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Nitrogen availability regulates the soil organic carbon sequestration by promoting microbial necromass and plant lignin phenol accumulation in orchard soil amended with organic residues

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Abstract: Plant carbon (C) inputs and their subsequent microbial transformation affect the soil organic C (SOC) net sequestration. However, the characteristics of plant- and microbial-derived C and SOC sequestration under organic matter plus different nitrogen (N) levels in orchard soils remain unclear. Therefore, a pot experiment over 120 days was conducted to investigate the plant and microbial biomarkers in soils under ^{13}C-labelled branches chip combined with N of 225 mg/kg (BRN1), 180 mg/kg (BRN2), 160 mg/kg (BRN3), 140 mg/kg (BRN4) and 0 mg/kg (BR). Branch residue and N addition increased the net SOC sequestration; the ^{13}C recovered in SOC under branch residue plus N treatments was higher than the BR treatment. The highest newly formed C was found under BRN1, followed by BRN2 and BRN3; BRN4 had the lowest newly formed C. Branch residue and N increased lignin phenol content, which promoted syringyl-to-vanillyl and decreased acid-to-aldehyde ratios of vanillyl phenol, indicating branch-C retention in the soil. The microbial necromass C content under residue plus N treatments was higher than under the branch alone treatment, and the highest values were found under the BRN2 treatment. Additional N supply resulted in a greater contribution of microbial necromass C to SOC in soil under branch residue amendment, rather than plant C. Accordingly, BRN2 is considered optimal for net SOC sequestration by plant-derived and fungal necromass C.

Keywords: amino sugar; pruned branch; plant-derived C; N fertiliser; net SOC content

Soil organic carbon (SOC) is an important component of soil quality and fertility, influencing soil physicochemical properties and nutrient cycling (Dinesh et al. 2022). Plant residues, including crop straw and horticultural crop residue, are the major source of organic carbon (C), and their return to the agricultural soil is fundamental for improving organic C storage (Aditi et al. 2023). Several organic residues, such as branches and leaves, are produced during this orchard management strategy (Germer et al. 2017). These residues are often discarded or incinerated, resulting in considerable environmental pollution. Consequently, these pruned branch residues could be effectively used as excellent organic matter and returned to the soil to improve SOC sequestration in orchards (Repullo-Ruibérriz de Torres et al. 2025). Generally, N fertiliser must be supplied when the plant residue is incorporated, as agricultural residues have a high C/N ratio (van der Sloot et al. 2022). However, the excessive application of inorganic N fertilisers has

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been resulted in serious environmental pollution and resource wastes (Wu et al. 2022). To address this situation, China is implementing a reduction in mineral fertiliser use in agriculture. Both the high and low levels impeded the coupling of organic matter and N fertiliser, leading to an imbalance in the soil C/N ratio. Finding the appropriate nitrogen (N) levels is crucial for organic C sequestration during organic matter return and for ensuring agricultural needs, food security, and environmental security. However, limited studies have investigated the characteristics of SOC sequestration in orchard soil amended with pruned branch residues co-applied with various N levels (Jakhro et al. 2025).

Plant residue is the major source for SOC; the recalcitrant plant residues, such as lignin, are a key component of plant-derived C (Sun et al. 2025). Lignin phenols are widely used as unique biomarkers of plant-derived C and are crucial in understanding the quality of organic matter inputs, as well as their use and stabilisation in various SOC (Duan et al. 2025). Three primary phenol groups have been extensively used in numerous studies to analyse the soil lignin content: vanillyl (V-type), syringyl (S-type), and cinnamyl (C-type) (Chen et al. 2024). The sum of V-, S-, and C-type lignin phenol is assessed as total lignin phenol content, which is subsequently used to evaluate the contribution of plant-derived C to the SOC (Sun et al. 2025). Wheat straw and N returning to soil increased soil lignin phenol contents (Meng et al. 2024). In contrast, for the inorganic fertilisation plus manure treatment, maize straw addition decreased lignin phenol content and its contribution to SOC (Liu et al. 2024). The relative change of lignin may vary with different soil qualities, fertiliser types, and amounts (Luo et al. 2025). Plant residues provide a direct energy source for soil microbes, plant C assimilated by microorganisms and transformed into microbial-derived C, which accounts for approximately 30–80% of the SOC (Zhao et al. 2025). Amino sugars are frequently used as microbial biomarkers to indicate microbial necromasses C (Paolo et al. 2025). In certain situations, N fertiliser increases microbial necromass C (Schwalb et al. 2024). Exogenous plant residue increases plant-derived C, whereas straw return plus N fertiliser enhances SOC mainly through microbial necromass C (Meng et al. 2024). In contrast, the application of inorganic fertiliser combined with manure resulted in a higher accumulation of plant phenol content than that of microbial necromass C (Liu et al. 2024). It remains unclear how interactions between branch-residue return and N fertilisation regulate plant-derived C and microbial-derived C contributions to SOC in orchard soil.

