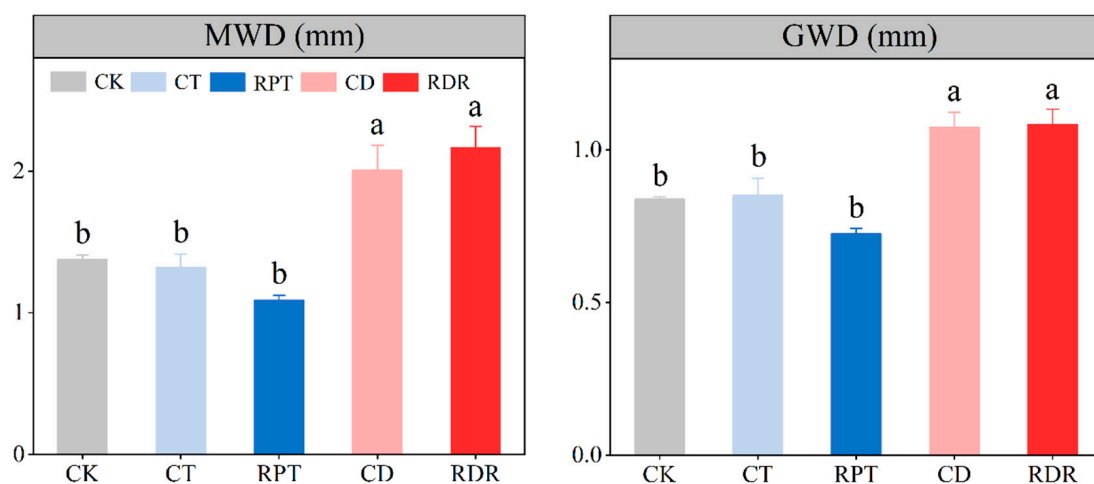


Figure 2. Mean weight diameter (MWD) and geometric mean diameter (GWD) in soils under different cropping systems. Error bars represent the standard deviation of three independent replicates ($n = 3$). Different lowercase letters indicate significant differences among cropping systems based on Tukey's test ($p < 0.05$). CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation.

3.2. Soil Organic Carbon in the Aggregate Fractions

Planting crops significantly increased the TOC, AOC, and ROC contents in all aggregate fractions ($p < 0.05$; Figure 1). Compared with the continuous cropping of tobacco, tobacco–pea rotation significantly decreased the TOC content in ME (-30.2%), the AOC content in ME and SC (-37.7 and -26.7% , respectively), the ROC content in MA and MI (-49.1 and -83.4% , respectively), and the ROC/TOC ratio in MI (-81.5%) but increased the AOC/TOC ratio in MI (27.5%) ($p < 0.05$; Figure 1). The AOC content and AOC/TOC ratio in SC decreased by 11.3 and 20.2% in the dasheen–ryegrass rotation relative to the continuous cropping of dasheen, while the ROC content in MA and SC increased by 21.7 and 157.2%, respectively ($p < 0.05$; Figure 1).

3.3. Soil Chemical-Bound Organic Carbon and Carbon Functional Groups in the Aggregate Fractions

Tobacco–pea rotation significantly decreased the Ca-OC content in ME and SC (-49.1 and -83.4% , respectively) compared with the continuous cropping of tobacco ($p < 0.05$; Figure 3). The Ca-OC and Fe/Al-OC contents in all aggregate fractions were similar between the dasheen–ryegrass rotation and the continuous cropping of dasheen ($p > 0.05$; Figure 3).

