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Nitrogen fertilization affected microbial carbon use efficiency and microbial resource limitations via root exudates

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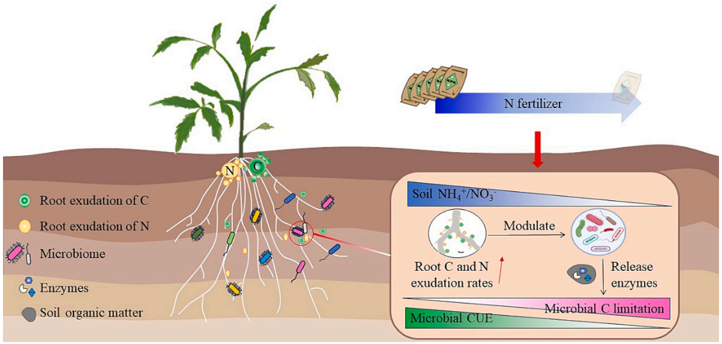
HIGHLIGHTS

- The rates of root C and N exudation decreased with N fertilizer application rates.
- Interaction between N fertilizer and plant root altered microbial C limitation, N/P limitations and CUE_{ST}.
- Microbial nutrient limitations varied from N to P when microbial habitat changed from bulk soils to rhizosphere soils.
- Root exudation rates and N fertilizer rates had contradicted effect on microbial C limitation in rhizosphere soils.
- Microbial CUE_{ST} in rhizosphere was positively related with N fertilizer application rates.

GRAPHICAL ABSTRACT



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ABSTRACT

Root exudation and its mediated nutrient cycling process driven by nitrogen (N) fertilizer can stimulate the plant availability of various soil nutrients, which is essential for microbial nutrient acquisition. However, the response of soil microbial resource limitations to long-term N fertilizer application rates in greenhouse vegetable systems has rarely been investigated. Therefore, we selected a 15-year greenhouse vegetable system, and investigated how N fertilizer application amount impacts on root carbon and nitrogen exudation rates, microbial resource limitations and microbial carbon use efficiency (CUE_{ST}). Four N treatments were determined: high (N3), medium (N2), low (N1), and a control without N fertilization (N0). Compared to the control (N0), the results showed that the root C exudation rates decreased significantly by 42.9 %, 57.3 % and 33.6 %, and the root N exudation rates decreased significantly by 29.7 %, 42.6 %, and 24.1 % under N1, N2, and N3 treatments, respectively. Interactions between fertilizer and plant roots altered microbial C, N, P limitations and CUE_{ST}; Microbial C and N/P limitations were positively correlated with root C and N exudation rates, negatively correlated with microbial CUE_{ST}. Random Forest analysis revealed that the root C and N exudation rates were key factors for soil microbial resource limitations and microbial CUE_{ST}. Through the structural equation model (SEM) analysis, soil NH_4^+

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content had significant direct effects on the root exudation rates after long-term N fertilizer application. An increase in root exudation rates led to enhanced microbial resource limitations in the rhizosphere soils, potentially due to increased competition. This enhancement may reduce microbial carbon use efficiency (CUE), that is, microbial C turnover, thereby reducing soil C sequestration. Overall, this study highlights the critical role of root exudation rates in microbial resource limitations and CUE changes in plant-soil systems, and further improves our understanding of plant-microbial interactions.

1. Introduction

Greenhouse vegetable production (GVP) can potentially increase farmers' income by high yield and year-round vegetable production (Hu et al., 2017; Yang et al., 2016). However, overfertilization is frequent in the GVP. Globally, an average of 1252 kg N ha^{-1} $year^{-1}$ is applied in GVP (Qasim et al., 2021), and N use efficiency (NUE) is <20 % (Ren et al., 2010; Ti et al., 2015). Excess N accumulates in soil, which not only leads to environmental pollution, but also changes microbial metabolism, and ultimately affects soil nutrient cycles (Chen et al., 2014; Ramirez et al., 2012).

Soil microbes regulate soil organic matter (SOM) decomposition by secreting extracellular enzymes, which are essential in the soil carbon (C), nitrogen (N) and phosphorus (P) biogeochemical cycles (Tapia-Torres et al., 2015). In turn, soil resource availability can influence microbial resource acquisition strategies and further regulate soil extracellular enzyme stoichiometry (Chen et al., 2023; Zhang et al., 2020). Soil enzyme stoichiometry reflects microbial energy and nutrient requirements and allows the evaluation of microbial resource limitations (Abay et al., 2024; Bi et al., 2022; Cui et al., 2022). Over the past two decades, numerous global or regional studies have adopted the ecoenzymatic stoichiometry theory (EEST) to assess these limitations. Within the EEST framework, "vector length" and "vector angle" are calculated to identify nutrient limitations patterns in soil ecosystems (Sinsabaugh and Follstad Shah, 2012; Cui et al., 2021). Therefore, comparing the relative nutrient requirements and acquisition strategies and the drivers of resource limitations through EEST in soil microbial community will provide a deeper understanding of long-term N fertilizer applications and their impact on soil microbial metabolic characteristics in greenhouse vegetable production system.

Microbial metabolism, such as resource limitations are controlled by multiple factors, in which N fertilization plays an essential role (Cui et al., 2021; Li et al., 2023; Zheng et al., 2020). Soil mineral N impacts plant-microbial nutrients competition through altering plant growth and root exudation and, consequently, regulates microbial resource limitations (Hodge et al., 2001; Morales et al., 2023; Yang et al., 2023a, 2023b). Root exudates, including a variety of dissolved compounds, provide C and nutrients for microbial growth, and changes plant-microbe interactions (Li et al., 2021; Sun et al., 2021). Long-term application of nitrogen fertilizer leads to a preferential use of nitrogen by soil microbes, altering the nutrient balance in the soil, and subsequently affecting the composition of root exudates (Phillips et al., 2011a). Moreover, root exudates exert a selection pressure on microbial community composition, and thus change microbial physiological metabolic strategies (Bi et al., 2022; Lareen et al., 2016). In addition, long-term nitrogen fertilizer application directly changes soil microbial abundance and community composition by decreasing soil pH (Chen et al., 2023; Li et al., 2023; Schwalb et al., 2023). Therefore, the balance between microbial nutrients requirements and actual composition in soil nutrient supply might be impacted (Sinsabaugh et al., 2009; Wang et al., 2021). However, the lack of research on the effects of N fertilizer application on root exudation rates and microbial resources limitations hinder our understanding of the mechanisms of microbial metabolism in response to N fertilizer application in greenhouse vegetable systems.

Another important parameter for accessing microbial metabolism is the carbon use efficiency (CUE), referring to microbial utilization of available C (Wu et al., 2022). In general, high CUE means more energy is allocated to biomass and less to respiration (Hagerty et al., 2018). Microbial CUE_{ST}, based on stoichiometry theory, provides a better link between enzyme activity, microbial biomass and soil nutrient resources (Cui et al., 2021; Ju et al., 2023; Sinsabaugh and Follstad Shah, 2012; Song et al., 2022). Thus, revealing the relationship between microbial resource limitations and CUE_{ST} may benefit our understanding for microbial physiological metabolism response to long-term nitrogen fertilizer application under greenhouse vegetable systems.

In this study, we collected samples from rhizosphere, bulk soil, and root exudates from tomato seedlings (15 days after tomato transplanting) to investigate how root exudation rates affected microbial metabolism under different N fertilizer application rates. We examined microbial resource limitations using an ecological enzyme vector model (Moorhead et al., 2016) and CUE_{ST} (Sinsabaugh et al., 2016). Here, we proposed three hypotheses: (1) The rates of root C and N exudation decrease with increasing N fertilizer application rates; (2) The resource limitations is alleviated by nitrogen (N) fertilizer application, similarly, lower root exudation rates are associated with reduced microbial resource limitations; (3) The microbial CUE_{ST} increases with the higher N fertilizer application rates.