Therefore, this study aimed to explore the origin and net sequestration of SOC in orchard soils amended with branch residues combined with different N levels. Identifying the source of SOC as plant-derived or microbe-derived C is critical for examining how different soil management techniques improve SOC sequestration.

MATERIAL AND METHODS

Preparation of ^{13}C-labelled branch residue. The ^{13}C labelled apple branches were prepared from the cv. Fuji apple trees are continually labelled with $^{13}CO_2$. After being labelled, the branches were pruned and ground into pieces ranging from 0.5 to 1 cm in size; the branch residues were then dried at 60 °C and incorporated into the soil. The branch residue contained 573.46 g/kg total C, 1.05% total N, a pH of 6.82, and the δ^{13}C value was 354.69‰.

Experimental design. A pot experiment with six treatments (three replicates each) was carried out. The six treatments including: (1) ^{13}C-labelled branch residue in soil without N fertiliser addition (BR); (2) ^{13}C-labelled branch residue in soil with 225 mg/kg N fertiliser addition (BRN1); (3) ^{13}C-labelled branch residue in soil with 180 mg/kg N fertiliser addition (BRN2); (4) ^{13}C-labelled branch residue in soil with 160 mg/kg N fertiliser addition (BRN3); (5) ^{13}C-labelled branch residue in soil with 140 mg/kg N fertiliser addition (BRN4); (6) soil without branch residue and N fertiliser (CK).

Before the experiment, the soil used in this pot experiment was collected from the 0–40 cm layer using a shovel in an apple orchard in Xinxiang City, Henan Province, China (35°34'21"N, 113°59'59"E; elevation 260.6 m a.s.l.). Six soil collecting sites were randomly selected in the rows of the cv. Fuji apple trees follow a serpentine line. All soil samples were transported to the laboratory and homogenised after visible plant debris and pebbles were removed. The soils are classified as Cambic Arenosol according to the FAO soil classification, and the chemical properties are as follows: pH, 7.34; SOC, 7.51 g/kg; available N, 53.47 mg/kg; available phosphorus, 13.93 mg/kg; available potassium, 48.71 mg/kg; cation exchange capacity, 63.37 $cmol_+$/kg. The homogeneous soil samples were air-dried, sieved through a 2 mm sieve, and used for the pot experiment.

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Each plastic pot (16 cm × 14 cm) contained 1 kg of homogenised soil, which was thoroughly mixed with ^{13}C-labelled branch residue at a soil: branch residue ratio of 1.5% of the soil's dry mass. The N fertiliser was applied as urea. The highest N level (225 mg/kg) in our study corresponds to a maximum N application rate of 10 g N per 1 kg of apple fruit produced, based on recommended N fertiliser management for apple orchards (Kangueehi 2008). Based on this maximum N level, the other N fertiliser rates were reduced by 20, 30, and 40%, respectively. During the treatment, all pots were randomly arranged in a greenhouse (20–25 °C), and watered daily to maintain the moisture at 60%.

After 120 days of treatment, soil samples from each pot were collected. The soil samples were air-dried and sieved; one part was used to measure the contents of SOC, amino sugars and ^{13}C-derived equivalents, and the others were used to analyse lignin phenol contents.

SOC contents and ^{13}C values. SOC contents were determined using previously described methods (Bao 2000). Briefly, a 0.3 g soil sample was wet digested with a mixture of potassium dichromate and sulfuric acid under heating, then titrated with ferrous ammonium sulfate. The δ^{13}C values of SOC were measured using an isotope ratio mass spectrometer (EA-IRMS, IsoPrime 100 Isotope Ratio Mass Spectrometer, Langenselbold, Germany).

The branch-derived newly formed C in SOC was calculated as follows (Liang et al. 2022):

$$\text{New } C_{sample} = C_{sample} \times (\delta^{13}C_{sample} - \delta^{13}C_{control})/(\delta^{13}C_{branch} - \delta^{13}C_{control}) \quad (1)$$

where: C_{sample} – organic C content of the corresponding C pool in the residue treatments; $\delta^{13}C_{sample}$ – δ^{13}C value of the C pool in residue-amended soil; $\delta^{13}C_{control}$ – δ^{13}C value of the corresponding C pool in the CK soil without residue addition; $\delta^{13}C_{branch}$ – δ^{13}C value of the residue.

$$\text{native C} = \text{total SOC} - \text{new } C_{SOC} \quad (2)$$

$$\text{net change in SOC} = SOC_a - SOC_b \quad (3)$$

where: new C_{SOC} – content of branch C in the SOC; native C – content of organic carbon derived from the native soil; SOC_a and SOC_b – SOC contents after and before treatment.

