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Nitrogen fertilizer builds soil organic carbon under straw return mainly via microbial necromass formation

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ABSTRACT

Carbon (C) and nitrogen (N) inputs strongly influence the formation, turnover and sequestration of soil organic carbon (SOC) in agricultural ecosystems. It is not clear, however, how N input regulates the contribution of plant- and microbial-derived C to SOC sequestration under straw return. To fill this gap, plant and microbial biomarkers, as well as enzyme activities were determined in a long-term (18 years) field experiment. Straw return and N fertilization increased SOC content by 20% and 10%, respectively. Specifically, straw return increased the proportion of total lignin (mainly vanillyl and syringyl) phenols in SOC by 16%, but decreased the proportion of cinnamyl in SOC by 7.5%. This implied that some plant residues were selectively preserved, while the compounds that were less stable than cinnamyl were easily decomposed. The increased phospholipid fatty acid (PLFA) content and enzyme activities with straw return indicated the acceleration of straw decomposition. Based on amino sugar content, straw return did not alter the proportion of microbial necromass to SOC. Together, lignin and amino sugars co-determined the stable contribution of plant- and microbial-derived C to SOC sequestration under straw return. N fertilization increased the portion of microbial necromass (especially bacterial necromass) C in SOC by 6% and thus decreased the plant residue contribution to SOC. Accordingly, N fertilization accelerated the microbial utilization of straw and consequently microbial necromass formation. In terms of PLFA composition, Ascomycota and Basidiomycota, Actinobacteria, and Gram-negative bacteria were the key groups forming microbial necromass and thus SOC. N fertilization increased N-acquiring enzyme activities and boosted the involvement of microbial necromass in nutrient cycling, which in turn may stimulate plant and microbial growth. Overall, straw return simultaneously increased plant- and microbial-derived C, while N fertilization stimulated microbial growth and enzyme activity and thus increased straw conversion to microbial necromass.

1. Introduction

It is critical to increase the sequestration and storage of soil organic carbon (SOC) in croplands to maintain and improve soil fertility (Lal, 2004; Yan et al., 2011; H. Zhao et al., 2018) as well as to mediate climate warming by reducing CO_2 emissions (van Wesemael et al., 2010). SOC is fundamentally derived from plants and stabilized in soil through complex biotic and abiotic processes (Lehmann and Kleber, 2015; Liang

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et al., 2017). Lignin is commonly used as a biomarker of plant residues (Hofmann et al., 2009; Hall et al., 2020) due to its chemical persistence, extremely low energy availability (Gunina and Kuzyakov, 2022) and longer turnover time. However, an increasing number of studies suggest that such recalcitrant lignin can be decomposed quickly at the early stage of decomposition (Lehmann and Kleber, 2015; Zhou et al., 2022), given that it is easily accessible and less protected by minerals or aggregates (Angst et al., 2021; Whalen et al., 2022; Xiao et al., 2023). Accessible plant residues can be converted into microbial-derived C through the *in vivo* turnover of microorganisms. The accumulation of microbial-derived C has recently been considered equally important as plant residues and even more important in some cases (Sokol and Bradford, 2019). More importantly, microbial necromass is closely associated and interacts with the soil mineral matrix to form relatively persistent organo-mineral associations. Accordingly, microbial necromass C tends to be more stable than plant-derived C (Liang et al., 2017, 2020; Sokol et al., 2022). Therefore, clarifying the contribution of plant-derived C (plant residues) and microbial-derived C (microbial necromass) to SOC is the key step in determining the mechanisms of SOC formation (Ma et al., 2018; Li et al., 2020; Luo et al., 2022).

The contribution of plant- and microbial-derived C to SOC in cropland can be influenced by human activities (Liang et al., 2020; Guhra et al., 2022). For example, straw return is a management regime with much higher C input than the input of only stubble (including rhizodeposits) C in croplands (Liu et al., 2014; Li et al., 2019; Berhane et al., 2020). Such massive plant residues must be degraded by enzymes to relatively small monomers or oligomers (typically less than 600 Da) before they can be actively transported across the cell walls of microorganisms (Weiss et al., 1991; Hedges and Oades, 1997). The hydrolysis of plant residues takes time, and thus the continuum from intact plant tissue to highly refined components increases the contribution of plant-derived C to SOC (Lawthef and Banksb, 1997; Lehmann and Kleber, 2015; Prescott and Vesterdal, 2021). Additionally, fresh plant residues may induce a priming effect to accelerate soil organic matter turnover (Mason-Jones et al., 2018). Especially, the high C:nitrogen (N) ratio of straw will stimulate microorganisms to excavate organic matter with high N content (e.g., microbial necromass) to balance N demand (Cui et al., 2020; Hao et al., 2021; Craig et al., 2022). Therefore, straw return may lead to an increase in the contribution of plant-derived C to SOC, but there is still a lack of direct evidence.

N fertilization maintains the stoichiometry of C:N balance under straw return and therefore prevents the decomposition of microbial necromass caused by N mining (Feng et al., 2022). In addition, N fertilization may induce an entombing effect: the accelerated transformation of plant residues into stable microbial products (Liang et al., 2017; Wang et al., 2018b; Xu et al., 2022), leading to an increase in microbial-derived C within SOC. Consequently, straw return combined with mineral fertilization can increase SOC by 0.12–0.16 Mg C $year^{-1}$ (Liu et al., 2014; Berhane et al., 2020). However, it remains elusive how the interactions between straw return and N fertilization regulate plant-derived C and microbial-derived C contributions to SOC.

Microorganisms and extracellular enzyme activities determine the conversion of plant residues into microbial C (Wang et al., 2018a; Zhang et al., 2018; Bao et al., 2020). Straw return and N fertilizer alter the symbiosis and competitive relationship between fungi and bacteria (Six et al., 2006). Straw favors fungal growth as they are low-quality substrates (high C:N) rich in lignin and cellulose, etc., while N fertilizers enhance bacterial utilization of low-molecular-weight plant debris, as N fertilizers provide available N and balance the high C:N ratio of straw (Six et al., 2006; Liu and Greaver, 2010; Hao et al., 2021). The selective degradation of plant residues by fungi and bacteria may accelerate the decomposition of plant residues (Glassman et al., 2018; Feng et al., 2022). In turn, active microorganisms and high level of enzyme activities induce fast conversion of plant-derived C into microbial-derived C (Liang et al., 2017; Luo et al., 2022). This means that the interaction between straw return and N fertilization may increase the contribution of microbial-derived C to SOC. On the contrary, the rapid growth of fungi and bacteria leads to the competition between them, and thus weakens their ability to utilize substrates (Glassman et al., 2018; Feng et al., 2022). In addition, N fertilization may also cause excessive ammonia toxicity or soil acidification, resulting in a decrease in microbial respiration and biomass (Wang et al., 2018a; Zhang et al., 2018). Altogether, the contrasting responses of microbial community and activity to straw and N input increase the uncertainty on the conversion of straw to microbial necromass.

Here, based on an ongoing 18-year field experiment conducted in the dryland area on the southern edge of the Loess Plateau, we aimed to 1) quantify the contribution of plant-derived and microbial-derived C to SOC under straw return and N fertilization in cropland; and further 2) determine the microbial mechanisms regulating plant residue conversion to microbial necromass. To accomplish these objectives, lignin phenols and amino sugars were used to assess plant-derived C and microbial-derived C in soil, respectively. Phospholipid fatty acid (PLFA) and C- and N-acquiring enzyme activities were determined to assess microbial community composition and activity. We hypothesized that: 1) the contribution of plant residue accumulation was higher than microbial necromass to the sequestration of new SOC under straw return because microorganisms may not be capable of consuming the excessive additional C at low N levels and; 2) N fertilization accelerated straw conversion to microbial biomass and increased microbial necromass formation by boosting enzyme activities and microbial growth.