2. Materials and methods

2.1. Experiment site and soil sampling

The greenhouse experiment was conducted from 2008 with cucumber and tomato rotation at Xinji Experimental Station, Hebei, China (115°17′53″E, 37°47′55″N). The area belongs to semi-humid continental monsoon climate with an average annual temperature of 11.5 °C and annual precipitation of 540 mm. Soil type is a loamy soil. The initial soil physicochemical properties were measured in 2008 and were as follows: pH 8.1 (soil-water ratio 2.5:1, 25 °C), soil organic matter (SOM) content of 5.0 g kg^{-1}, Olsen-P (AP) content of 82.9 mg kg^{-1}, available potassium (AK) content of 60.0 mg kg^{-1}, nitrate nitrogen (NO_3^-) content of 5.5 mg kg^{-1} and ammonium nitrogen (NH_4^+) content was 19.4 mg kg^{-1}. Electrical conductivity (EC) at a water-soil ratio of 5:1 at 25 °C was 307.4 μS cm^{-1}. The soil bulk density was around 1.35 g cm^{-3} and the field water holding capacity was 25 %. The greenhouse is 40 m long and 7.5 m wide, covered with polyethylene film. The experiment was a completely randomized block design, with a plot size of 10.8 m^2 (1.8 m wide × 6 m long) and three replicates for each treatment.

Four N fertilizer application rates were selected for tomato: 0, 102, 327, and 552 kg N ha^{-1} per year, applied in the form of urea. The phosphorus (P_2O_5) application remained constant at 0 kg ha^{-1}, while potassium (K_2O) was applied at a rate of 210.6 kg ha^{-1} per year for all treatments. Additionally, chicken manure was applied at a rate of 1560.4 kg ha^{-1} per year, providing 22.85 kg N ha^{-1}, 50.63 kg P_2O_5 ha^{-1}, and 36.01 kg K_2O ha^{-1} (on a dry weight basis). Wheat straw was applied at a rate of 14,599 kg ha^{-1} per year, containing 100.02 kg N ha^{-1}, 24.39 kg P_2O_5 ha^{-1}, and 203.36 kg K_2O ha^{-1} (on a dry weight basis).

On September 8, 2022, 15 days after transplantation of tomato seedlings (4–5 leaf stage), soil samples were collected from 0 to 20 cm layer. Rhizosphere soil samples within each plot were collected using the shaking method, following established protocols (Edwards et al., 2015; Li et al., 2020). In brief, the soil in the root zone (0–20 cm depth) was excavated, and the soil that was loosely bound to the roots was removed by shaking. The remaining soil, which was tightly adhered to the roots, was collected and defined as rhizosphere soil. Non-rhizosphere (bulk) soil samples were obtained from an adjacent open area without vegetation, also at a depth of 0–20 cm.

Three samples were collected from each plot and mixed to form a composite sample. Sub-samples of rhizosphere and bulk soil for the analysis of enzyme and microbial biomass were stored at 4 °C. The remaining samples was air-dried for determination of soil physical and chemical properties.

2.2. Soil properties

Air-dried soil samples were sieved through a 2.0 mm sieve. Soil pH was determined using a glass combination electrode with a soil/water ratio of 1:2.5. Soil organic matter (SOM) was determined using the potassium dichromate oxidation method (Walkley and Black, 1934). Total N (TN), Olsen phosphorus (Olsen-P), and available potassium (AK) were measured using Kjeldahl digestion method (Bremner and Mulvaney, 1983), Olsen P method (Olsen and Sommers, 1983) and flame photometry (Jackson, 1973), respectively. Ammonium and nitrate (NH_4^+–N and NO_3^-–N) concentrations were measured using a continuous flow analyzer after sample extraction with 2 M KCl (Kachurina et al., 2000). Microbial biomass C (MBC) and N (MBN) were determined using chloroform fumigation method (Brookes et al., 1985; Wu et al., 1990). The C and N contents of the extracts were measured using a TOC/TN analyzer (Multi N/C 3100, Analytik Jena, Germany). The calculation of MBC and MBN involved assessing the difference in total extractable C and N content between fumigated and non-fumigated soil samples, which was then divided by a correction factor of 0.45 for MBC and 0.54 for MBN (Brookes et al., 1985; Wu et al., 1990).

2.3. Sampling root exudates

We collected root exudates in situ following the method described by Phillips et al. (2008) with a slight adjustment. Briefly, intact tomato root systems were carefully washed and placed in a 100 mL centrifuge tube filled with 80 mL C-free nutrient solution (composition (μM) 200 $MgCl_2$, 100 KCl, 600 $CaCl_2$ and 5 H_3BO_3; Liu et al., 2016). Four tomato seedlings were selected in each plot. The 100 mL tubes were then covered with aluminum foil to protect the contents from UV light and maintained for 24 h in situ. Then the roots were flushed twice with ultrapure water before they were removed. Following this, the liquids in the 100 mL tubes were filtered using a 0.45 μM membrane. The filtered liquids from the four tubes were combined and the total volume was adjusted to 500 mL with sterile distilled water. This combined sample was then analyzed by the TOC analyzer (Multi N/C 2100; Analytic Jena, Jena, Germany). The plants were further dried at 110 °C for 30 min and then at 60 °C for 24 h to determine its dry mass.

2.4. Enzymatic activities analyses

Extracellular enzyme activities (EEAs), which participate in carbon (C) (β-1,4-glucosidase, BG), nitrogen (N) (L-leucine aminopeptidase and β-1,4-*N*-acetylglucosaminidase, LAP and NAG) and phosphorus (P) (alkaline phosphatase) cycles, were measured using fluorometric techniques (Marx et al., 2001; Saiya-Cork et al., 2002). The substrate used in this experiment was a 200 μM solution of BG, NAG, AP, and LAP. The buffer solution used was a 50 mM acetate buffer (pH 7.0). The reference standard for BG, NAG, and AP analyses was 10 μM 4-Methylumbelliferone (MUB), and 10 μM 7-Amino-4-methyl coumarin (AMC) was used as reference standard solution for LAP analysis. Buffer, substrate and reference standards solution were added to a black polystyrene 96-wells microplates plate in strict accordance with the method described by DeForest (2009). First, 250 μL buffer, 200 μL buffer plus 50 μL standard solution, and 200 μL buffer plus 50 μL substrate solution were added into the first three columns of the 96-wells microplates as blank controls, reference standards and negative controls, respectively. Subsequently, 1 g fresh soil was added to 100 mL of 50 mM acetate buffer (pH 7.0) homogenized with a magnetic stirrer and then 200 μL continuously stirred soil suspension was added to the 96-wells microplates with 50 μL substrate solution, 50 μL buffer or 50 μL standard solution for sample, blank, and quench standard, respectively. The eight wells of each column corresponded to eight replicates. The microplates were incubated in the dark at 25 °C for 4 h, and then the reaction was stopped by adding 10 μL of 1.0 M NaOH to each well. Fluorescence was measured using a microplate reader with λ365 nm excitation and λ450 nm emission (Synergy H1M, BioTek Instruments, VT, USA). The enzymatic activity was expressed as nanomoles of substrate released per hour per gram of dry soil (nmol g^{-1} h^{-1}).

2.5. Ecoenzymatic stoichiometry and CUE estimation

Microbial resource/nutrient limitations were quantified by calculating the vector length and vector angle based on the stoichiometry of extracellular activity (Cui et al., 2021; Moorhead et al., 2016, 2013). A larger value for vector length indicates greater carbon (C) limitation for microbes, and vector angle for <45° or > 45° indicate nitrogen (N) or phosphorus (P) limitations, respectively (Moorhead et al., 2013). Microbial P limitation increases with an increase in vector angle, whereas microbial N limitation increases with a decrease in vector angle. Vector length and vector angle were calculated as follows:

$$x = \frac{lnBG}{ln(LAP + NAG)}$$

$$y = \frac{lnBG}{lnAP}$$

$$\text{Vector length} = \sqrt{x^2 + y^2}$$

$$\text{Vector angle} = \text{Degrees}\{\text{ATAN2}[y, x]\}$$

where β-1,4-glucosidase (BG) is involved in the carbon cycle, L-leucine aminopeptidase (LAP) and β-1,4-*N*-acetylglucosaminidase (NAG) are involved in the nitrogen (N) cycle, and alkaline phosphatase (AP) is involved in the phosphorus (P) cycle. x and y represent the relative activities of C- versus N-acquiring enzymes and C- versus P-acquiring enzymes, respectively.