Analysis of lignin phenols. The lignin phenol monomer content in the soil was determined using CuO oxidation and the gas chromatography-mass spectrometry (GC-MS) method (Otto and Simpson 2006). Lignin phenols included vanillyl (V-type [V]: vanillin, acetovanillone, and vanillic acid), syringyl (S-type [S]: syringaldehyde, acetosyringone, and syringic acid), and cinnamyl (C-type [C]: p-coumaric acid and ferulic acid). The total lignin phenol concentration was calculated as the sum of S-, C-, and V-type monomers. The S-type-to-V-type (S/V) and C-type-to-V-type (C/V) ratios were used to determine the degree of lignin biotransformation. The acid-to-aldehyde ratios of the V-type [(Ad/Al)v] and S-type [(Ad/Al)s] were calculated to determine the degree of lignin degradation (Liu et al. 2025).

Assuming that the release efficiency of C-unit phenols was 100%, the ratio of plant-derived C to SOC (Pr) was calculated using the following formula:

$$Pr = \frac{\frac{S}{33.3\%} + \frac{V}{90\%} + C}{8\% \times SOC} \times 100\% \quad (4)$$

where: V, S, and C – carbon contents associated with V-, S-, and C-type phenols, respectively, and 8% is the lowest amount of lignin found in the major plant debris.

Analysis of amino sugar contents and ^{13}C enrichment. The content and ^{13}C enrichment and contents of glucosamine (GlcN), galactosamine (GalN), mannosamine (ManN), and muramic acid (MurA) were measured using the GC-MS method (Zhang and Amelung 1996, Glaser and Gross 2005). The soil samples were hydrolysed with 6 mol/L HCl at 105 °C for 8 h, then filtered and adjusted to pH 6.6–6.8. After a 10 min centrifugation at 1 000 × *g*, the supernatant was freeze-dried.

The newly formed fungal necromass (^{13}C-FNC) and bacterial necromass (^{13}C-BNC) were calculated as follows (Qu et al. 2024):

$$^{13}\text{C-FNC} = (^{13}\text{C-GlcN}/179.17 - 2 \times {}^{13}\text{C-MurA}/251.23) \times 179.2 \times 9 \quad (5)$$

$$^{13}\text{C-BNC} = {}^{13}\text{C-MurA} \times 45 \quad (6)$$

$$\text{Microbial necromass C (MNC)} = \text{FNC} + \text{BNC} \quad (7)$$

Statistical analysis. Statistical analysis was performed using PASW Statistics version 18 (SPSS, Chicago, USA). The results are presented as the mean and standard deviation of three replicates. The treatments were compared using one-way ANOVA and Tukey's test at a significance level of $P < 0.05$. Other charts were created using Origin 2025 software (Origin Lab Corporation, Northampton, USA).

RESULTS AND DISCUSSION

Soil lignin phenol content and biotransformation. Lignin phenols in soil are unique biomarkers of plant-derived C, which is a major source of SOC

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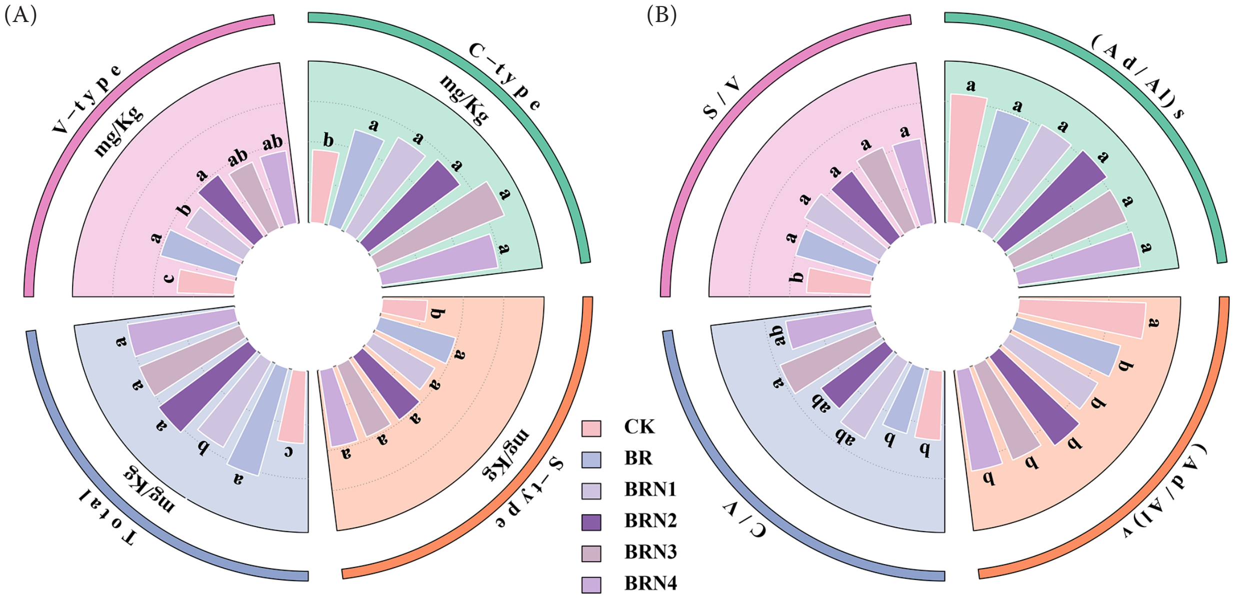