2. Materials and methods

2.1. Study site

A long-term field experiment was conducted in 2002–2020 on the southern margin of the Loess Plateau in Yangling, Shaanxi, China (34°17′N, 108°04′E). The mean annual temperature and mean annual precipitation were 13 °C and 635 mm, respectively. The long-term field experiment was a monoculture agroecosystem of winter wheat (*Triticum aestivum* L.) sown in mid-October and harvested in mid-June of the following year. The calcareous clay loam (29.5% clay, 53.9% silt, and 16.6% sand) was developed from loess parent material and classified as Eum-Orthic Anthrosol (or Udic Haplustalf in the USDA system). The initial soil properties (0–20 cm) in 2002 were as follows: pH 8.2, SOC 8.0 g kg^{-1}, available N 7.8 mg kg^{-1}, and Olsen P 9.1 mg kg^{-1}.

2.2. Experimental design

The field experiment was a two-factor randomized block design. The two factors were wheat straw return (Str) and N fertilization (N), both with three levels (Table 1). In total, there were 36 plots (9 treatments × 4 replicates) with a plot size of 9.9 m × 6 m.

Straw mulching was adopted in 2002–2016. During these years, 80% of wheat stubble was left in the inter-rows after harvest in all plots, and the straw mulching rates in the Str7.5 and Str15 treatments were 2250

Table 1
Experimental treatment design, straw and nitrogen input rates.

Treatments	Straw return rate (Mg ha^{-1})		N fertilization rate (kg N ha^{-1}, urea)	
Str0N0	Str0	0	N0	0
Str0N120		0	N120	120
Str0N240		0	N240	240
Str7.5N0	Str7.5	7.5	N0	0
Str7.5N120		7.5	N120	120
Str7.5N240		7.5	N240	240
Str15N0	Str15	15	N0	0
Str15N120		15	N120	120
Str15N240		15	N240	240

Note: Str is straw, N is nitrogen, Str0, Str7.5, Str15, N0, N120, and N240 are the different straw return and N fertilization rates from 2016 to 2020.

and 4500 kg ha^{-1}, respectively. In 2016–2020, only 40% of the stubble was left and the remaining aboveground biomass was removed and stored. This stored straw was crushed to a size <5 cm and was evenly distributed in the plots of treatments Str7.5 (7500 kg ha^{-1}) and Str15 (15,000 kg ha^{-1}) before wheat sowing. Calcium superphosphate (100 kg ha^{-1}, 3.5% P) was applied as basal fertilizer on all plots. Potassium fertilizer was not applied because the loamy soil was naturally rich in potassium. Prior to wheat sowing, a rotary tiller was used to incorporate mineral fertilizers and straw into the soil to a depth of 0–20 cm.

2.3. Crop yield and stubble measurement

Crop yields and soil C and N inputs were assessed annually. Before harvest, the aboveground biomass of winter wheat was sampled in the middle six rows of each plot (20% of each plot). The dry weight of grain and straw was determined after drying at 105 °C for 30 min and thereafter at 60 °C for 8 h. The C and N contents of the straw were 40% and 0.63%, respectively (C:N = 63). Underground parts (roots + rhizodeposits) were estimated to account for 19.6% of total plant C (Bolinder et al., 2007). Atmospheric N deposition was 22 kg ha^{-1} year^{-1} (Liang et al., 2016). Total C and N inputs from straw, stubble (including both above ground and underground parts), N fertilizer, and atmospheric N deposition from 2002 to 2020 are shown in Table 2.

2.4. Soil sampling, SOC and total N analyses

Soil samples (0–20 cm) was collected at the wheat harvest stage (mid-June 2020), and 5 soil cores were collected using a soil auger (3 cm in diameter) from each plot and mixed to form a comprehensive sample. A total of 36 soil samples were collected (9 treatments × 4 replicates), immediately placed in an ice box and shipped to the laboratory. Subsequently, visible rocks and plant debris were removed with tweezers. Each sample was divided into two aliquots. One aliquot was passed through a 2.0 mm sieve, thoroughly mixed and stored at 4 °C for the analyses of PLFA and soil enzyme activities. The other aliquot was air-dried, grounded and all passed through a 0.125 mm sieve to determine SOC, total nitrogen (TN), amino sugars and lignin phenols. SOC was determined by wet oxidation-redox titration and TN was determined by the semi-micro Kjeldahl method (Cao et al., 2022). The calculation of the C:N ratio in the soil was based on the ratio of SOC and TN.

2.5. Lignin phenol analysis

The content of soil lignin monomers was determined according to the method proposed by Otto et al. (2005). Samples were digested with alkaline CuO in Teflon vessels under high pressure to break down lignin macro-molecules into monomer phenols. Octadecyl solid phase extraction column (SampliQ C_{18} columns, Agilent Technologies, Santa Clara, CA, USA) was used to collect lignin-derived phenols, which were then eluted and derivatized with pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide. Phenols derived from lignin were analyzed using a DM-1 column (30 m × 0.25 mm × 0.25 μm) on an Agilent 6890A GC equipped with an FID. Ethylvanillin was added as an internal standard before oxidation and phenylacetic acid was added before derivatization to determine the recovery efficiency of the lignin products.

The lignin phenol included vanillyl (V-type: vanillin, acetovanillone and vanillic acid), syringyl (S-type: syringaldehyde, acetosyringone and syringic acid), and cinnamyl (C-type: p-coumaric acid and ferulic acid). The total content of lignin phenol was calculated as the sum of vanillyl, syringyl and cinnamyl monomers. In addition to the lignin monomer content, the S-type: V-type ratio and the C-type: V-type ratio were calculated to estimate the degree of lignin biotransformation. The decreasing trend of these ratios indicated that lignin biotransformation was in progress. Acid/aldehyde ratios of vanillyl (Ad/Al)v and syringyl (Ad/Al)s were calculated to assess the degree of lignin degradation, with higher ratio values indicating greater degradation (Otto and Simpson, 2006).

2.6. Analysis of amino sugars

Amino sugars were analyzed according to the method described by Zhang and Amelung (1996). Briefly, soil samples were hydrolyzed with 6 M HCl (105 °C, 8 h), filtered, adjusted to pH 6.6–6.8, and centrifuged (1000×*g*, 10 min). The supernatant was freeze-dried and the amino sugars in the residue were redissolved in methanol. The reconstituted amino sugars were converted to malononitrile derivatives and determined on an Agilent 6890A GC equipped with FID using an HP-5 capillary column (25 m × 0.25 mm × 0.25 μm). Amino sugars were quantified using myoinositol added before purification as an internal standard. The recovery efficiency of the amino sugars was quantitatively monitored using methylglucamine which was added to the sample before derivatization as a recovery standard.

Glucosamine (GlcN) and muramic acid (MurN) were selected from the detected amino sugars as representatives to estimate the accumulation of fungal and bacterial necromass (Appuhn and Joergensen, 2006; Engelking et al., 2007; Liang et al., 2019). Fungal necromass C was determined by subtracting bacterial GlcN from total GlcN, assuming that MurN and GlcN occurred in bacterial cells in a 1:2 M ratio. The methods of the calculation were as follows:

$$\text{Fungal necromass C (mg g}^{-1}\text{ soil)} = (\text{GlcN (mmol g}^{-1}\text{soil)} - 2 \times \text{MurN (mmol g}^{-1}\text{ soil))} \times 179.2\text{ g mol}^{-1} \times 9 \quad (1)$$

$$\text{Bacterial necromass C (mg g}^{-1}\text{ soil)} = \text{MurN (mg g}^{-1}\text{ soil)} \times 45 \quad (2)$$

Where 179.2 is the molecular weight of GlcN; 9 is the conversion factor of fungal GlcN to fungal necromass C; and 45 is the conversion factor of bacterial MurN to bacterial necromass C. Microbial necromass C was estimated as the sum of fungal and bacterial necromass C. Maximal plant residue C was the difference between SOC and microbial necromass C (i.e., SOC − microbial necromass C). It should be noted that

Table 2
Total C and N input derived from straw, crop stubble, N fertilizer and atmospheric N deposition during 2002–2019.