Microbial carbon use efficiency (CUE) derived from stoichiometry theory (CUE_{ST}) was calculated using the following equations (Schimel et al., 2022; Sinsabaugh et al., 2016; Sinsabaugh and Follstad Shah, 2012). CUE_{ST} is a fundamentally different way of viewing resource use efficiency than classical CUE estimates. Rather, it reflects the capability of microbes to modify the disparity between the composition of microbial biomass and the basic composition of the available resources by the allocation of enzymatic activities rather than resource use efficiency, which classical CUE estimates.

$$CUE_{ST} = CUE_{MAX} \times \frac{S_{C:N}}{S_{C:N} + k_{C:N}}$$

$$S_{C:N} = \frac{B_{C:N}}{L_{C:N}} \times \frac{1}{EEA_{C:N}}$$

where CUE_{MAX} is set at 0.6 (Sinsabaugh et al., 2016). The value of half-saturation constant $K_{C:N}$ is 0.5. $S_{C:N}$ is a scalar ratio that reflects the capability of the microbes to modify the disparity between the composition of microbial biomass and the basic composition of the available resource by the allocation of enzymatic activities. $B_{C:N}$ is the elemental C:N ratio of microbial biomass. $L_{C:N}$ was estimated as SOC/TN. $EEA_{C:N}$ was calculated as BG/(NAG + LAP).

2.6. Data analysis

A two-way ANOVA was used to assess the effects of plants root, N fertilization application, and their interaction on soil extracellular enzymatic activities, soil properties, microbial biomass, microbial resource limitations, and microbial CUE_{ST} (Gomez and Gomez, 1984). Duncan's significance test was used to determine the differences in soil properties, root exudation rates, enzyme activities, and microbial biomass and resource metabolism among the different N treatments (Duncan, 1955). The significance of rhizosphere and bulk was conducted using paired-samples *t*-test. All analysis were carried out by SPSS 16.0 (SPSS Inc., Chicago, USA).

Linear regression was used to determine the relationship between vector length, vector angle, and CUE_{ST} and root C and N exudation rates. A Pearson correlation heatmap was generated to examine the relationships between different variable. Both linear regression and the Pearson correlation heatmap were created using Origin software (OriginLab Corporation, Northampton, MA, USA).

The main factors (i.e., plant, soil properties, and N fertilizer) for vector length, vector angle, and CUE_{ST} were identified by random forest (RF) analysis using the "randomForest" package in R software (v4.3.2, R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/) (Breiman, 2001). The significance of RF models and factors was tested using the "rfPermute" and "rfUtilities" packages, respectively (Archer, 2016; Murphy, 2018).

Structural equation model (SEM) was used to evaluate how N fertilizer affected microbial resource limitations and CUE_{ST}. The selected variables were significant predictors identified by RF analysis. Structural equation modeling (SEM) was performed using Amos 21.0 software package (Smallwaters Corporation, Chicago, IL, USA). The best-fit model was obtained using a chi-square test (χ^2), low χ^2 /df (< 2), P values ($0.05 < P < 1$), high goodness-of-fit index (GFI > 0.9), low root mean square errors of approximation (RMSEA <0.05), and low Akaike Information Criteria (AIC) (Hu and Bentler, 1999).

3. Results

3.1. Impacts of different N fertilizer application rates on tomato dry biomass and root exudation

Tomato biomass (dry weight) was significantly reduced only under N3 treatment at seedling stage, compared with N0 treatment (Fig. 1A, $P < 0.05$). Root C exudation rates reduced by 42.9 %, 57.3 % and 33.6 % under N1, N2, and N3 treatments, respectively (Fig. 1B and C). Root N exudation rates decreased by 29.7 %, 42.6 % and 24.1 %, respectively, compared with N0 treatment. Both root C and N exudation rates were the highest in the N0 treatment, followed by N1 and N3 treatments, which were not significantly different from each other, and lowest in the N2 treatment ($P < 0.05$).

3.2. Soil extracellular enzymatic activities, soil properties and microbial biomass

The C, N and P-acquiring enzyme activities i.e., BG, LAP+NAG, and AP, were significantly affected by plant roots (Fig. 2). Compared with bulk soils, C-acquiring enzyme activity in rhizosphere soils significantly increased by 108.7 % and 254.9 % under N0 and N1 treatments while decreased by 40.0 % and 31.1 % under N2 and N3 treatments (Fig. 2A, $P < 0.05$); P-acquiring enzyme activity enhanced by 1.3–2.3 times among different N treatments (Fig. 2B, $P < 0.05$); N-acquiring enzyme activity decreased by 22.5 %–52.1 % among different N treatments (Fig. 2C, $P < 0.001$).

In rhizosphere soils, compared with the N0 treatment, C-acquiring enzyme activity decreased with the increase of N fertilizer application rates by 44.0 %, 72.9 % and 82.1 % under N1, N2 and N3 tr, respectively ($P < 0.05$); N-acquiring enzyme activities increased by 33.0 % only under the N1 treatment ($P < 0.05$), which was 1.2–1.3 times higher than under other treatments, with no significant differences among N2, N3, and N0 treatments ($P > 0.05$). P-acquiring enzyme activity was enhanced by 22.6 % and 58.4 % under N2 and N3 treatments ($P < 0.05$), while there was no significant difference under N1 treatment ($P > 0.05$). In bulk soils, the lowest C and P acquiring enzyme were under N1 treatment, while the lowest N- acquiring enzyme activity was under N3 treatment.

Compared to bulk soils, total phosphorus (TP) significantly increased by an average of 22.7 % in rhizosphere soils across all treatments (Fig. S1, $P < 0.05$). However, available nutrients, i.e., Olsen-P, AK and NH_4^+ all significantly decreased by 21.1 %, 20.7 %, and 16.0 %, respectively ($P < 0.05$). N2 treatment increased rhizosphere SOM compared with bulk soils (ΔSOM) (Fig. S2). However, other treatments (N0, N1 and N3 treatments) reduced rhizosphere SOM.

Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) reduced by 17.3 % and 19.6 %, respectively, in rhizosphere soils compared to bulk soils (Fig. S3, $P < 0.01$). In the bulk soils, the MBC in the N3 treatment was significantly higher than in the N0 and N2 treatments ($P < 0.05$), with no significant difference compared to N1 treatment ($P > 0.05$). However, in the rhizosphere soils, there were no significant differences among the four fertilizer treatments ($P > 0.05$).