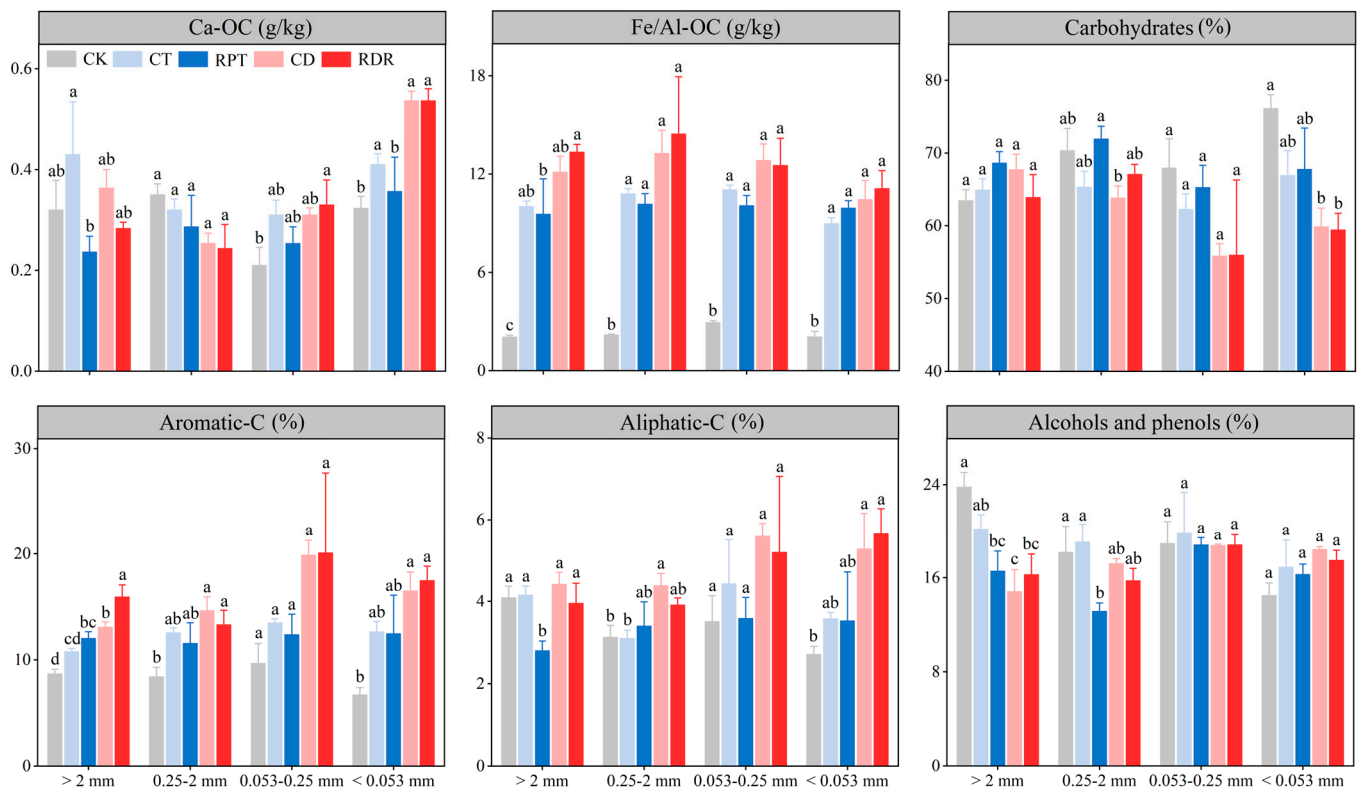


Figure 3. Chemically bound organic carbon and carbon functional groups in the soil aggregate fractions under different cropping systems. Error bars represent the standard deviation of three independent replicates ($n = 3$). Different lowercase letters indicate significant differences among cropping systems based on Tukey's test ($p < 0.05$). CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation; Ca-OC, Ca-bound organic carbon; Fe/Al-OC, iron/aluminum-bound organic carbon; C, carbon.

The positions of the absorption peaks of the different cropping systems were the same (1037 , 1638 , 2926 , 3441 , and 3619 cm^{-1}), but the intensity of these peaks varied (Figure 4). The relative content of carbohydrate was highest across different cropping

systems (55.8–76.1%), with an absorption peak at 1037 cm^{-1} (Figure 4). The relative contents of aliphatic C in ME and alcohol and phenol in MA decreased by 32.5 and 31.1%, respectively, in the tobacco–pea rotation relative to the continuous cropping of tobacco ($p < 0.05$; Figure 3). Additionally, the dasheen–ryegrass rotation significantly increased the relative content of aromatic C in ME (21.8%) compared with the continuous cropping of dasheen ($p < 0.05$; Figure 3).