Treatments	C input			N input			
	Straw	stubble	Total C input	N fertilizer	N deposition	stubble	Total N input
	Mg C ha^{-1}	Mg C ha^{-1}	Mg C ha^{-1}	kg N ha^{-1}	kg N ha^{-1}	kg N ha^{-1}	kg N ha^{-1}
Str0N0	0	30.9	30.9	0	390	520	910
Str0N120	0	46	46	2160	390	775	3325
Str0N240	0	48.1	48.1	4320	390	811	5520
Str7.5N0	24.6	30.5	55.1	0	390	514	904
Str7.5N120	24.6	49.3	73.9	2160	390	832	3381
Str7.5N240	24.6	53.4	78	4320	390	901	5611
Str15N0	49.2	30.1	79.3	0	390	506	896
Str15N120	49.2	44.6	93.8	2160	390	753	3303
Str15N240	49.2	50.1	99.3	4320	390	846	5556

there are many assumptions in the determination of microbial necromass, which may over or underestimate maximal plant residue C (Liang et al., 2019; Whalen et al., 2022).

2.7. Soil PLFA analysis

The composition of the soil microbial community was assessed by PLFA biomarker analysis. PLFAs were isolated according to the procedure described by Frostegård and Bååth (1996). Briefly, soil PLFAs were extracted from 8 g freeze-dried soil in a single-phase chloroform-methanol-citrate buffer solution. Phospholipids were separated from nonpolar lipids and converted to fatty acid methyl esters (FAMEs) before analysis. FAMEs were quantified using a gas chromatograph (Agilent 7890, Santa Clara, USA) equipped with a flame ionization detector and HPUltra 2 column (25.0 m × 200 μm × 0.33 μm). FAMEs were identified using a MIDI Sherlock Microbial Identification System (MIDI Inc.). Quantification of each fatty acid was performed by comparing the area of individual peaks with that of the internal standard (methyl nonadecanoate; C19:0), and the amount was expressed in nmol g^{-1} dry soil.

The PLFAs were assigned to different microbial groups based on Joergensen (2022). Soil bacteria consist of Gram-positive and Gram-negative bacteria, and the former was formed by Firmicutes and Actinobacteria. PLFAs i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0 were markers of Firmicutes, and PLFAs 10Me16:0, 10Me17:0, and 10Me18:0 were used to represent Actinobacteria. PLFAs 16:1ω9c, 16:1ω7c, cy17:0, 18:1ω7c, and cy19:0 were markers of Gram-negative bacteria. In addition, PLFA 16:1ω5c was a marker of arbuscular mycorrhizal fungi (AMF) and 18:2ω6c was a marker of Ascomycota and Basidiomycota. AMF, Ascomycota and Basidiomycota were fungi.

2.8. Enzyme activities assays

The activities of five soil hydrolytic enzymes were determined according to the method of German et al. (2011). They included three enzymes involved in C-acquisition (β-1,4-glucosidase (BG), cellobiohydrolase (CBH) and β-xylosidase (XYL)), and two involved in N-acquisition (β-1,4-N-acetylglucosaminnidase (NAG) and leucine aminopeptidase (LAP)). Briefly, 96-well microplates were used for the enzyme assays. For each sample, 1 g of fresh soil was suspended in 50 ml of 50 mM sodium acetate buffer (pH 8.0). 200 μl of soil suspension and 50 μl of 200 μM enzyme substrate were added to each microplate well. 200 μl of soil suspension and 50 μl of sodium acetate were added to the control wells. Quench control wells were filled with 50 μl standard substrate of 7-amino-4-methylcoumarin or 4-methylumbelliferyl (10 μM) and 200 μl soil suspension. Then, 50 μl of 200 μM enzyme-specific substrate was added to 200 μl sodium acetate buffer in blank substrate wells. Eight replicates of each plate were incubated in the dark at 25 °C for 2.5 h. Fluorescence was measured using a microplate reader (M200PRO, TECAN Company, Switzerland) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The activity of the hydrolytic enzymes was expressed in μmol g^{-1} dry soil h^{-1}.

2.9. Statistical analysis

All data in figures are presented as mean ± standard errors (SE) of the four replicates (n = 4). All statistical analyses were conducted in JMP 11 for Windows (SAS Institute Inc., Cary, NC) or R (version 3.6.1). The Shapiro-Wilk test was used to test for normality, and the Levene test was used to test the homogeneity of variance. Data for SOC, soil C:N ratio, fungal necromass C, fungal necromass C in SOC, microbial necromass C in SOC, and total lignin phenols met the requirements of normality and homogeneity. The two-way analysis of variance (ANOVA) was used to reveal the effects of straw return, N fertilization, and their interactions on the above parameters at $p < 0.05$. The Tukey's HSD test was followed to determine the statistical significance of these parameters at $p < 0.05$. Bacterial necromass C, bacterial necromass C in SOC, and total lignin phenols in SOC were found to be out of normality or homogeneity. The Scheirer-Ray-Hare test was used to reveal the effects of straw return, N fertilization, and their interactions on these parameters at $p < 0.05$. Dunn test was employed to determine the statistical significance of these parameters at $p < 0.05$. If the effects of both straw return and N fertilization were significant, η^2 was calculated as a parameter to represent the contribution of each factor to the total effect size.

$$\eta^2 = SS_A/SS_{total} \quad (3)$$

Where SS_A is the variance between treatments due to factor A and SS_{total} is the total sum of squares.

The relationships among SOC, fungal necromass C, bacterial necromass C, lignin phenols, the degree of lignin degradation, PLFA, and enzyme activities were evaluated using Spearman's correlation. Random forest (RF) analysis was applied to identify the main predictors (fungal necromass C, bacterial necromass C, and lignin phenols) of SOC sequestration. The percentage increases in the mean square error (MSE) of variables were calculated to estimate the importance of these predictors. The higher MSE% values indicate relatively more important variables. The significance of each predictor for the response variables was assessed with the "rfPermute" package in R.

3. Results

3.1. Soil organic carbon content and C:N ratio

Compared with Str0N0, straw return and N fertilization increased SOC by 4.8%–38.4% (Fig. 1a). The increased effect was more pronounced at high straw return rates than at other rates (Str0 < Str7.5 < Str15). Similarly, compared to the soil without N fertilization (N0), N fertilization (N120 and N240) increased SOC by 8.4% and 11%, respectively. The effect size of straw return on SOC content was greater than that of N fertilizer. The C:N ratio was higher in soil with straw return than in soil without straw return, but N fertilization had no effect on the C:N ratio (Fig. 1b).

3.2. Soil lignin phenols in soil organic carbon

Compared with the soil without straw return (Str0), straw return (Str7.5 and Str15) increased the content of total lignin phenols, V-type, and S-type phenols as well as their proportion in SOC (Str0 < Str7.5 < Str15, Fig. 2a–c, e-g). The C-type phenol content was higher under Str15 than under Str7.5 and Str0, while the proportion of C-type phenols in SOC was lower under Str7.5 and Str15 than under Str0 (Fig. 2d and h). Thus, higher straw returns increased the contribution of V-type and S-type phenols to SOC but decreased the contribution of C-type phenols.

Straw return and the interactions between straw return and N fertilization affected lignin biotransformation (Fig. 3a). Compared to Str0, straw return decreased the C-type: V-type ratio by 24% and 28% under Str7.5 and Str15, respectively. The ratio of S-type: V-type was higher under Str0N240 than under Str0N120 and Str0N0, while those under Str7.5 and Str15 were similar regardless of the N fertilization rate. Furthermore, the $(Ad/Al)_V$ and $(Ad/Al)_S$ ratios decreased with increasing straw return (Fig. 3b) and were negatively correlated with the content of V-type and S-type phenols (Fig. 3c and d), respectively. The $(Ad/Al)_S$ ratio under N240 was lower than that under N0.