3.3. Microbial resource limitations

The vector length and angle of enzymatic stoichiometry were calculated to quantify the relative microbial C and N/P limitations (Fig. 3). Vector angle under N0, N1 and N3 treatments was above the 1:1 line, suggesting P limitation for microbes in rhizosphere soils. While vector angle under N2 treatment in rhizosphere soils and all data points in bulk soils were below the 1:1 line, suggesting N limitation for microbes (Fig. 3A). For rhizosphere soils, vector length significantly decreased with larger N fertilizer application rates, which means

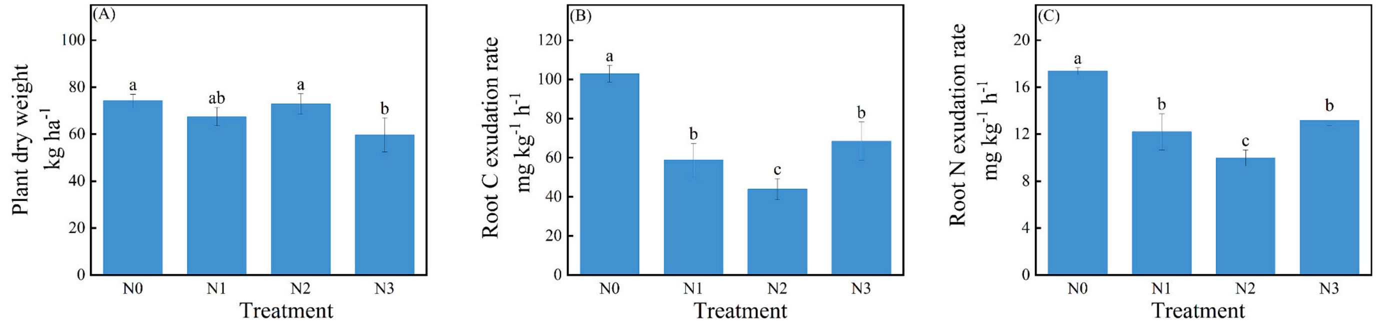

Fig. 1. Tomato dry weight (A) and root carbon and nitrogen exudation rates (B and C) under four nitrogen amount treatments. Different letters indicate significant differences ($P < 0.05$) among treatments. The error bars indicate the standard errors (n = 3). Fertilizer treatments: N0 (0 kg N ha^{-1}), N1 (102 kg N ha^{-1}), N2 (327 kg N ha^{-1}), N3 (552 kg N ha^{-1}).

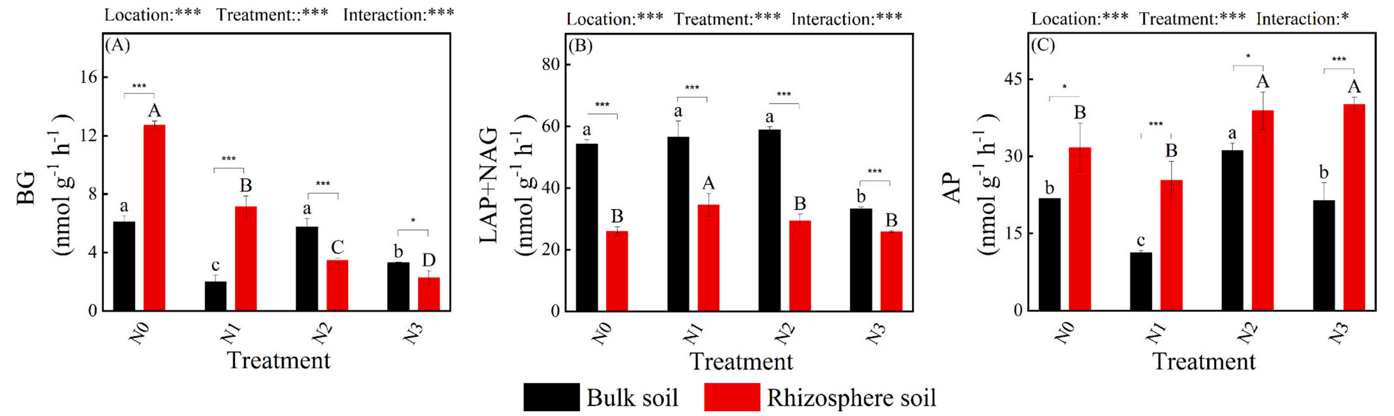

Fig. 2. Extracellular enzymatic activities (EEAs) in rhizosphere (red color) and bulk (black color) soils under four nitrogen fertilizer treatments. Different letters indicate significant differences ($P < 0.05$) among treatments. The error bars indicate the standard errors (n = 3). Results of two-way ANOVA and *t*-test are presented ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Fertilizer treatments: N0 (0 kg N ha^{-1}), N1 (102 kg N ha^{-1}), N2 (327 kg N ha^{-1}), N3 (552 kg N ha^{-1}). BG, β-1,4-glucosidase (A); NAG, β-1,4-*N*-acetylglucosaminidase (B); LAP, L-leucine aminopeptidase (B); AP, alkaline phosphatase (C).

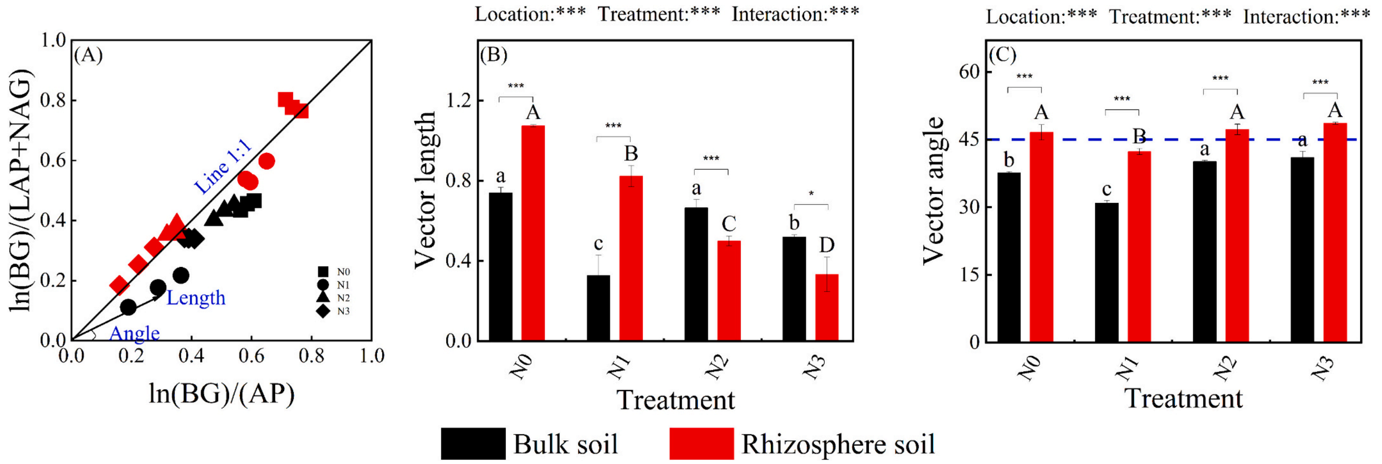

Fig. 3. The exoenzymatic vector model quantifies microbial resource limitation in rhizosphere and bulk soils under four treatments. Relationships between ln (BG)/ ln (LAP+NAG) versus ln (BG)/ln (AP) (A). Vector length represents microbial C limitation (B) and vector angle represents microbial N or P nutrient limitation (C). BG, β-1,4-glucosidase; NAG, β-1,4-*N*-acetylglucosaminidase; LAP, L-leucine aminopeptidase; AP, alkaline phosphatase. Different letters indicate significant differences ($P < 0.05$) among treatments. The error bars indicate the standard errors (n = 3). Results of two-way ANOVA and t-test are presented ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Fertilizer treatments: N0 (0 kg N ha^{-1}), N1 (102 kg N ha^{-1}), N2 (327 kg N ha^{-1}), N3 (552 kg N ha^{-1}).

microbial C limitation increased with N fertilizer application rates.

Compared with the bulk soils, the vector length representing microbial C limitation significantly increased for N0 and N1 treatments, while significantly decreased for N2 and N3 treatments in rhizosphere soils (Fig. 3B, $P < 0.01$ and $P < 0.05$, respectively). Meanwhile the vector angle representing microbial N and P limitations significantly increased for all treatments (Fig. 3C, $P < 0.001$).

In rhizosphere soils, the vector angle of N0, N2 and N3 treatments were significantly higher than the N1 treatment due to the low N-acquiring enzyme activities and high P-acquiring enzyme activity which combined with >45° vector angles, suggesting P limit to microbial metabolism (Fig. 3C, $P < 0.05$). In bulk soils, N1 treatment had the lowest vector length with the lowest C-acquiring enzyme activity, indicating the lowest microbial C limitation ($P < 0.05$). The vector angles of all treatments were <45°, indicating those treatments had N limitation.