Figure 1. The lignin phenol contents and biotransformation in branch-amended soils subjected to different nitrogen (N) levels. S- – syringyl; C- – cinnamyl; V- – vanillyl. Lignin degradation indices were described by acid/aldehyde ratios of vanillyl (Ad/Al)v and syringyl (Ad/Al)s. Data are expressed as means ± standard deviation (*n* = 3). Lowercase letters indicate significant differences among all treatments at $P < 0.05$. CK – soil without branch residue and N fertiliser; BR – ^{13}C-labelled branch residue in soil without N fertiliser addition; BRN1 – 225 mg/kg N fertiliser addition; BRN2 – 180 mg/kg N fertiliser addition; BRN3 – 160 mg/kg N fertiliser addition; BRN4 – 140 mg/kg N fertiliser addition

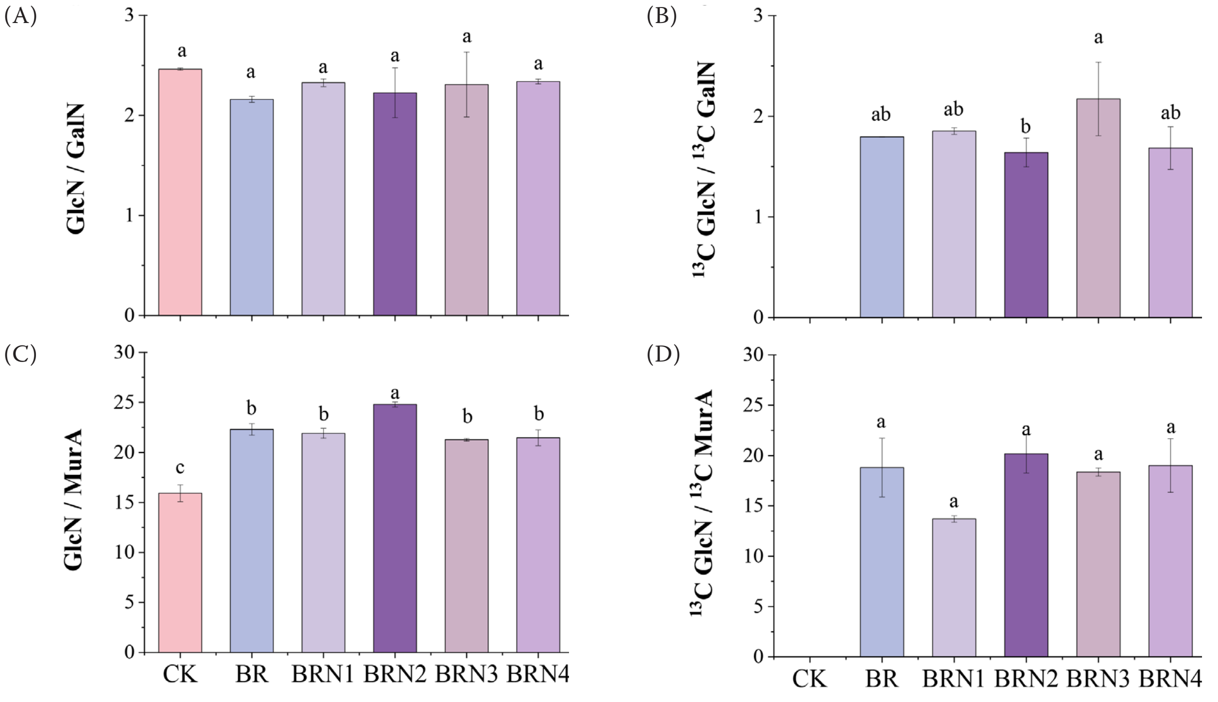

Figure 2. The amino sugars characteristics in branch-amended soils subjected to different nitrogen (N) levels. GlcN – glucosamine; GalN – galactosamine; MurA – muramic acid. Data are expressed as means ± standard deviation (*n* = 3). Lowercase letters indicate significant differences among all treatments at $P < 0.05$. CK – soil without branch residue and N fertiliser; BR – ^{13}C-labelled branch residue in soil without N fertiliser addition; BRN1 – 225 mg/kg N fertiliser addition; BRN2 – 180 mg/kg N fertiliser addition; BRN3 – 160 mg/kg N fertiliser addition; BRN4 – 140 mg/kg N fertiliser addition