3.3. Microbial necromass C in soil organic carbon

Compared with Str0N0, straw return and N fertilization increased fungal and bacterial necromass C by 2.2%–35.9% and 13.8%–68.4%, respectively (Fig. 4a and b). Fungal necromass C content was increased by both straw return (Str0 < Str7.5 < Str15) and N fertilization (N0 <

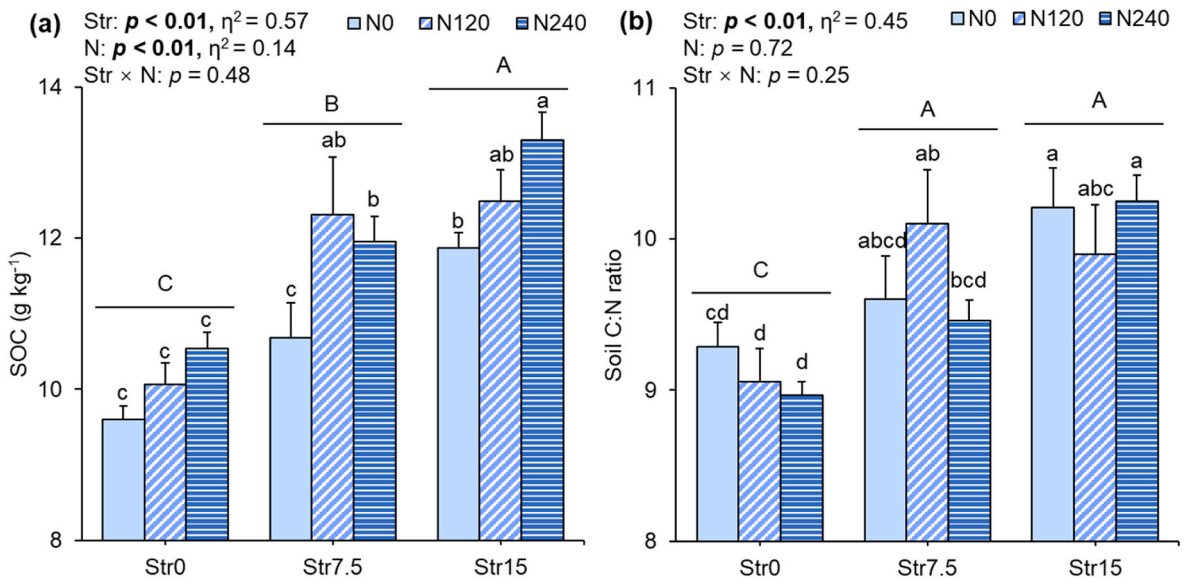

Fig. 1. Effects of long-term straw return and N fertilization on SOC content (a) and soil C:N ratio (b). Error bars represent standard errors of the mean (n = 4). Str0, no straw return; Str7.5, low straw return rate; Str15, high straw return rate; N0, no N fertilization; N120, low N fertilization rate; N240, high N fertilization rate. Upper case letters indicate significant differences among three straw return rates at $p < 0.05$. Lower case letters indicate significant differences among all treatments at $p < 0.05$. η^2, the contribution of each factor to the total effect size.

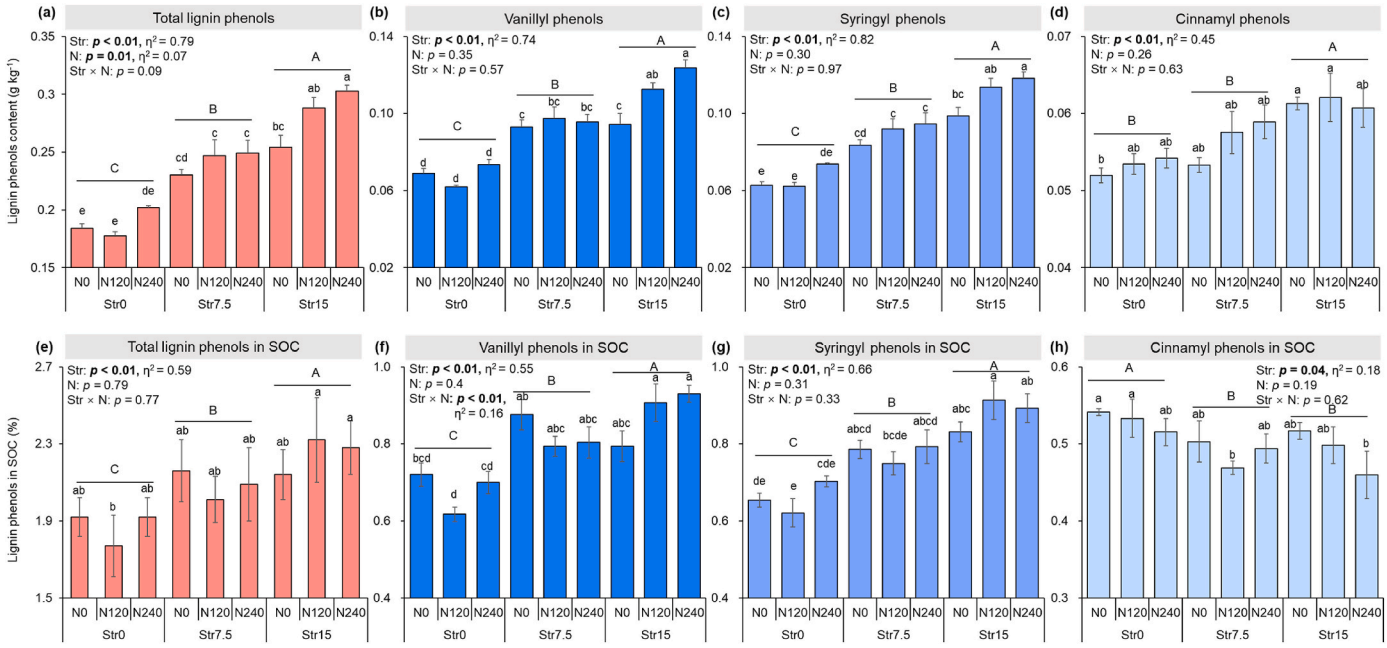

Fig. 2. Effects of long-term straw return and N fertilization on total (a) and individual (b–d) lignin phenol contents in soil, and the proportion of total (e) and individual (f–h) lignin phenols in SOC. Str0, no straw return; Str7.5, low straw return rate; Str15, high straw return rate; N0, no N fertilization; N120, low N fertilization rate; N240, high N fertilization rate. Error bars represent standard errors of the mean (n = 4). Upper case letters indicate significant differences among 3 straw return rates at $p < 0.05$. Lower case letters indicate significant differences among all treatments at $p < 0.05$. η^2, the contribution of each factor to the total effect size.

N120 = N240) (Fig. 4a). In contrast, straw return and N fertilization had no effects on the proportion of fungal necromass C in SOC (Fig. 4d). N fertilization rather than straw return increased bacterial necromass C content and its contribution to SOC (Fig. 4b and d). N120 and N240 substantially increased the proportion of bacterial necromass C in SOC compared to N0. N240 reduced the proportion of the maximal plant residue C in SOC compared to N0 (Fig. 4c). The random forest model indicated that fungal necromass C, bacterial necromass C and lignin phenols were the reliable predictors of SOC, and among them, fungal necromass C contributed the most to SOC (Fig. 5).

3.4. Microbial community composition and enzyme activities

Straw return and N fertilization increased the PLFA contents and changed the microbial community composition (Table 3). The PLFAs of Ascomycota and Basidiomycota, Actinobacteria and Gram-negative bacteria were higher under Str7.5 and Str15 than under Str0. Similarly, they were higher under N120 and N240 than under N0 (Table 3).