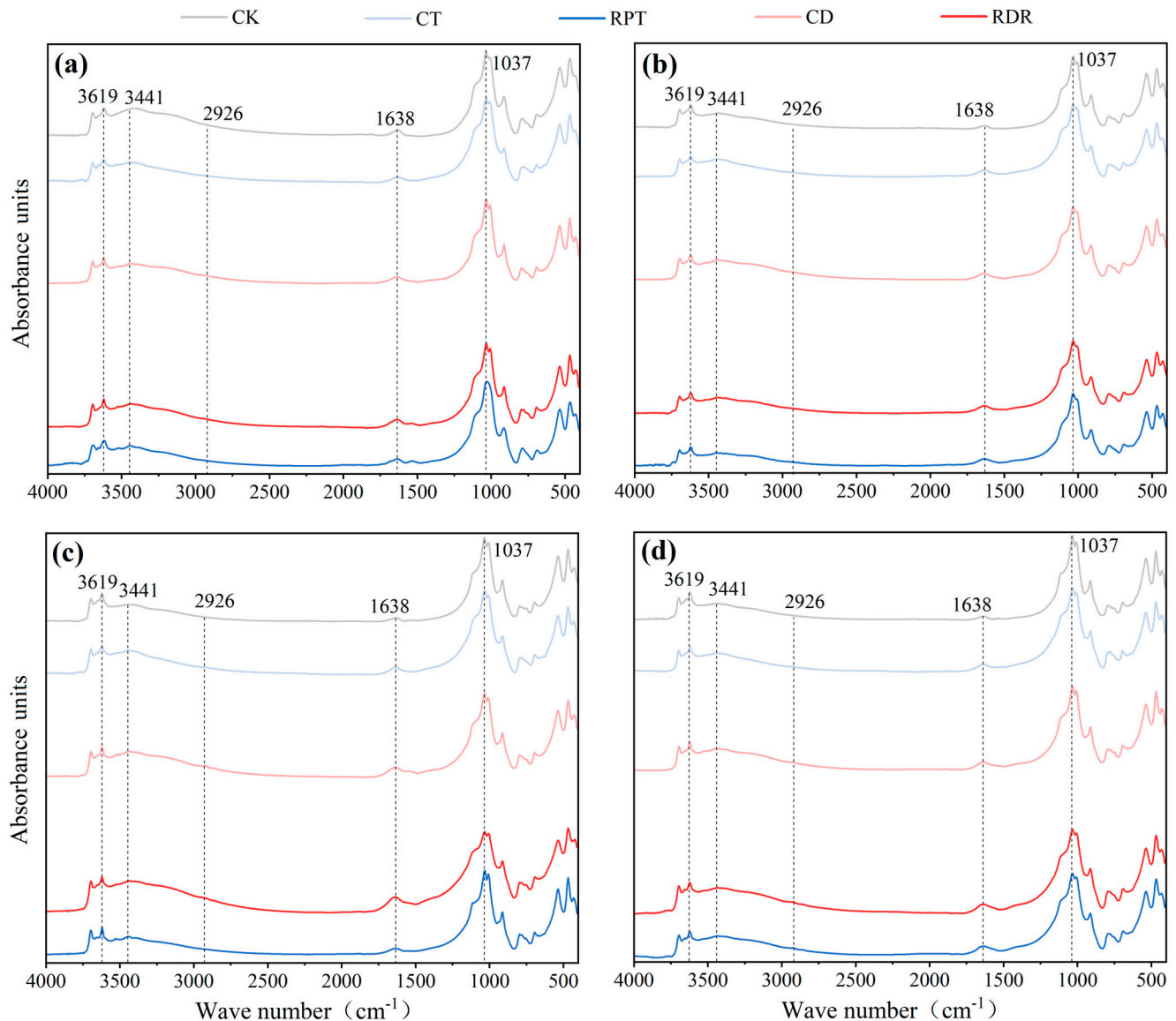


Figure 4. Fourier-transform infrared (FTIR) spectroscopy of $>2\text{ mm}$ (a), $0.25\text{--}2\text{ mm}$ (b), $0.053\text{--}0.25\text{ mm}$ (c), and $<0.053\text{ mm}$ (d) aggregates under different cropping systems. CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation.

3.4. Soil Microbial Properties in the Aggregate Fractions

Cropping systems altered the soil's C-cycling enzymes in the aggregate fractions (Figure 5). Specifically, compared with the continuous cropping of tobacco, tobacco–pea rotation significantly decreased the β -GC activity in MA and SC (−35.6 and −61.8%, respectively); the PPO activity in ME, MI, and SC (−50.8, −34.0, and −53.2%, respectively); and the IVT activity in ME, MA, and MI (−63.6, −86.0, and −92.3%, respectively) but increased the POD activity in MA and SC (54.1 and 33.5%, respectively) ($p < 0.05$; Figure 5). Moreover, the inclusion of ryegrass increased the β -GC activity in ME and MI (75.9 and

76.3%, respectively), the POD activity in SC (30.4%), the PPO activity in MA and SC (70.2 and 52.0%, respectively), and the IVT activity in MI (203.3%) but decreased the IVT activity in MA (−73.3%) ($p < 0.05$; Figure 5).