(Wang et al. 2025). Compared with the CK, the concentrations of V-, C-, and S-type phenols, as well as total lignin phenols in soil, were increased by the addition of branch residues, and this was combined with N treatments (Figure 1A). This result is consistent with that of Meng et al. (2024), who observed that returning wheat straw and N increased lignin phenol content in soil. The increased V-type and total lignin phenol in the BRN2 treatment could be attributed to more branch residues remaining in the soil and the V-type lignin monomers being preferentially preserved. Compared to CK, branch residues plus N increased the S/V, lowered the $(Ad/Al)_V$ ratio, but no notable differences were observed among the four N levels (Figure 1B). The increased S/V ratio and decreased (Ad/Al)v indicate a reduced level of oxidative decomposition of lignin (Luo et al. 2025). In such cases, microorganisms produce less oxidase to decompose recalcitrant SOC to acquire N, particularly when degrading N-containing substrates are shielded by recalcitrant organic matter, such as lignin (Craine et al. 2007). These findings show that the addition of branch residues combined with N increased plant lignin input and decreased the extent of plant-derived C degradation in the soil, thereby favouring the formation of plant-derived C.

Microbial necromass C contents and formation. The GlcN/GalN and ^{13}C-GlcN/^{13}C-MurA ratios did

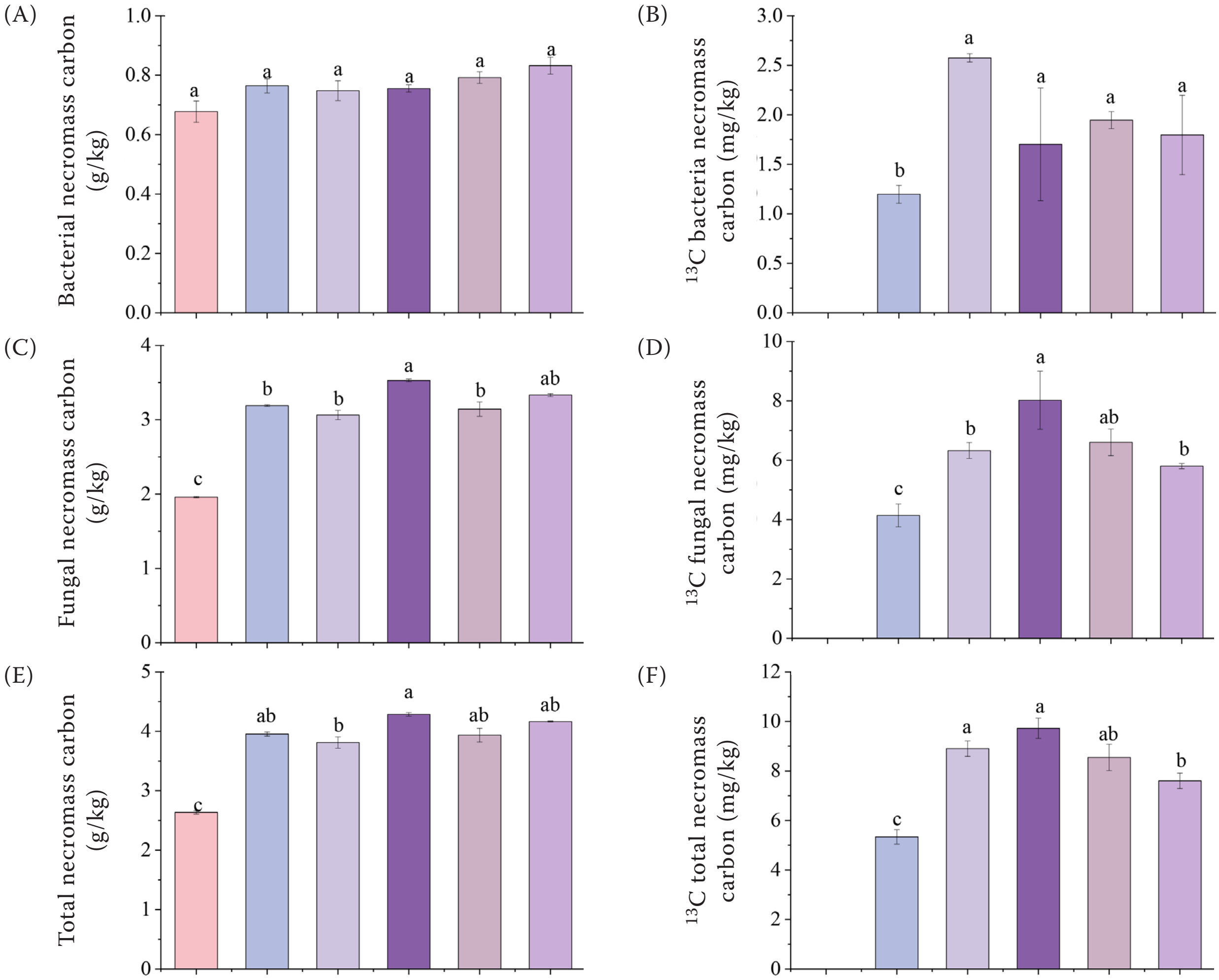

Figure 3. The microbial necromass carbon content and branch-derived ^{13}C incorporation in branch-amended soils subjected to different nitrogen (N) levels. Data are expressed as means ± standard deviation (n = 3). Lowercase letters indicate significant differences among all treatments at $P < 0.05$. CK – soil without branch residue and N fertiliser; BR – ^{13}C-labelled branch residue in soil without N fertiliser addition; BRN1 – 225 mg/kg N fertiliser addition; BRN2 – 180 mg/kg N fertiliser addition; BRN3 – 160 mg/kg N fertiliser addition; BRN4 – 140 mg/kg N fertiliser addition