Pearson correlation analysis showed that vector length was significantly positively correlated with root C and N exudation rates ($P < 0.05$, Fig. S4). Furthermore, linear regression analysis also indicated a positive correlation among vector length with root C and N exudation rates ($P < 0.05$, Fig. 4A and B).

3.4. Soil microbial CUE$_{ST}$.

The assessment of stoichiometric CUE$_{ST}$ was significantly different between bulk and rhizosphere soils (Fig. 5, $P < 0.001$). Compared with bulk soils, the average CUE$_{ST}$ significantly decreased by 8.4 % in rhizosphere soils. In rhizosphere soils, the CUE$_{ST}$ significantly increased with N fertilizer application rates by 28.8 %, 29.5 % and 36.2 %, respectively, under N1, N2 and N3 treatments, compared with N0 treatment. In bulk soils, the CUE$_{ST}$ significantly increased under N1 and N3 treatments by 4.7 %, compared with N0 and N2 treatments (Fig. 5A, $P < 0.05$).

There was a negative correlation between CUE$_{ST}$ and vector length (Fig. 5B, $P < 0.01$), vector angle (Fig. 5C, $P < 0.05$), as well as root C and N exudation rates (Fig. 5D and E, $P < 0.01$).

3.5. Drivers of root exudation rates, microbial resource limitations and microbial CUE$_{ST}$

SOM, NO_3^-, NH_4^+, N fertilizer and DON were the key drivers for root C and N exudation rates according to RF analysis (Fig. S5). SOM was the most important factor for both (7.7 and 8.5, $P < 0.01$). For root C exudation rates, the relative importance of NH_4^+, NO_3^- and N fertilizer

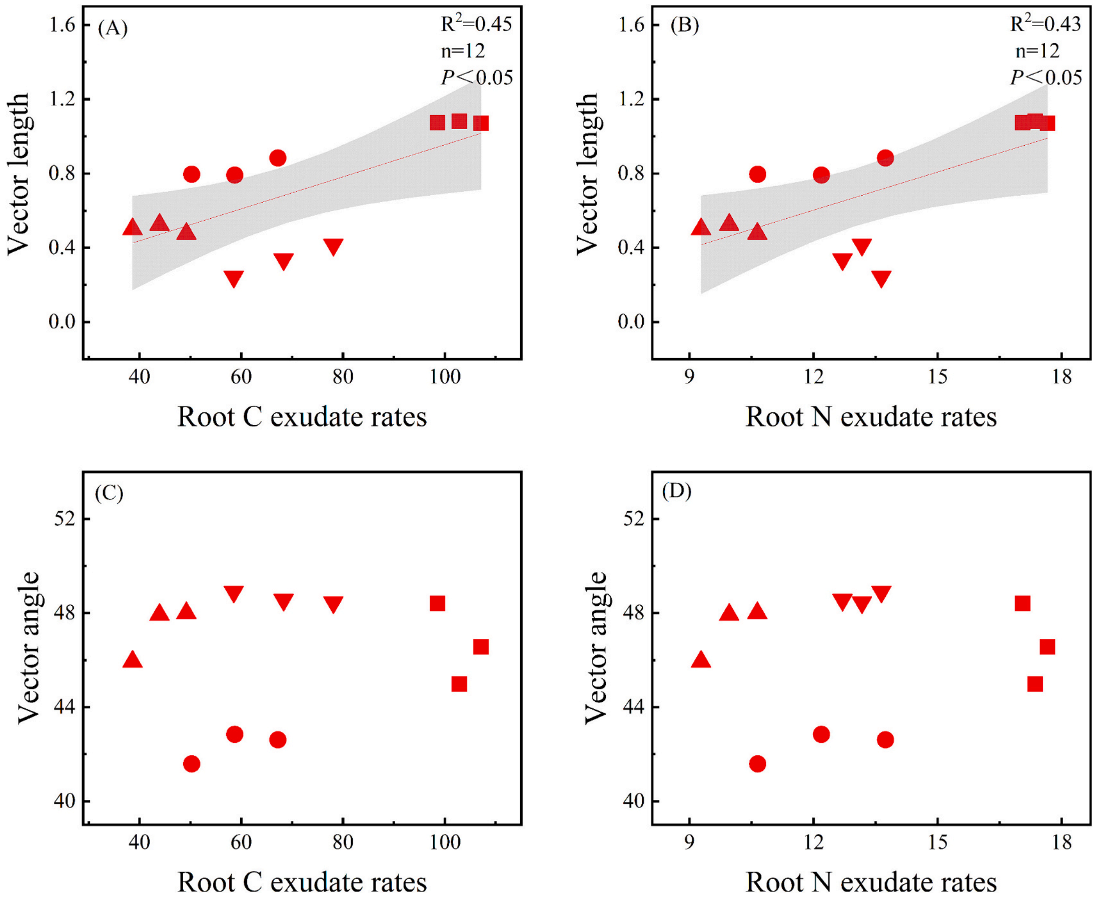

Fig. 4. Linear relationships between vector length, vector angle and root carbon and nitrogen exudation rates.

were 7.1, 6.7 and 6.0, respectively (Fig. S5A, $P < 0.01$). For root N exudation rates, the relative importance of N fertilizer, NH_4^+ and NO_3^- were 7.5, 6.9 and 6.4 respectively (Fig. S5B, $P < 0.01$). The importance of DON for both was 4.5 and 4.1 ($P < 0.05$).

Root N exudation rates, root C exudation rates, and N fertilizer were the key drivers for vector length, vector angle and CUE_{ST} according to RF analysis (Fig. 6). For vector length, NO_3^- was the most important factor (14.7, $P < 0.01$) followed by TP and N fertilizer application rates (Fig. 6A). For vector angle, root N exudation rates, soil location (rhizosphere or bulk soil), root C exudation rates and N fertilizer were the most important factors with relative importance of 9.1, 8.6, 8.6 and 6.6, respectively ($P < 0.01$). In addition, AP (5.5, $P < 0.05$) was also a significant factor for the vector angle (Fig. 6B). For CUE_{ST}, vector length was the most important factor (8.2, $P < 0.01$); Root C and N exudation rates were also extremely important (7.6 and 6.8, $P < 0.01$), as well as NO_3^- (7.5, $P < 0.05$); NH_4^+, SOM and N fertilizer were significantly important for CUE_{ST} with relative importance of 7.2, 6.6 and 3.6, respectively (Fig. 6C, $P < 0.05$).

N fertilizer application decreased the root exudation rates by providing more NH_4^+ in the soil (−0.83, $P < 0.001$), with an explanation of 64 % (Fig. 7). While reduction of root exudation rates directly affected vector angle, which means microbial N/P limitations (−0.76, $P < 0.01$). In addition, N fertilizer application rates had a negative effect on vector length by providing more NO_3^-, (−0.65, $P < 0.01$). Structural equation modeling explained 59 % and 72 % of the variation in vector length and vector angle. The direct negative effect of vector length on microbial CUE_{ST} was greater than that of root exudation rates. Moreover, microbial C limitation had the greater direct negative impact on microbial CUE_{ST} (−0.59, $P < 0.001$), followed by root exudation rates (−0.40, $P < 0.05$). The model explains 71 % of the variation in microbial CUE_{ST}.