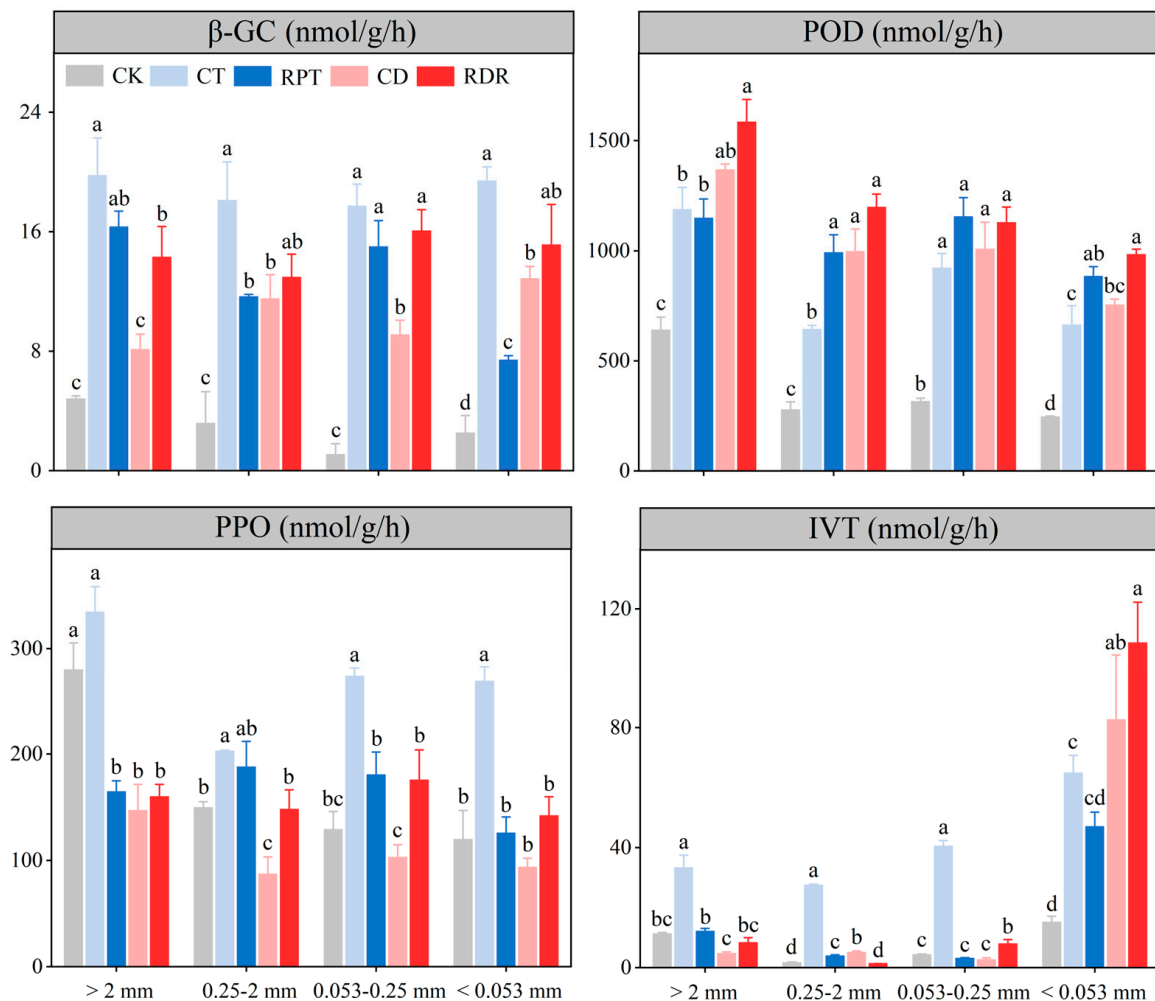


Figure 5. The C-cycling enzyme activities in the soil aggregate fractions under different cropping systems. Error bars represent the standard deviation of three independent replicates ($n = 3$). Different lowercase letters indicate significant differences among cropping systems based on Tukey's test ($p < 0.05$). CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation; β-GC, β-glucosidase; POD, peroxidase; PPO, polyphenol oxidase; IVT, invertase.

Cropping systems had positive effects on the microbial biomass in the soil aggregate fractions. For example, when averaged across the soil aggregate fractions, planting crops resulted in an 83.7–595.0% increase in the microbial biomass content compared with that in abandoned farmland ($p < 0.05$; Figure 6). Generally, the inclusion of pea had a minimal effect on the total microbial, bacterial, and actinomycete biomass across the soil aggregate fractions ($p > 0.05$; Figure 6). However, the fungal biomass in SC was reduced by 60.7% in the tobacco–pea rotation relative to the continuous cropping of tobacco ($p < 0.05$; Figure 6). In addition, the dasheen–ryegrass rotation significantly increased the total microbial, bacterial, and fungal biomass in MA (34.6, 31.3, and 52.4%, respectively) and the actinomycete biomass in ME and MA (46.6 and 44.5%, respectively) compared with the continuous cropping of dasheen ($p < 0.05$; Figure 6).