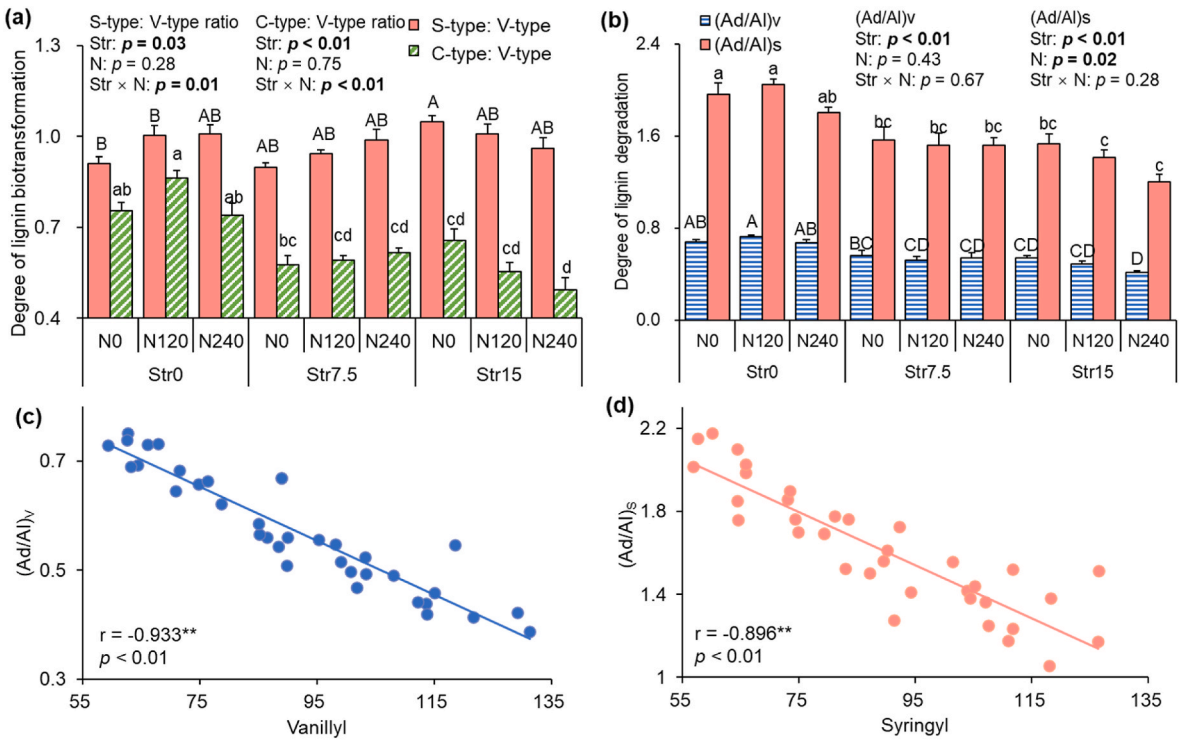

Fig. 3. Effects of long-term straw return and N fertilization on lignin biotransformation and degradation. The degree of lignin biotransformation is represented by the S-type: V-type and the cinnamyl: vanillyl ratio (a). Lignin degradation indices were described by acid/aldehyde ratios of vanillyl (Ad/Al)v and syringyl (Ad/Al)s (b). Spearman correlations of Ad/Al ratios for vanillyl with vanillyl content (c) and syringyl phenols with syringyl content (d). Str0, no straw return; Str7.5, low straw return rate; Str15, high straw return rate; N0, no N fertilization; N120, low N fertilization rate; N240, high N fertilization rate. Upper case letters indicate significant differences of the S-type: V-type ratio or (Ad/Al)v among all treatments at $p < 0.05$. Lower case letters indicate significant differences of the C-type: V-type ratio or (Ad/Al)s among all treatments at $p < 0.05$.

The effect size of N fertilization on the ratio of Ascomycota and Basidiomycota: AMF was higher than that of straw return. Fungal necromass C increased with the PLFAs of Ascomycota and Basidiomycota and AMF (Fig. 6a), while bacterial necromass C increased with the biomass of Actinobacteria, Gram-negative bacteria, and Firmicutes (Fig. 6b). Soil enzyme activities increased with straw return and N fertilization (Table 4). Straw return had a greater impact on BG and LAP, while N fertilization had a greater impact on XYL, CBH, and NAG. Fungal and bacterial necromass C showed positive effects on NAG and LAP (Fig. 6c and d).

4. Discussion

4.1. Straw return did not change the plant-derived C contribution to soil organic carbon

Straw may be acting as a precursor of SOC since straw return increased the content of SOC, as well as plant-derived (represented by lignin) and microbial-derived (estimated from amino sugar) C (Figs. 1, 2 and 4). However, it remains unclear if straw return increased total plant-derived C or only some of the lignin. The proportion of total lignin phenols to SOC increased with straw inputs (Fig. 2) because straw residues could be protected within aggregates and by minerals (Lehmann and Kleber, 2015; Angst et al., 2021; Xiao et al., 2023). Increasing evidence has proven that straw input accelerated the formation and stabilization of aggregates (Huang et al., 2018; Y. Zhao et al., 2018). In this process, straw residues acted as the core of aggregates, and thus they were disconnected from microorganisms and enzymes. In addition, products of lignin oxidation and hydrolysis may be adsorbed by minerals (Angst et al., 2021; Xiao et al., 2023). Straw inputs increased the proportion of V-type and S-type phenols in SOC, and simultaneously decreased their decomposition rate (Fig. 3b–d). This finding indicated the selective preservation of these two monomer types (Angst et al., 2021). Thus, if lignin phenols were used as plant-derived biomarkers (Chen et al., 2021; Yang Yang et al., 2022), then our results indicated that more plant-derived C accumulated in soil under straw return (Figs. 2 and 3). However, straw return decreased the proportion of C-type phenols in SOC (Figs. 2h and 3) because C-type phenols are preferentially located in the outermost cell wall layer (Li et al., 2020) and are easily accessed by microorganisms. These microorganisms mainly included Ascomycota and Basidiomycota, which were stimulated by straw return (Table 3). Some of them e.g., white-rot and brown-rot fungi, are capable of degrading lignin by the production of lignin peroxidase and manganese-dependent peroxidase and laccase (Granja-Travez et al., 2020; Atiwesh et al., 2022). Moreover, lignin is only a small portion of straw (approximately 6–15% of the dry weight of wheat straw) and is commonly interconnected with large amounts of cellulose and hemicellulose to form a lignin-carbohydrate complex (Tarasov et al., 2018). Cellulose and hemicellulose are degraded faster than C-type phenols (Mühlbachová et al., 2021) due to their location on the outside of the lignin-carbohydrate complex and the increased activities of β-1,4-glucosidase, cellobiohydrolase and β-xylosidase (Table 4). Together, these results indicated that the contribution of plant-derived C to SOC may not increase under straw return. Therefore, we conclude that only using lignin as a biomarker of plant residues cannot accurately provide an answer to whether straw return affects the contribution of plant-derived C to SOC.