4. Discussion

4.1. Effects of N fertilizer application on root C and N exudation rates

Variation of belowground C allocation is a physiological adjustment for plants to maintain nutrient acquisition in contrasting environments (Dijkstra et al., 2009; Phillips et al., 2012). In this long-term experiment, root exudation rates of C and N were significantly reduced under N treatments (N1, N2 and N3 treatments) compared to N0 treatment (Fig. 1). Because plants have more available nitrogen (NH_4^+ and NO_3^-) for their growth after N fertilizer application, they do not need extra root exudates for nutrient acquisition (Fig. S1). These results are in line with Dijkstra et al. (2009) and Phillips et al. (2011a, 2011b, 2009), who found that plants invest more photosynthates belowground in N-limited environments and release more enzymes from the root system into the rhizosphere to acquire nutrients for plant growth (Preece and Peñuelas, 2016; Song et al., 2022). On the other hand, we hypothesize that changes in resource availability have altered the composition and amount of root exudates in greenhouse, such as sugars and organic acids, as well as the relative proportion of organic compounds containing N, such as amino acids and proteins. Furthermore, the amount of root exudates is also influenced by the nutritional status of the plant (Vives-Peris et al., 2020). For example, root exudates decreased under higher N fertilizer application rates (Fig. 1), which might due to nutrient imbalance of the plant (Dorais et al., 2000). Meanwhile, high nitrogen remaining in the soil was another reason for the decrease of root C and N exudation rates (Fig. S4 and Fig. 7). In GVP, low N use efficiency (NUE) due to oversupply, low N absorption capacity from sparse root systems, and the short growing cycles of vegetables increase nitrogen leftover (Ren et al., 2010; Ti et al., 2015), which indirectly affected root exudation rates (Fig. S4).

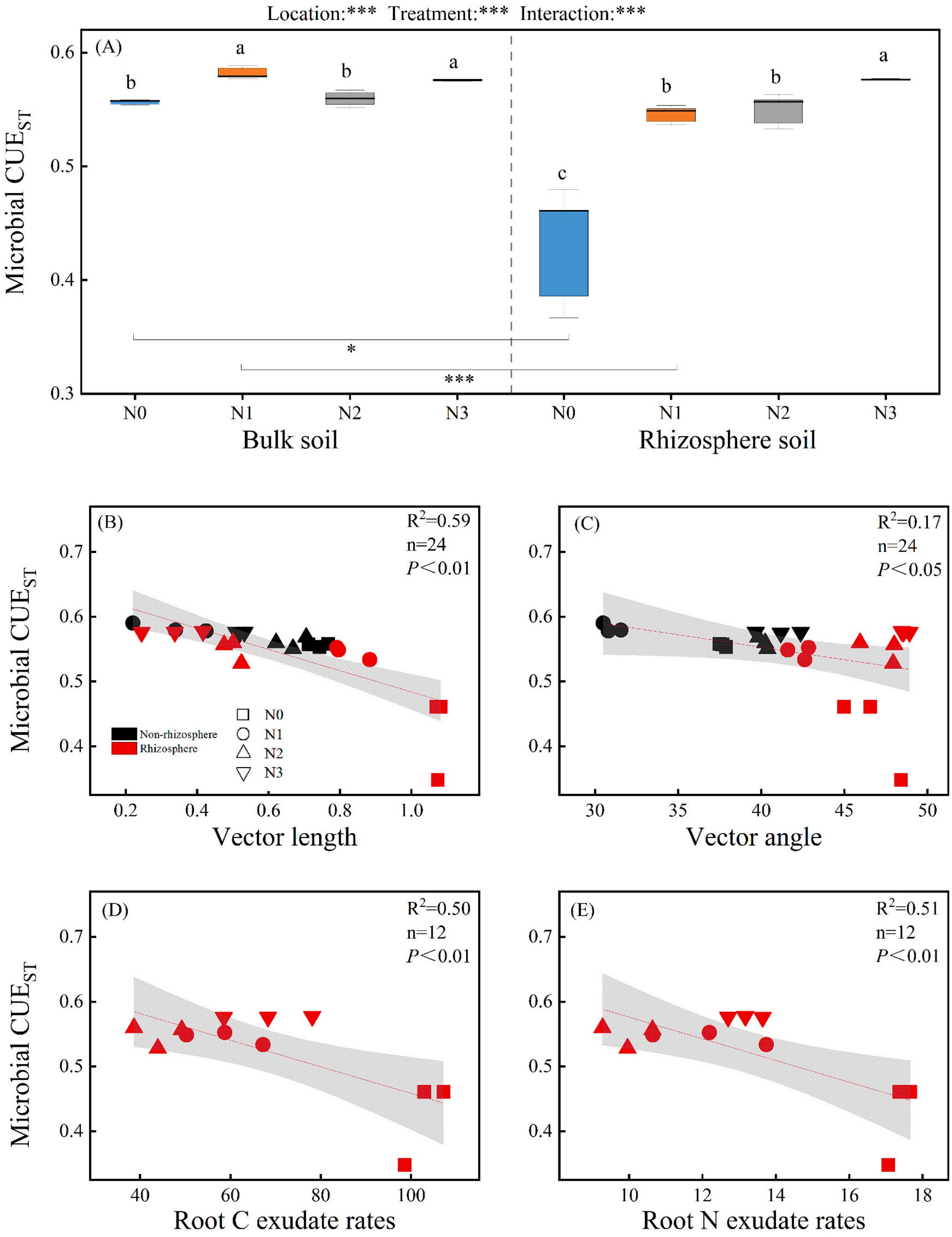

Fig. 5. Microbial carbon use efficiency of rhizosphere and bulk soils under four N treatments (A) and its linear relationship with vector analysis (B and C) and root exudation rates (D and E). Different letters indicate significant differences ($P < 0.05$) among four N treatments. The error bars indicate the standard errors (n = 3). Results of two-way ANOVA and t-test are presented (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Fertilizer treatments: N0 (0 kg N ha^{-1}), N1 (102 kg N ha^{-1}), N2 (327 kg N ha^{-1}), N3 (552 kg N ha^{-1}).

Plants allocate approximately half of the photosynthesized C belowground into the soil as rhizodeposition where it is incorporated into soil microbial biomass and organic matter (Lynch and Whipps, 1990; Pausch and Kuzyakov, 2018; Schenck zu Schweinsberg-Mickan et al., 2012). Root exudates are part of the rhizodeposition process, which is the major source of soil organic carbon released by roots (Nguyen, 2009). Increased belowground C and N allocation may promote the preferential utilization of root exudates by rhizosphere microbes, leading to increase SOM content due to surplus C being allocated to microbial growth (Jones et al., 2009; Pausch and Kuzyakov, 2018). However, our study found that higher rates of root exudation increased SOM decomposition (ΔSOM, rhizosphere-bulk soil) (Fig. S2). Although root exudation rates were the lowest under the N2 treatment, soil SOM content increased in rhizosphere soil compared with bulk soils, while the other treatments reduced SOM content (N0, N1 and N3 treatments) (Fig. S1). The increase in SOM content under the N2