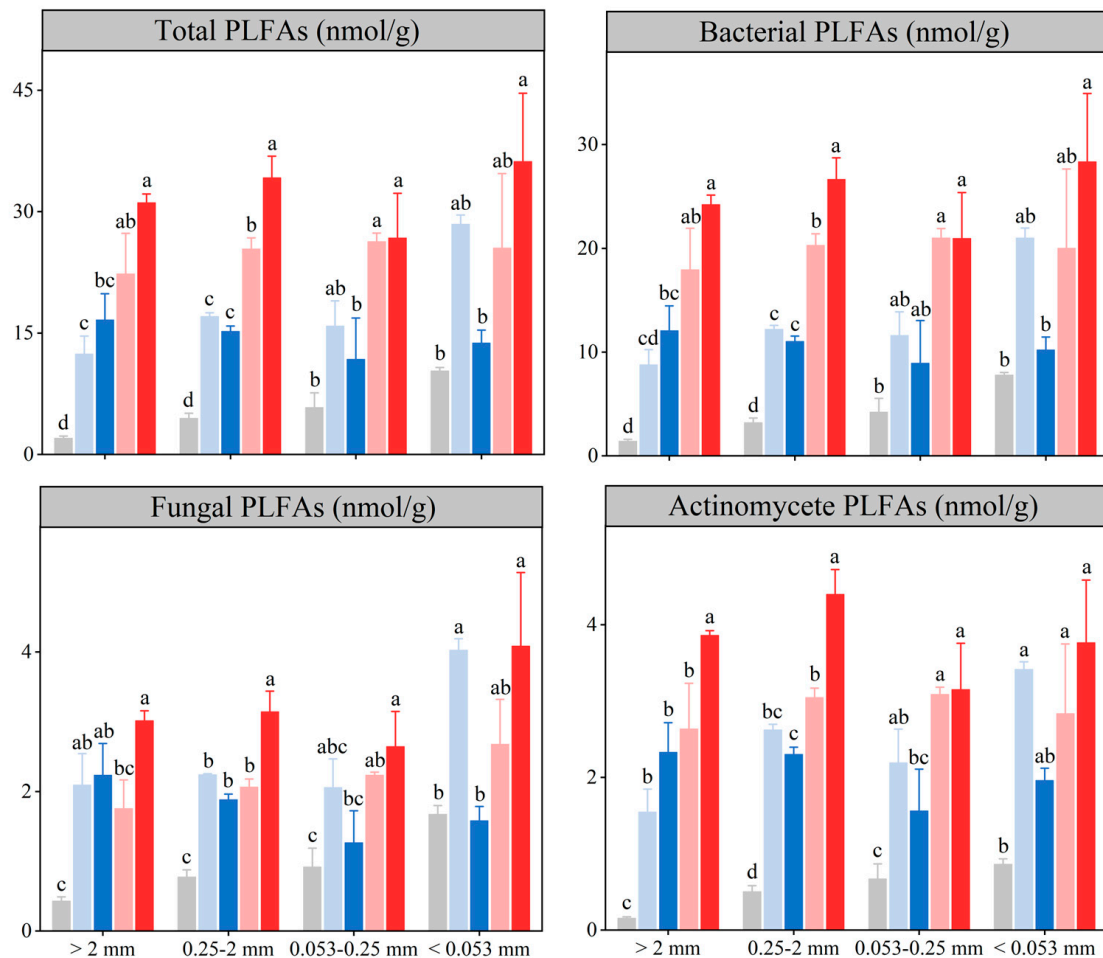


Figure 6. The microbial PLFA abundances in the soil aggregate fractions under different cropping systems. Error bars represent the standard deviation of three independent replicates ($n = 3$). Different lowercase letters indicate significant differences among cropping systems based on Tukey's test ($p < 0.05$). CK, abandoned farmland; CT, continuous cropping of tobacco; RPT, tobacco–pea rotation; CD, continuous cropping of dasheen; RDR, dasheen–ryegrass rotation; PLFA, phospholipid fatty acid.

3.5. Relationships of Soil Organic Carbon with Aggregate Size Distribution and Biochemical Properties at the Aggregate Level

Medium-term crop rotation significantly altered the correlations between soil aggregate size distribution, biochemical properties, and OC content (Figure 7). A correlation analysis showed that, for the continuous-cropping soils, the TOC, AOC, and ROC contents were positively correlated with POD activity but negatively correlated with β -GC, PPO, and IVT activities ($p < 0.05$; $n = 24$; Figure 7a). For the crop rotation soils, the TOC, AOC, and ROC contents were positively related to the Fe/Al-OC, aromatic-C, and aliphatic-C contents and microbial biomass but negatively related to the carbohydrate content ($p < 0.05$; $n = 24$; Figure 7b). The PLS-PM results also showed that soil aggregate size distribution and chemical metrics have positive effects on the OC content, and these effects were enhanced by medium-term crop rotation. Additionally, the increased OC content was mainly driven by the microbial biomass in the aggregate fractions. Medium-term crop rotation changed the negative effect of microorganisms on the OC content into a positive effect at the aggregate level ($p < 0.01$; Figure 8).