From another perspective, SOC is commonly divided into two parts: plant-derived and microbial-derived C (Huang et al., 2022; Whalen et al., 2022). Accordingly, the difference between SOC and microbial-derived C is considered as the maximum content of plant-derived C. Therefore, the unchanged proportion of microbial

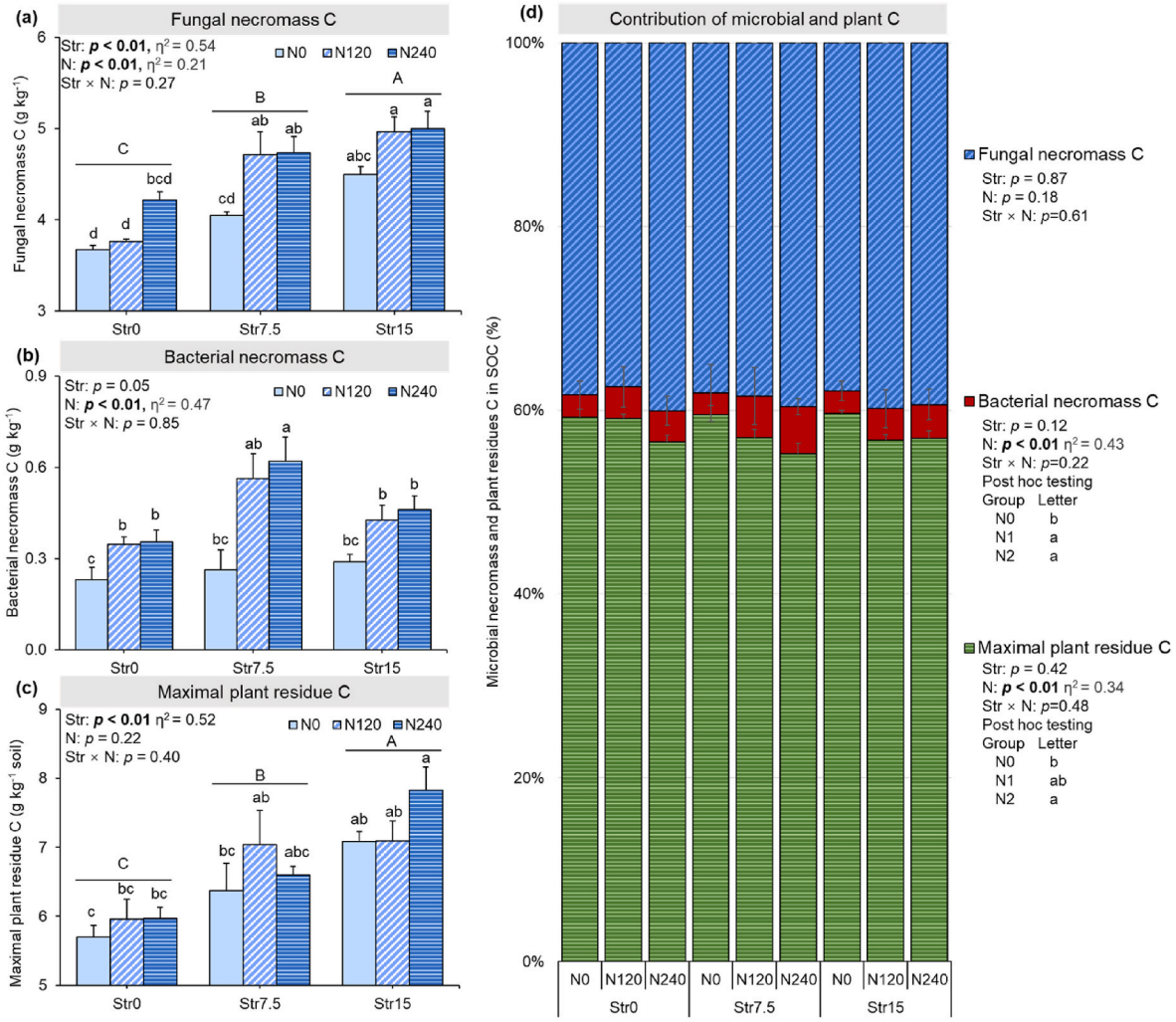

Fig. 4. Effects of long-term straw return and N fertilization on the content of fungal (a), bacterial (b) necromass C, and maximal plant residue C (c) contents. Contribution of microbial necromass C and maximal plant residue C in SOC sequestration (d). Error bars in a-c represent standard errors of the mean (n = 4). Upper case letters indicate significant differences of fungal necromass C among straw return rates (a and c) at $p < 0.05$. Lower case letters indicate significant differences among all treatments at $p < 0.05$.

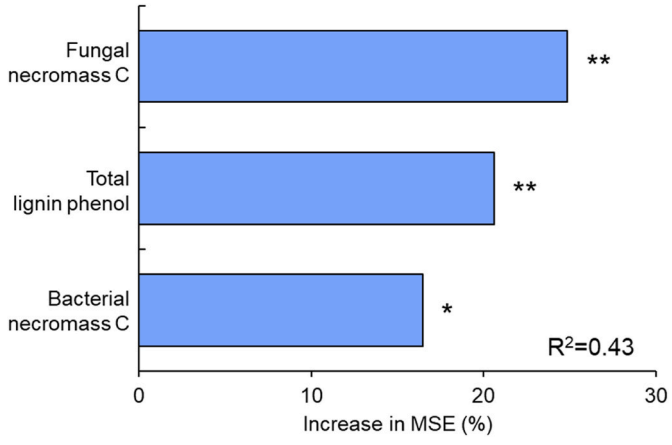

Fig. 5. Relative importance of lignin phenols and microbial necromass C by the percentage increase of the mean squared error (MSE%) using random forests models. **and * in (d) indicates $p < 0.01$ and $p < 0.05$, respectively (n = 36).

necromass C in SOC (Fig. 4d) indicated that straw return did not increase the proportion of plant-derived C as expected, inconsistent with Hypothesis 1. Straw return strongly increased SOC accumulation and this was mainly related to the conversion of straw into fungal biomass which was further subsequently accumulated in the soil as fungal necromass (Fan et al., 2020; Yali Yang et al., 2022). This finding was attributed to the substantial fungal biomass, especially under the conditions of sufficient C sources (Blagodatskaya and Kuzyakov, 2013; Wang et al., 2021; Zhu et al., 2021). In particular, Ascomycota and Basidiomycota contributed more than AMF to fungal necromass, because AMF are commonly low in bulk soil (outside of the rhizosphere), and the incorporation of large amounts of straw and N fertilizer into bulk soil can stimulate the growth of saprophytic fungi over that of AMF (Medina et al., 2020; Karst et al., 2021). In addition, the fungal necromass can be further preserved by physical protection within the aggregates and by adsorption on minerals (Angst et al., 2021). The more active growth of the fungi in turn can stimulate the formation of soil aggregates (Six et al., 2006; Witzgall et al., 2021; Agnihotri et al., 2022; Wei et al., 2022). As a consequence, the coincident increase in both SOC and microbial necromass under straw return resulted in the unchanged microbial necromass proportion in SOC. In addition, the higher relative importance of fungal necromass than lignin phenols to the increase in SOC content with straw return (Fig. 5) indirectly proved that using lignin phenols

Table 3
Effect of long-term straw return and N fertilization on PLFA concentrations (nmol g^{-1}) and microbial community composition.