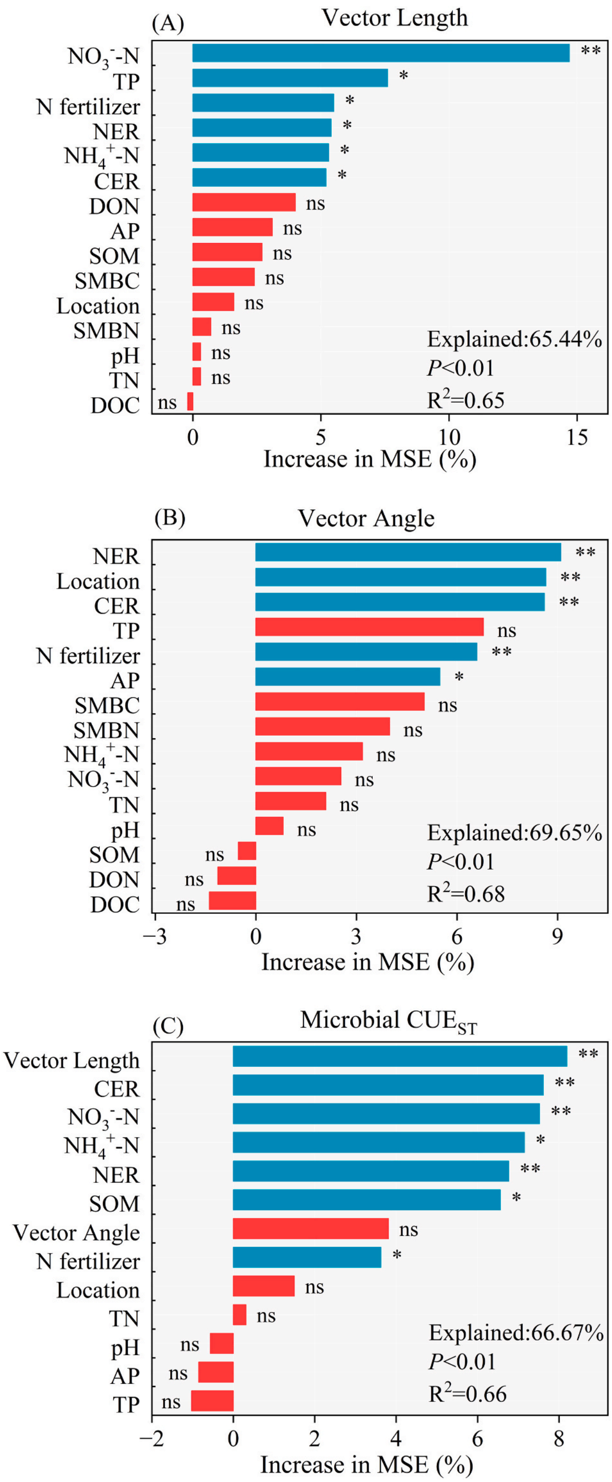

Fig. 6. The importance for vector length, vector angle and microbial carbon use efficiency (CUE) characterized by random forest analysis. The estimation of the importance of predictors was based on the percentage increases in mean squared error (MSE). Variables with higher MSE% values were identified as more importance. Significance is indicated by $P < 0.01$** and $P < 0.05$*.

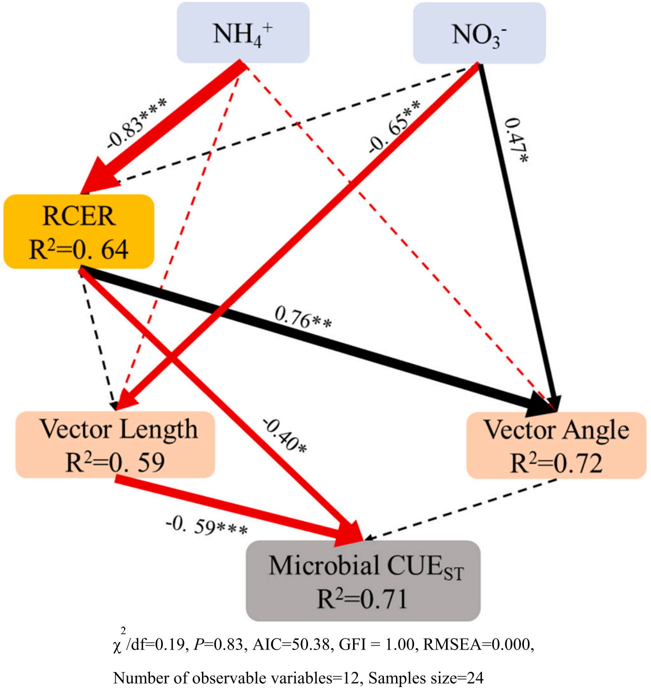

Fig. 7. Structural equation model (SEM) showing how nitrogen fertilizer affected microbial resource limitation and carbon use efficiency via root exudation. RCER was root C exudation rates due to root C and N exudation rates is high collinearity (Pearson correlation coefficient $R^2 = 0.99$). NH_4^+ and NO_3^- refer to ammonium and nitrate content in the soil. Microbial CUE$_{ST}$ represents microbial carbon use efficiency from stoichiometry theory. Solid and dashed arrows indicate significant and insignificant pathways, respectively (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Black and red lines represent positive and negative relationships, respectively. Numbers along the arrows indicate standardized path coefficients. R^2 represents the proportion of variance.

treatment is consistent with findings in the literature, suggesting appropriate nitrogen levels promote organic matter accumulation (Dorais et al., 2000; Cotrufo et al., 2013; Panchal et al., 2022). While in soils with low N availability (such as the N0 treatment), root C exudation from photosynthate is utilized by microbes to generate extracellular enzymes, stimulating microbial N mining from the SOM, which reduce SOM accumulation (Asmar et al., 1994; Bengtson et al., 2012; Dijkstra et al., 2013). However, the highest rates of N fertilizer application (N3) still reduced SOM content, which means that plants and microbes may be affected by limitation of other nutrients, such as available P (Cui et al., 2024). In response, plants will increase root exudation rates leaching more phosphorus-soluble organic compounds into the soil (Canarini et al., 2019; Shen et al., 2018), stimulating microbes to mineralize P from SOM through improved phosphatase activity (Dijkstra et al., 2013; Spohn et al., 2013; Spohn and Kuzyakov, 2013). Thus, excessive N inputs might not benefit the accumulation of root-derived carbon (C) exudates into the soil (Kuzyakov and Xu, 2013; Lynch et al., 2023; Norton and Firestone, 1996).

4.2. Effects of rhizosphere and N fertilizer application on microbial carbon, nitrogen and phosphorous limitations

Both N fertilizer application and sampling location (rhizosphere and bulk soil) had significant effects on vector length and vector angle of the ecoenzymatic stoichiometry ($P < 0.001$, Fig. 3), suggesting that microbial C and N/P limitations were regulated by both N fertilization and plant root activity. Random forest analysis further confirmed that N fertilizer, root C and N exudation rates had a significant effect on both microbial C and N/P limitations ($P < 0.05$, Fig. 6). In rhizosphere soils, microbial C limitation significantly decreases with the increase of N fertilizer application rates (N0 > N1 > N2 > N3, Fig. 3B). This result was in line with our hypothesis. On the one hand, with the increase of N fertilizer application rates, the N supply in the rhizosphere environment becomes more adequate, and rhizosphere microorganisms no longer need to rely on the decomposition of SOM to obtain nitrogen. On the other hand, the production of root exudates alleviates the need for soil carbon by rhizosphere microorganisms.

We also found that vector length was significantly correlated with the rise of root C and N exudation rates (Fig. 4), indicating that higher root exudation rates actually increased microbial C limitation. The reason may be due to competition between plants and soil microorganisms. Although rhizosphere deposition can alleviate C-limitation by providing additional organic carbon sources, the growth of plant root system limited the C acquisition of rhizosphere microorganisms (Cui et al., 2018). Furthermore, higher root exudation also creates a greater demand for microbial enzymes to process them, thereby intensifying microbial C limitation. Additionally, plants and microbes have similar nutrient requirements, leading to strong competition in a rhizosphere resource-limited environment (Kuzyakov and Xu, 2013). In other words, competition for root nutrients can reduce soil nutrient availability and hinder microbial access to nutrients. As a result, microbial nutrient limitations are associated with greater nutrient competition between microbes and plants (Cui et al., 2022; Inselsbacher et al., 2010). According to economic theories of microbial metabolism, enzyme production generally increases under nutrient limitations and decreases under conditions of excess in soils, that is, the resource allocation theory (Allison and Vitousek, 2005; Carrara et al., 2018). Therefore, under low nitrogen application rates (N0 and N1 treatments), roots exudate more organic matter to acquire available nutrients from the soil rather than for microbial utilization (i.e., increased root exudates), limiting the nutrients available for microbes which, in return, tend to secrete more carbon-related enzymes to decompose soil organic carbon for nutrient acquisition (Cui et al., 2022; Inselsbacher et al., 2010). In contrast, under nitrogen-sufficient conditions (N2 and N3 treatments), rhizosphere microbes do not need to rely on decomposing recalcitrant SOM for nitrogen acquisition but rather preferentially utilize easily decomposable root exudates (Kuzyakov et al., 2000; Kuzyakov and Cheng, 2004). This leads to a decrease in soil microbial C limitation, i.e., a reduction in the release of β-glucosidase enzyme activity (Figs. 2A and 3B). In turn, microbial nutrient limitations may also inhibit the production of new substances in plants and the subsequent input of new C (Cui et al., 2020).