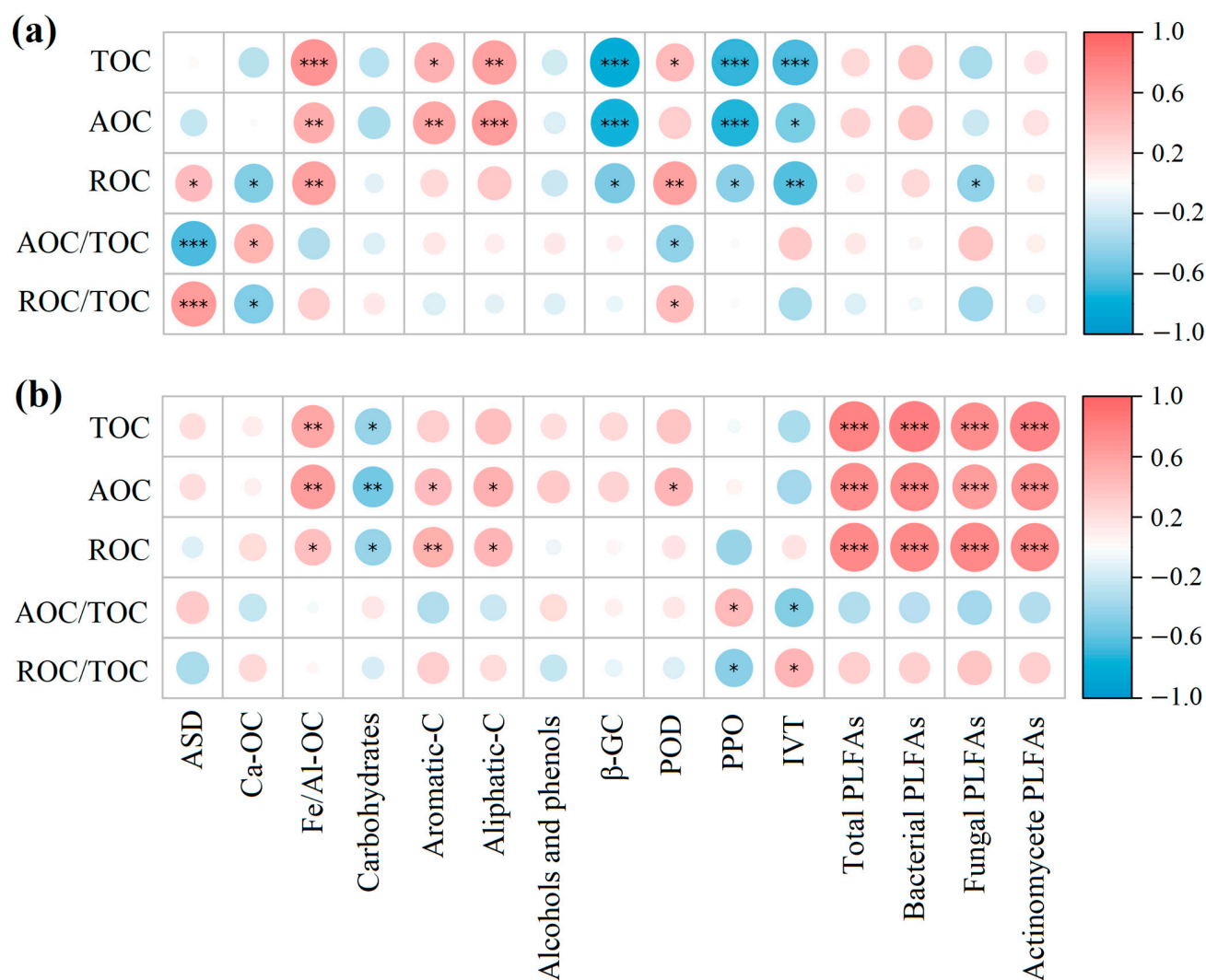


Figure 7. The Spearman correlation between soil aggregate size distribution, biochemical properties, and organic carbon at the aggregate level in the continuous-cropping (a) and rotation (b) treatments ($n = 24$). Asterisks indicate significance at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. TOC, total organic carbon; AOC, active organic carbon; ROC, recalcitrant organic carbon; ASD, aggregate size distribution; Ca-OC, Ca-bound organic carbon; Fe/Al-OC, iron/aluminum-bound organic carbon; β -GC, β -glucosidase; POD, peroxidase; PPO, polyphenol oxidase; IVT, invertase; PLFA, phospholipid fatty acid.

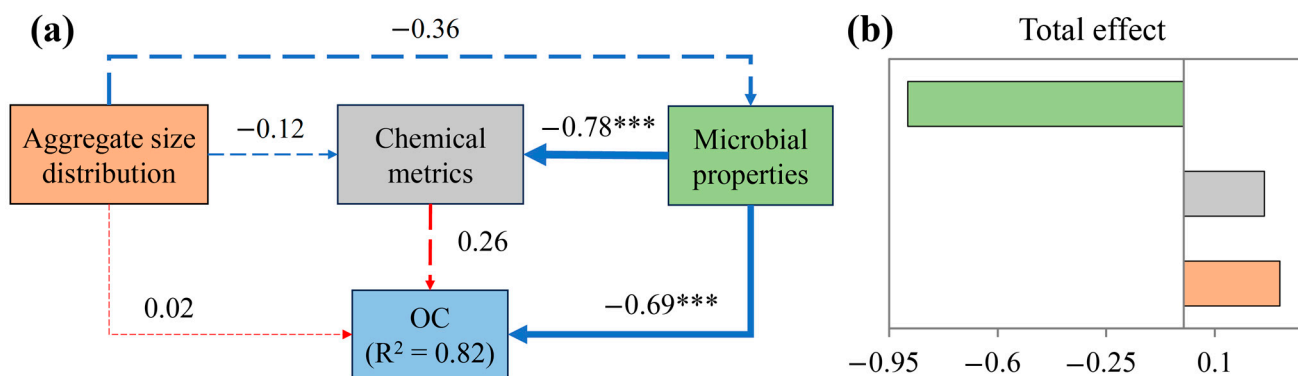


Figure 8. Cont.

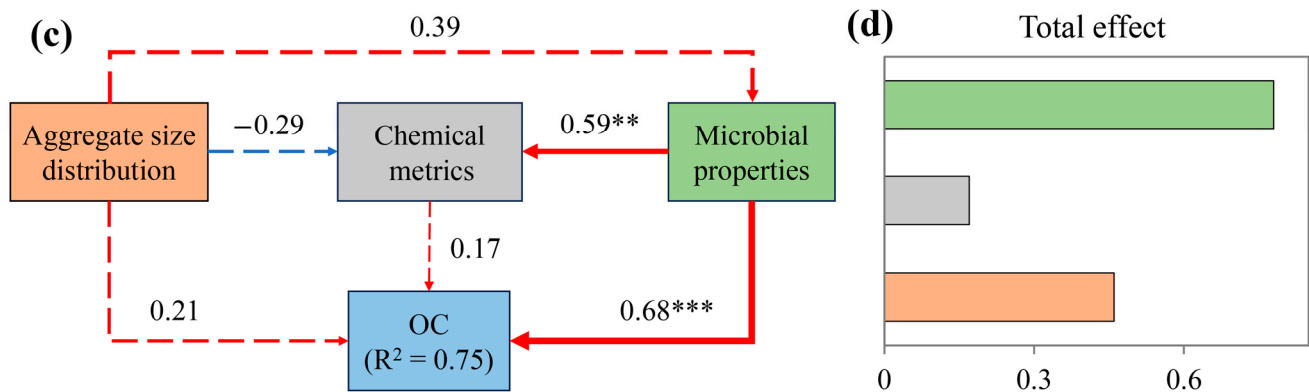


Figure 8. Partial least squares path modeling assessing the direct and indirect effects of soil aggregate size distribution and biochemical properties on organic carbon at the aggregate level in the continuous-cropping (a) and rotation (c) treatments. Numbers adjacent to arrows show standardized path coefficients. Red and blue lines indicate positive and negative relationships, respectively, with the thickness representing the extent of the influence. The R^2 values indicate the proportion of variance explained for each endogenous variable. Path coefficients are calculated after 999 permutations. Asterisks indicate significance at ** $p < 0.01$ and *** $p < 0.001$. Total effect represents the relative importance of soil aggregate size distribution and biochemical properties on organic carbon at the aggregate level in the continuous-cropping (b) and rotation (d) treatments.