not alter significantly across treatments (Figure 2A, D). The ratio of ^{13}C-GlcN to ^{13}C-GalN under BRN3 was markedly higher than that under BRN2 (Figure 2B). In comparison to CK, branch residues combined with N treatment increased the GlcN to MurA ratio, and the highest GlcN/MurA ratio was observed under the BRN2 treatment (Figure 2C). Both GlcN/MurA and ^{13}C-GlcN/^{13}C-MurA exceeded five (Figure 2C, D), indicating that FNC was more important than BNC in increasing SOC content when branch residues and N addition. The FNC and MNC contents under branch residue plus N were higher than those under CK (Figure 3A, C, E). Organic residues and N addition provide C and N sources for soil microbial growth and biomass accumulation, which are then transformed into microbial residue C (Qu et al. 2024). Soil fungi are better adapted to digest low-quality plant residue C and efficiently increase FNC accumulation, most likely due to increased soil N content and dissolved organic C (Meng et al. 2024). These findings suggest that branch residues combined with N increased microbe-derived C, particularly fungal-derived C.

The newly formed MNC decreased with lower levels of N when N was less than 180 mg/kg (BRN2) (Figure 3B, D, F). This indicates a deficiency in N required for microbial growth, and soil microorganisms may be unable to consume excess exogenous organic matter at suboptimal soil C: N ratios (Gaudel et al. 2024). Ding et al. (2023) observed that newly formed amino sugars from rice straw-derived ^{13}C were substantially reduced in soils without enough N. Furthermore, MNC can be degraded and reused by microorganisms to supply their N demand during growth when nutrients are limited (Shahbaz et al. 2018). The maximum ^{13}C-FNC and total ^{13}C-MNC in the BRN2 treatment indicate that the microbial-derived C in this treatment may be more stable, whereas those in the other treatments are more sensitive to disturbance.

Net SOC sequestration. External organic residues may act as SOC precursors by increasing SOC concentrations (Liu et al. 2024, 2025). Branch residue, combined with N, induced a net gain in SOC (Figure 4). Co-application of branch residue with N resulted in higher newly formed SOC than branch residue alone. The BRN1 treatment had the highest content of the newly formed SOC. The newly formed SOC was similar between the BRN2 and BRN3 treatments, and was 80.87% and 69.79% higher than that in the BRN4 treatment, respectively.