	Firmicutes	Gram-negative bacteria	Actinobacteria	Ascomycota and Basidiomycota	AMF	Firmicutes: Gram-negative bacteria	Ascomycota and Basidiomycota: AMF	Fungi: bacteria
Str0N0	3.15 ± 0.67 b	2.69 ± 0.31 c	2.26 ± 0.34 bc	0.89 ± 0.12 c	0.61 ± 0.03 b	1.17 ± 0.17 ab	1.46 ± 0.15 e	0.19 ± 0.013 a
Str0N120	3.06 ± 0.64 b	2.62 ± 0.76 c	2.16 ± 0.54 c	0.95 ± 0.21 bc	0.48 ± 0.10 b	1.19 ± 0.15 ab	2.00 ± 0.04 c	0.18 ± 0.011 ab
Str0N240	3.82 ± 0.83 b	3.58 ± 0.66 abc	2.82 ± 0.34 abc	1.41 ± 0.26 abc	0.61 ± 0.08 b	1.06 ± 0.06 b	2.29 ± 0.14 ab	0.20 ± 0.005 a
Str7.5N0	3.87 ± 0.84 ab	2.72 ± 0.66 c	2.50 ± 0.44 bc	0.92 ± 0.18 c	0.57 ± 0.11 b	1.44 ± 0.18 a	1.63 ± 0.04 de	0.16 ± 0.004 b
Str7.5N120	6.10 ± 0.78 a	4.89 ± 0.38 a	3.79 ± 0.32 a	1.83 ± 0.19 a	0.98 ± 0.09 a	1.24 ± 0.12 ab	1.87 ± 0.05 cd	0.19 ± 0.004 a
Str7.5N240	3.79 ± 0.91 b	3.31 ± 0.52 bc	2.73 ± 0.46 bc	1.30 ± 0.25 abc	0.57 ± 0.13 b	1.14 ± 0.11 b	2.31 ± 0.08 ab	0.19 ± 0.004 a
Str15N0	4.11 ± 1.22 ab	3.52 ± 0.74 abc	2.74 ± 0.43 abc	1.15 ± 0.21 bc	0.75 ± 0.13 ab	1.16 ± 0.11 ab	1.53 ± 0.06 e	0.18 ± 0.009 ab
Str15N120	4.48 ± 1.03 ab	4.04 ± 0.79 abc	2.97 ± 0.41 abc	1.47 ± 0.26 ab	0.73 ± 0.15 ab	1.11 ± 0.08 b	2.03 ± 0.10 bc	0.19 ± 0.009 a
Str15N240	4.73 ± 1.28 ab	4.53 ± 0.83 ab	3.31 ± 0.62 ab	1.78 ± 0.31 a	0.72 ± 0.20 ab	1.03 ± 0.11 b	2.52 ± 0.26 a	0.20 ± 0.010 a
Source of variance and multiple comparisons								
Straw return								
p	0.01	0.01	0.01	0.01	0.049	0.02	0.07	0.01
η^2	0.22	0.21	0.21	0.15	0.17	0.21	0.02	0.13
Str15	A	A	A	A	A	B		A
Str7.5	A	A	A	A	A	A		B
Str0	B	B	B	B	B	B		AB
N fertilization								
p	0.11	0.01	0.02	0.01	0.58	0.02	0.01	0.01
η^2	0.08	0.18	0.13	0.38	0.03	0.2	0.87	0.37
N240		A	A	A		A	A	A
N120		A	A	A		AB	B	A
N0		B	B	B		B	C	B
Str*N								
p	0.02	0.01	0.01	0.13	0.01	0.85	0.05	0.04
η^2	0.23	0.26	0.28	0.2	0.37	0.04	0.03	0.15

Note: AMF, arbuscular mycorrhizal fungi. Data are means of 4 replicates ± standard deviation (n = 4). The differences of these microbial parameters were analyzed by two-way ANOVA and Tukey's HSD test. Lower case letters indicate significant differences between various treatments at $p < 0.05$. Upper case letters indicate significant differences between 3 straw return rates or 3 N application rate at $p < 0.05$.

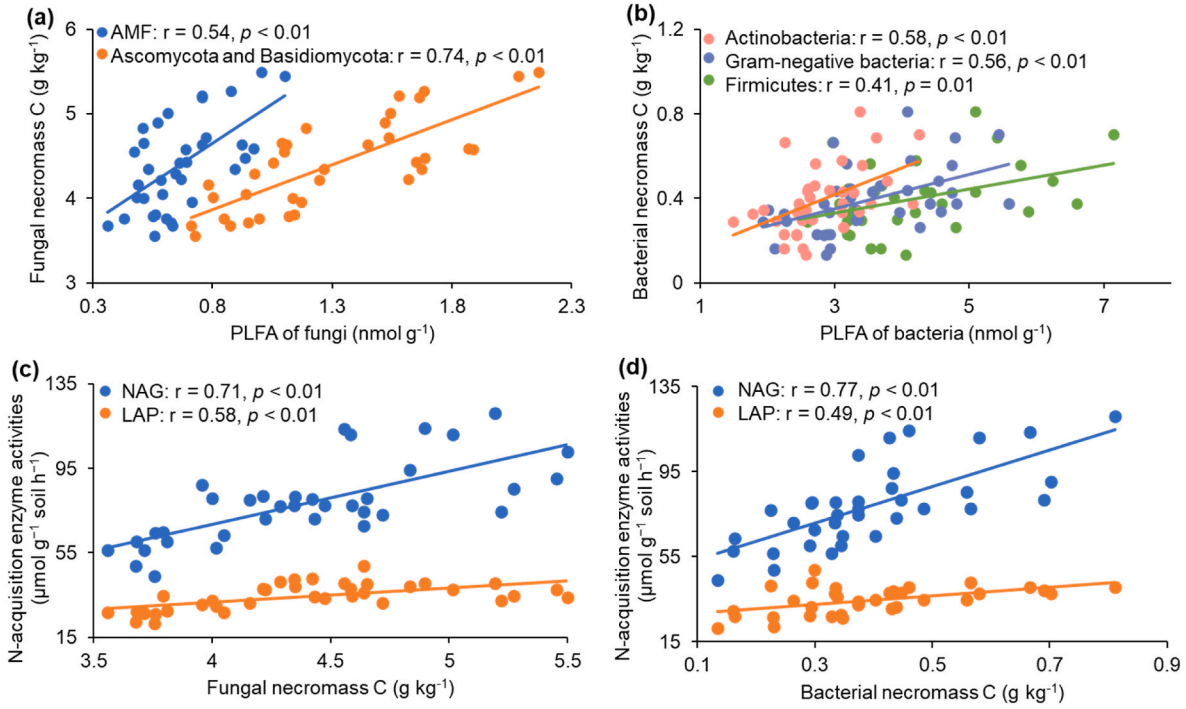

Fig. 6. Spearman correlations of microbial necromass C with PLFA (a, b) or with N-acquiring enzyme activities (c, d). Gram-negative bacteria, gram negative bacteria; AMF, arbuscular mycorrhizal fungi; NAG, β-1,4-N-acetylglucosaminnidase; LAP, leucine aminopeptidase.

Table 4
Effect of long-term straw return and N fertilization on enzyme activities (nmol g^{-1} dry soil h^{-1}).

	β-1,4-glucosidase	β-xylosidase	Cellobiohydrolase	β-1,4-N-acetylglucosaminnidase	Leucine aminopeptidase
Str0N0	74.1 ± 4.5 e	14.4 ± 2.8 d	20.9 ± 5.2 cd	51.4 ± 6.1 d	24.3 ± 2.8 c
Str0N120	82 ± 5.2 de	27.1 ± 6.2 b	23.9 ± 5.3 bcd	62.6 ± 2.5 cd	29 ± 3.9 bc
Str0N240	91.8 ± 10.8 cde	32.2 ± 1.3 ab	32.2 ± 2.4 ab	76.3 ± 6 c	35.6 ± 2.7 ab
Str7.5N0	97.8 ± 7.2 bcd	17.3 ± 1.7 cd	20.8 ± 2.5 d	72.2 ± 13.9 cd	29.6 ± 2 bc
Str7.5N120	113.7 ± 7.8 ab	26.7 ± 1.3 bc	26.4 ± 7.6 bcd	81.7 ± 6 bc	36.3 ± 2.6 ab
Str7.5N240	112.6 ± 4.8 ab	28.9 ± 5.8 ab	30.7 ± 3.1 abc	101.7 ± 19.6 ab	40.8 ± 1.5 a
Str15N0	110.6 ± 13.7 abc	29.9 ± 3.3 ab	28.2 ± 3.5 abcd	75 ± 5.4 c	42.2 ± 5.2 a
Str15N120	118.9 ± 4.6 a	36 ± 3.1 ab	32.4 ± 1 ab	78.5 ± 5.6 c	34.6 ± 3.9 ab
Str15N240	119.4 ± 10.9 a	38.3 ± 5.8 a	37.6 ± 2.1 a	109.8 ± 4.8 a	37.6 ± 2.8 a
Source of variance and multiple comparisons					
Straw return					
p	0.01	0.01	0.02	0.01	0.01
η2	0.69	0.38	0.23	0.36	0.31
Str15	A	A	A	A	A
Str7.5	A	B	B	A	A
Str0	B	B	B	B	B
N fertilization					
p	0.01	0.01	0.01	0.01	0.04
η^2	0.12	0.41	0.45	0.39	0.19
N240	A	A	A	A	A
N120	A	A	B	B	B
N0	B	B	C	B	B
Str*N					
p	0.58	0.92	0.95	0.99	0.04
η^2	0.02	0.03	0.02	0.01	0.29