4. Discussion

Our results demonstrated that the tobacco–pea and dasheen–ryegrass rotation systems had opposite effects on the soil OC content and biochemical metrics in the aggregate fractions, partially supporting H1. Moreover, the increased OC content was mainly driven by the microbial biomass in the aggregate fractions, and medium-term crop rotation changed the negative effect of microorganisms on the OC content into a positive effect at the aggregate level, supporting H2.

4.1. Effects of Medium-Term Crop Rotation on Soil Aggregate Size Distribution and Stability

Soil aggregation is an important parameter for understanding soil functional and structural quality [56,57]. The results showed that soil texture was directly influenced by the dominance of MA in the soil mass distribution throughout the experiment (Figure 1). The low sand content ($24 \pm 10\%$) in the studied soils (Table 1) promoted soil particle aggregation, resulting in a higher proportion of MA. This was supported by the significant positive correlation between sand and MA (Pearson's $r = 0.727$, $p < 0.01$). This finding is consistent with the results of the study by Zhang et al. [58], where the 0.25–2 mm fraction (over 40.7%) dominated the soil mass in a sandy loam. Moreover, we found that medium-term crop rotation changed the soil textural fractions' percentages (Table 1), mainly because the soil has an important local variability [59]. Another possible reason is that the differences in tillage depth between cropping systems also result in varying degrees of soil mixing, which in turn, leads to differences in soil particle distribution [60].

Our analysis revealed that both dasheen-based cropping regimes markedly increased the ME distribution and the aggregate stability indices (MWD and GMD), while reducing the MI fraction relative to abandoned farmland (Figures 1 and 2). The primary (root physical entanglement) and secondary (fungus-mediated biochemical adhesion) mechanisms were combined to enhance the formation of aggregates [32,61]. The increase in the OC content after crop cultivation (Table 1) might also contribute to soil particle aggregation [62], as supported by a positive relationship between soil OC and the aggregate stability indices ($MWD = 0.87 + 0.02 \times OC$, $p < 0.01$). Moreover, tobacco–pea rotation significantly decreased the proportion of MA (−19.8%) but increased the proportion of MI (57.7%) compared

with the continuous cropping of tobacco (Figure 1). This is mainly due to frequent soil disturbances and the destruction of the arbuscular mycorrhizal fungal network caused by intensive tillage under tobacco–pea rotation, which leads to the decomposition of macro-aggregates into fine aggregates [63,64].

4.2. Effects of Medium-Term Crop Rotation on Soil Organic Carbon in the Aggregate Fractions

Planting crops significantly increased the TOC, AOC, and ROC contents in all aggregate fractions ($p < 0.05$; Figure 1). We ascribed such positive effects to high levels of organic and inorganic fertilizer application on the cropland [65,66]. The nutrient consumption needed for vegetation reconstruction might also explain the decline in OC content in abandoned farmland [67,68]. Another possible explanation is that crops increase OC storage by elevating C inputs (e.g., litter and roots), increasing the diversity and activity of the soil microbial community and suppressing the C losses from decomposition [69–71]. Overall, the significant decline in the OC content in abandoned farmland soils suggests that medium-term natural fallow might not be a wise agricultural management practice in Southwest China.

Compared with the continuous cropping of tobacco, tobacco–pea rotation significantly reduced the TOC content in ME, the AOC content in ME and SC, and the ROC content in MA and MI (Figure 1). The more frequent use of agricultural management practices in tobacco–pea rotation results in the destruction and reorganization of existing aggregates, leading to the release and loss of OC [5,11,20], which is supported by the decrease in the proportion of macro-aggregates and the increase in the proportion of micro-aggregates (Figure 1). However, an increase in plant-derived C from legumes also stimulates soil OC decomposition [72,73]. Additionally, the TOC, AOC, and ROC contents in aggregate fractions were slightly increased in the dasheen–ryegrass rotation compared to the continuous cropping of dasheen (Figure 1). We attributed this result to the fact that dasheen–ryegrass rotation results in diversified litter and substrate inputs, which increases soil microbial biomass (Figure 6) and decomposition rates [73,74]. These opposing effects of increasing the C input and stimulating decomposition might partially offset each other [75,76]. Therefore, the positive responses of the OC content to crop rotation were insignificant in most aggregates. However, the AOC content and AOC/TOC ratio in SC were reduced by 11.3 and 20.2%, respectively, in the dasheen–ryegrass rotation relative to those in the continuous cropping of dasheen (Figure 1), which is attributed to the lower ability of the SC fraction to interact with organic binders derived from residues and microbial activity [24].

4.3. Effects of Medium-Term Crop Rotation on Soil Chemical-Bound Organic Carbon and Carbon Functional Groups in the Aggregate Fractions

In the present study, tobacco–pea rotation decreased the contents of Ca-OC and Fe/Al-OC in aggregate fractions compared with the continuous cropping of tobacco (Figure 4), which could be attributed to the fact that the inclusion of pea inputs more root exudates (e.g., citric acid and oxalic acid), which directly disrupts the binding of metal oxides and C, resulting in the release of protected C for mineralization by microorganisms [77,78]. Organic acids can also dissolve metal minerals and cause them to leach, thus decreasing the contents of Ca-OC and Fe/Al-OC in aggregate fractions [79,80]. Moreover, aliphatic C is unstable, easily decomposed by microorganisms, and typically protected within macro-aggregates [81,82]. The aliphatic-C content in ME was reduced by 32.5% for the tobacco–pea rotation, relative to the continuous cropping of tobacco (Figure 4), mainly due to the decline in macro-aggregates (Figure 1), thus resulting in weaker protection for aliphatic C. However, the decreased aliphatic-C content in the tobacco–pea rotation soil did not lead to an increase in aromatic C (which is readily adsorbed by metal oxides) but to an increase in carbohydrates (Figure 4), indicating that aliphatic C is more easily decomposed by microorganisms than

carbohydrates. Few studies have reported on selective carbohydrate adsorption by metal oxides [83–85]. Therefore, we speculate that the high affinity for carbohydrates in tobacco–pea rotation soil is not correlated with the low contents of Ca-OC and Fe/Al-OC.