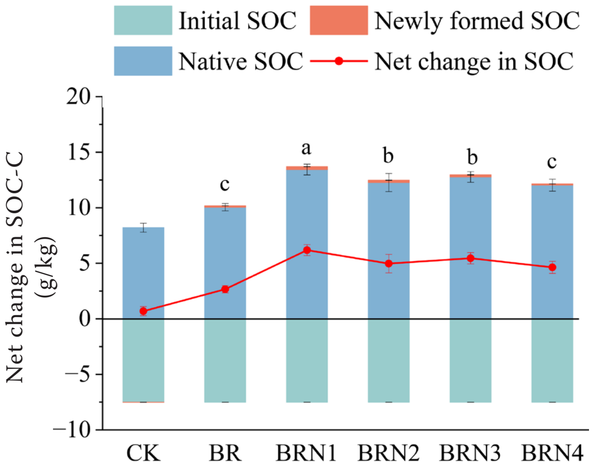

Figure 4. The net change of soil organic carbon (SOC) in branch-amended soils subjected to different nitrogen (N) levels. Data are expressed as means ± standard deviation (n = 3). Lowercase letters indicate significant differences among all treatments at P < 0.05. CK – soil without branch residue and N fertiliser; BR – ^{13}C-labelled branch residue in soil without N fertiliser addition; BRN1 – 225 mg/kg N fertiliser addition; BRN2 – 180 mg/kg N fertiliser addition; BRN3 – 160 mg/kg N fertiliser addition; BRN4 – 140 mg/kg N fertiliser addition

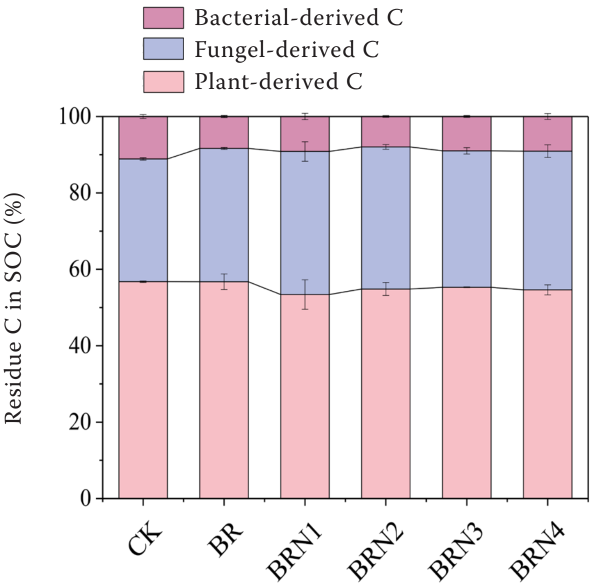

Figure 5. The contribution of plant- and microbe-derived carbon to soil organic carbon (SOC) in branch-amended soils subjected to different nitrogen (N) levels. CK – soil without branch residue and N fertiliser; BR – ^{13}C-labelled branch residue in soil without N fertiliser addition; BRN1 – 225 mg/kg N fertiliser addition; BRN2 – 180 mg/kg N fertiliser addition; BRN3 – 160 mg/kg N fertiliser addition; BRN4 – 140 mg/kg N fertiliser addition