Note: Data are means of 4 replicates ± standard deviation (n = 4). The differences of these enzyme parameters were analyzed by two-way ANOVA and Tukey's HSD test. Lower case letters indicate significant differences between various treatments at $p < 0.05$. Upper case letters indicate significant differences between 3 straw return rates or 3 N application rate at $p < 0.05$.

alone as a biomarker is not suitable for predicting the proportion of plant-derived C in SOC. Therefore, we suggest considering differences between necromass and SOC for quantifying the proportion of plant residues, and phenol monomers for characterizing the composition of the plant residues.

4.2. N fertilization accelerated microbial utilization of plant residues and necromass formation

N fertilization under straw return increased the ratio of fungi to bacteria, Ascomycota and Basidiomycota content (Table 3). In addition, the interaction of straw return and N fertilization increased Actinobacteria and Gram-negative bacteria content (Table 3). The enrichment of Actinobacteria, Ascomycota and Basidiomycota under straw return helped to increase the utilization efficiency of straw because it is generally believed that fungi (especially Ascomycota and Basidiomycota rather than AMF) and Actinobacteria are the main participants in straw decomposition (Six et al., 2006; Zhao et al., 2016). In addition, the greater amount of Actinobacteria, Gram-negative bacteria, Ascomycota and Basidiomycota were associated with higher enzyme activities (e.g., β-1,4-glucosidase, cellobiohydrolase and β-xylosidase) to acquire nutrients, which may in turn promote their growth. Thus, N fertilization accelerated the conversion of straw into microbial biomass.

N fertilization increased the proportion of bacterial necromass C in SOC (Fig. 4d). In addition to the reasons mentioned above, the low background value of the contribution of bacterial necromass C to SOC (2.4% in Str0N0) made the increase more pronounced compared with that of fungal necromass (38%) (Fig. 4). Surprisingly, the proportion of bacterial necromass C in SOC was much lower than that in other studies (15% on average) (Liang et al., 2019; Xu et al., 2022). This finding may be due to the local soil properties and climatic conditions of the study area (Buckeridge et al., 2020). The high soil pH (8.2), low nutrient content and low soil moisture did not favor bacterial growth (Patoine et al., 2022), while high temperature stimulated the decomposition of necromass (Donhauser et al., 2021; Li et al., 2021). This was further illustrated by the lower bacterial biomass in this study (10.5 nmol g^{-1}, Table 3) than that in other studies (Li et al., 2020; Xu et al., 2022).

Higher N-acquiring enzyme activities were also associated with an increase in microbial necromass C (Fig. 6c–d). This indicated that the consumption of microbial necromass C could be accelerated with its content and percentage increase. Specially, bacteria have a lower C:N ratio and are more dispersed in soil. Thus, their necromass is more accessible to living microorganisms and enzymes and may have been degraded more quickly (Wang et al., 2021; Hartmann and Six, 2022). This was supported by the higher correlation of bacterial necromass with β-1,4-N-acetylglucosaminnidase activity than that of fungal necromass (Fig. 6). Consequently, the increased amount of bacterial necromass and its rapid decomposition suggested that bacteria play an important role in both N sequestration and mineralization (Mason-Jones et al., 2021; Ni et al., 2021; Ma et al., 2022).

5. Conclusions

This study demonstrated the role of plant- and microbial-derived C in SOC sequestration under straw return and N fertilization based on an 18-year field experiment (Fig. 7). Although straw return strongly contributed to SOC sequestration, it did not change the contribution of plant residues or microbial necromass C to SOC. Comparatively, N fertilization accelerated the conversion of plant residues into microbial necromass by boosting the growth of Actinobacteria, Gram-negative bacteria, Ascomycota and Basidiomycota and increasing enzyme activities. As a result, N fertilization built SOC mainly through the accelerated accumulation of microbial necromass. The greater accumulation of microbial necromass increased N-acquiring enzyme activities and thus in turn contributed to the decomposition of necromass. Overall, a comprehensive analysis of the biomarkers of living microbial biomass (PLFA), microbial necromass (amino sugars), and plant residue biomass (lignin phenols) enabled us to trace plant residue conversion to microbial biomass and necromass formation and to demonstrate the importance of microorganisms for SOC sequestration.

Author contributions

Xiangtian Meng, Xiaohong Tian and Yakov Kuzyakov conceived and

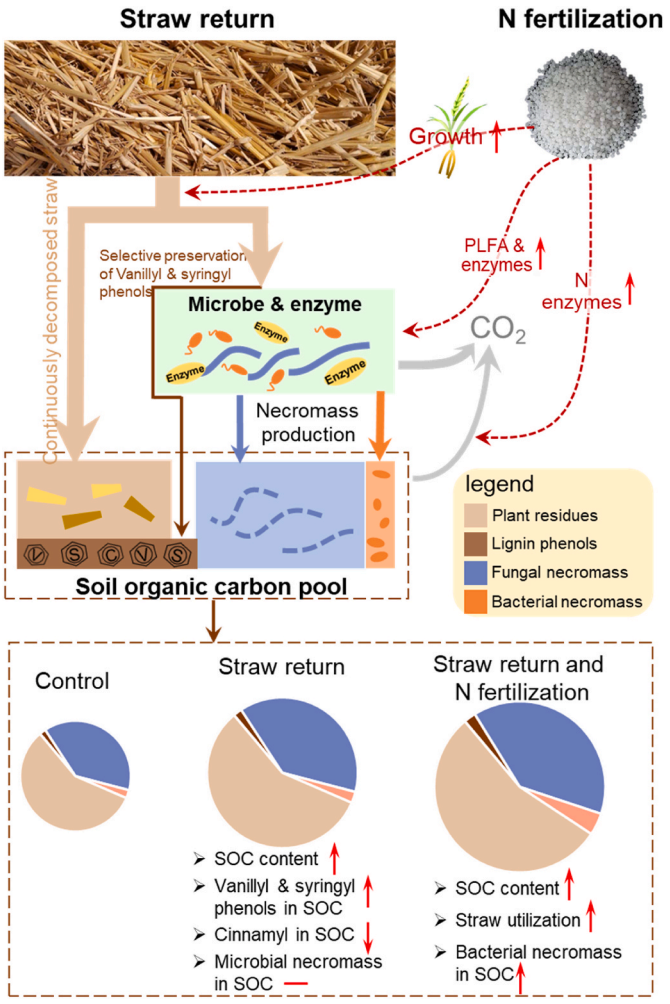

Fig. 7. Conceptual diagram of the impact of straw return and N fertilization on SOC formation. The solid red arrows within the frame indicate an increase, and other colored solid arrows within the frame indicate the accumulation or transformation processes of crop residues or microbial necromass; red dashed arrows within the frame indicate increasing N fertilization.

designed this experiment. Yunuo Li, Yapeng Jiao, Yuhan Jiang, Chunyan Qu and Jianglan Shi performed the field and laboratory work. Xiangtian Meng and Xuechen Zhang drafted the original manuscript. Ekaterina Filimonenko, Yakov Kuzyakov, Lichao Fan and Yuji Jiang contributed to the evaluation of the results. All authors read, corrected, and approved the manuscript.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgments

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