We found that dasheen–ryegrass rotation had minimal effects on the contents of Ca-OC and Fe/Al-OC, but significantly increased the aromatic-C content in ME (21.8%) compared with the continuous cropping of dasheen (Figure 4). The increased microbial biomass—especially fungi and actinomycetes (Figure 6)—promotes Ca-OC and Fe/Al-OC accumulation, leading to increased microbial metabolites and residues, which are the main precursors forming Ca-OC and Fe/Al-OC [86–88]. This is further supported by a significant positive relationship between microbial properties and chemical metrics in rotational soils (Figure 7).

4.4. Effects of Medium-Term Crop Rotation on Soil Microbial Properties in the Aggregate Fractions

Our results showed that, compared with the continuous cropping of tobacco, tobacco–pea rotation decreased almost all soil C-cycling enzyme activities and the total microbial, bacterial, fungal, and actinomycete biomass in macro- or micro-aggregates (Figures 5 and 6). We ascribed this to the fact that the inclusion of pea (a legume plant) improves soil N levels by providing litter with a low C/N ratio and biological N fixation [89–91], which increases microbial C limitation [92]. Alternatively, the more frequent use of agricultural management practices in the tobacco–pea rotation results in the destruction of the fungal mycelial network and the deterioration of the microbial living environment (e.g., frequent moisture and temperature changes) [23,93,94], which results in decreased microbial metrics in tobacco–pea rotation soils.

In contrast, the inclusion of ryegrass increased almost all soil C-cycling enzyme activities and the total microbial, bacterial, fungal, and actinomycete biomass in all aggregate fractions (Figures 5 and 6). The root deposits from crops and the decomposition of crop litter directly increase dissolved OC, organic acid, and organic matter in dasheen–ryegrass rotation soils, thereby promoting microbial growth [95–97]. However, these increases were greater in macro-aggregates than in micro-aggregates (Figures 5 and 6), possibly because the dasheen–ryegrass rotation preferentially allocates labile carbon substrates for microbial biomass synthesis within macro-aggregates rather than micro-aggregates [98,99], ultimately promoting medium-term C stabilization through microbial-mediated processes [74].

4.5. Implications

This study investigated the effects of medium-term crop rotation on soil aggregates and aggregate-associated OC content and biochemical properties, providing key insights for crop management in sustainable agriculture. The results revealed significant crop specificity in rotation benefits (Figures 1–6), indicating that crop rotation benefits are not universal but strongly dependent upon the complementarity of the crop functional groups. For instance, leguminous crops enhanced OC storage through symbiotic nitrogen fixation and C inputs, while deep-rooted grasses enhanced physical protection via aggregate formation. Therefore, crop combinations with biochemical synergistic effects need to be scientifically selected based on regional soil conditions (e.g., sand/clay ratio) and management goals (e.g., C sequestration, structural enhancement, or nutrient cycling). Additionally, the increased OC content in the crop rotation system was mainly driven by the microbial metrics in aggregate fractions, and medium-term crop rotation reversed the microbial influence on the OC from negative to positive (Figure 8), underscoring the need (i) to maintain microbial community diversity through diversified crop rotations to stimulate humus synthesis and binding agents and (ii) to strengthen the protection of aggregates' physical structures by minimizing mechanical tillage and retaining surface cover to sustain

microbial microenvironments. Overall, medium-term crop rotation not only enhances soil OC storage by improving aggregate stability and microbial communities (particularly through the physical protection of readily decomposable C fractions) but also restructures C-nutrient coupling pathways (e.g., the positive correlation between OC and microbial biomass). Therefore, we propose incorporating medium-term crop rotation systems into fallow land management and climate-smart agriculture frameworks to achieve agricultural C neutrality goals via the accumulation of stabilized and aggregate-associated OC.

5. Conclusions

Overall, our results showed that the effect of medium-term crop rotation on soil aggregate-associated OC content and biochemical properties varied between crop types. The tobacco–pea and dasheen–ryegrass rotation systems showed opposite effects on the soil OC content and biochemical metrics in the aggregate fractions. Furthermore, for the continuous-cropping soils, the OC contents were positively correlated with POD activity but negatively correlated with other enzymes. For the crop rotation soils, the OC contents were positively related to the Fe/Al-OC, aromatic-C, and aliphatic-C contents and microbial biomass but negatively related to the carbohydrate content. Additionally, the effects of soil chemical metrics on the OC content in crop rotation systems were greater in rotation systems than in continuous-cropping systems. The increased OC content was mainly driven by the microbial properties in the aggregate fractions, and medium-term crop rotation changed the negative effect of soil microorganisms on the OC content into a positive effect at the aggregate level. Medium-term crop rotation enhances OC storage by improving soil structural stability and microbial community dynamics.

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