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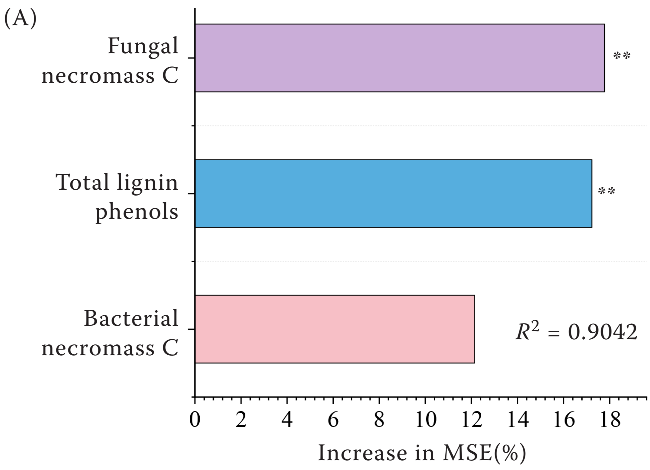
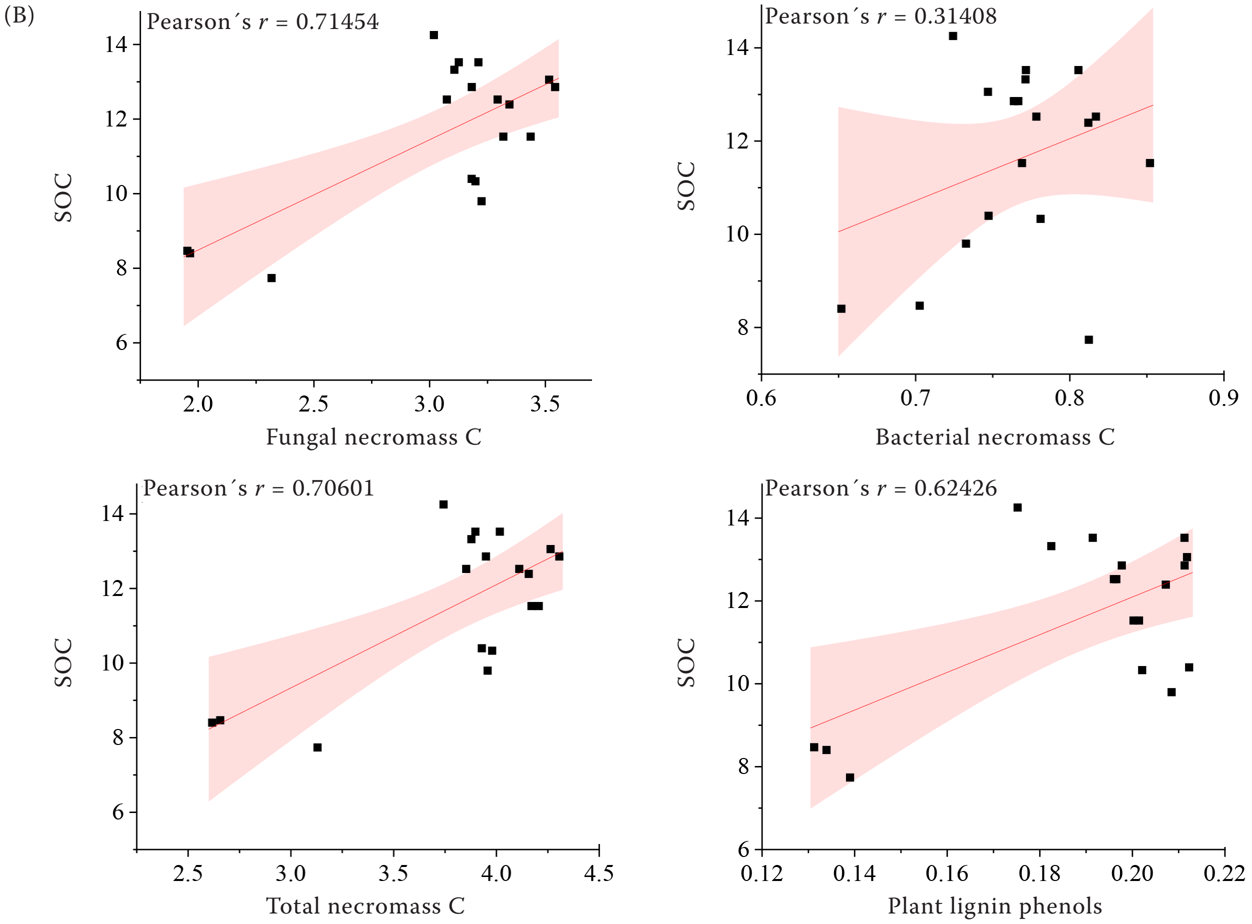
Figure 6. Relative importance of lignin phenols and microbial necromass carbon by the percentage increase of the mean squared error (MSE%) using random forest models, and the relationships between soil organic carbon (SOC) and microbial- or plant-derived carbon





The accumulation of plant lignin phenols and their efficient transformation into microbial residue can enhance SOC sequestration. The contributions of microbial- and plant-derived C to SOC varied with N level (Figure 5). Most of the plant-derived C in SOC accounted for 31.72–50.08%. The proportion of FNC in the SOC ranged from 22.25% to 30.78%. Compared to CK, the branch residues combined with N treatment increased the fraction of FNC in SOC, with BRN2 treatment yielding the highest value. BNC made the smallest contribution to SOC, accounting for 5.44–8.03%.

The random forest model revealed that FNC and lignin phenols were reliable predictors of SOC. FNC contributed the most to SOC, followed by lignin phenols (Figure 6A). A significant positive correlation was observed among plant lignin phenols, FNC, total MNC, and SOC content, whereas BNC showed no significant correlation with SOC content (Figure 6B). This result indicates that incorporating branch residues plus N into the soil accelerates fungal anabolism and necromass production, resulting in a high proportion of FNC in SOC accumulation (Hupperts et al. 2025). The results suggest that N addition through increased plant C and microbial necromass C accumulation is an effective strategy for achieving SOC sequestration.

Therefore, branch residues plus N reduction levels increased net SOC sequestration. Branch residues combined with N increased the lignin phenol concentration but did not significantly affect the contribution of plant-derived C to SOC, regardless of N levels. Additionally, the higher incorporation of ^{13}C-branch into fungal necromass than into bacterial necromass suggests that the increase in SOC sequestration was primarily attributable to the accumulation of fungal necromass C. BRN2 had the highest microbial-derived C. In contrast, fungal- and bacterial-derived C decreased as N levels reduced. These insights may facilitate the promotion of SOC sequestration potential in orchard soils by returning pruned branch residue, particularly when combined with reduced N application. This study demonstrates the value of plant- and microbial-derived C in providing targeted insights for revealing the source of SOC. However, evidence of SOC contribution is based on microplot experiments, and in the future, we still need to observe SOC sequestration in the field.

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