The vector angles for soil enzymatic stoichiometry in rhizosphere for all treatments were higher than in bulk soils (Fig. 3), suggesting that plants alter microbial N/P limitations. In the rhizosphere, the vector angles of N0, N1 and N3 treatments were >50°, indicating that plant P uptake caused microbial P limitation under these three conditions. Therefore, microbes produced more P acquiring extracellular enzymes for their growth. This result was comparable with Deng et al. (2019). Plant can solubilize inorganic P (e.g., calcium phosphates) by acidifying soil pH, releasing protons, citrate, malate and other root exudates, and could mineralize organic P into available P for microbes. However, Olsen-P contents in the soil ranged from 26.74 mg kg^{-1} in the rhizosphere soils to 45.54 mg kg^{-1} in the bulk soils among the four treatments (Fig.S1). Therefore, plants can directly uptake available phosphorus in soil, rather than obtain inorganic phosphorus or organic phosphorus in soil through root exudates (Zhang et al., 2023). Our results showed that microbial P limitation was more affected by root system (vs. bulk soil) than by N fertilizer (Figs. 6 and 7). Although N fertilizer can alter nutrient supply in the soil, root activities (such as the release of root exudates) have a more significant impact on microbial phosphorus acquisition. In bulk soils, the microbe is N limited rather than P limited because there are no plants competing with microbes for phosphorus and microbial uptake of phosphorus is lower (Bell et al., 2010). On the other hand, microbes tended to decrease their acquisition of the most limiting P to maintain stoichiometric homeostasis (Cui et al., 2020).

4.3. Effects of rhizosphere and N fertilizer application on microbial CUE_{ST}

Microbial carbon use efficiency (CUE_{ST}) in the greenhouse soils ranged from 0.35 to 0.59 (Fig. 5), higher than the mean microbial CUE of 0.26 observed from a broad range of ecosystems (Sinsabaugh et al., 2016). Nitrogen fertilizer increased microbial CUE_{ST}, especially in the rhizosphere (Fig. 5), by reducing respiration and promoting microbial growth (Li et al., 2021; Wang et al., 2023; Yuan et al., 2019), which is in line with previous studies (Poeplau et al., 2019; Spohn et al., 2016). On the one hand, microorganisms may consume less adenosine triphosphate (ATP) for the metabolism associated with N acquisition in N enriched soils; therefore, the excess C was allocated to growth, leading to the increase of microbial CUE (Manzoni et al., 2012). On the other hand, it has been confirmed that N fertilizer addition can alter decomposer community composition to acquire more labile substrates. In this process, soil microbial metabolism focuses towards phosphorus acquisition and soil N becomes a key element that promotes the production of microbial enzymes, especially those involved in phosphorus acquisition, such as acid phosphatase. This shift can reduce the amount of energy that microbes need to allocate into producing enzymes such as acid phosphatase to get nutrients, allowing more energy (or carbon) to be allocated to growth rather than maintenance or nutrient acquisition. Thus, this can lead to an increase in microbial CUE, as more carbon is being used efficiently for biomass production rather than being consumed in metabolic processes associated with nutrient stress or access (Allison and Vitousek, 2005; Liu and Zhang, 2019).

However, strong nutrient competition between roots and microbes in the rhizosphere decreased CUE_{ST}. Our result finds that the average microbial CUE_{ST} in the rhizosphere was lower than that in the bulk soils (Fig. 5). And plants were more important for microbial CUE_{ST} than fertilization (Fig. 6). There are two main reasons for this fierce competition. First, N uptake by roots can produce a strong depletion zone in rhizosphere (Kuzyakov and Razavi, 2019; Kuzyakov and Xu, 2013), leading to low N availability. Microbes and roots in rhizosphere compete for N and other nutrients (Fusseder and Kraus, 1986; Tinker and Nye, 2000). Such strong competition is confirmed by higher N uptake rates at lower N concentrations (Tinker and Nye, 2000). At this time, the limitations of rhizosphere microbial resources increases (Fig. 3) and microbial growth decreases (Fig. S3), thus reducing microbial CUE_{ST}. Our correlation analysis and structural equation models confirmed this (Fig. S4 and Fig. 7). Second, roots release a large amount of exudates, most of which are labile soluble molecules that can be easily taken up and utilized by soil microbes. Therefore, the abundance and activity of microbes are much higher in the rhizosphere than in the bulk soil (Kuzyakov and Blagodatskaya, 2015), resulting in increased demand for available nutrients (Available N and P) and stronger competition for nutrients with roots. Microbial growth results in N mineralization from SOM, meaning that the microbes allocate more energy to nutrient acquisition rather than growth, reducing rhizosphere soil microbial CUE_{ST}. Meanwhile, microbes stimulated the priming effect, which is not conducive to soil C retention (Kuzyakov and Xu, 2013). This phenomenon is more pronounced under low nitrogen conditions, with the greatest reduction in SOM (Fig. S1) and microbial CUE_{ST} (Fig. 5) in the rhizosphere soil compared with bulk soil under the N0 treatment.

Interestingly, we observed that the higher the root C and N exudation rates, the lower the microbial CUE_{ST} (Fig. 5). While increased belowground C and N allocation may promote the preferential utilization of root exudates by rhizosphere microbes, leading to decrease soil organic matter (SOM) content and microbial CUE due to surplus C being allocated to microbial growth (Jones et al., 2009; Pausch and Kuzyakov, 2018). Our study also found that higher rates of root exudation decreased organic matter content (Fig. S3). Higher root exudation rates led to increased organic matter decomposition, further lowering CUE_{ST} due to enhanced nutrient competition and microbial energy allocation to nutrient acquisition. Various studies have shown that the increase in root exudates can be caused by high nitrogen input (Baptist et al., 2015; Henry et al., 2007; Neumann et al., 2014) or by low nitrogen availability (Chowdhury et al., 2014; Kuzyakov and Domanski, 2000), or not be influenced by nitrogen levels (Johansson, 1992). It is noteworthy that the increase in root exudation rates in our study was caused by a decrease in fertilizer application. Our results also found that root C and

N exudation rates were more influential on CUE_{ST} than N availability and SOM (Fig. 6). It can be evident that changes in microbial CUE were caused by changes in root exudates induced by nitrogen fertilizers. It has been shown that labile substrates (such as root exudates) tend to induce higher microbial CUE and cause more efficient accumulation of MAOM through microbial transformation (Cotrufo et al., 2015; Sokol and Bradford, 2019).

Vector length was the main controlling factor for microbial CUE_{ST} (Fig. 6), indicating that microbial metabolism can control microbial CUE (Geyer et al., 2019; Ju et al., 2023; Mooshammer et al., 2014). Moreover, the pattern of microbial CUE_{ST} was opposite to that of microbial C and P limitations (Fig. 5, Fig. 7). This pattern was particularly evident in the rhizosphere. An increase in root exudates from plant can provide energy to microbes and stimulate the priming effect, in turn enhancing the microbial conversion of C (Blagodatskaya and Kuzyakov, 2008; Shahzad et al., 2015). In conclusion, when soil N is limited, root exudates more organic matter to obtain N, which enhance the competition between plant and microbes, thus the microbes release more C-related acquiring extracellular enzymes that is increase C limitation (Fig. 4), thus making the microbes increased the utilization of soil carbon i.e. a decrease in SOM content which is also decrease microbial CUE_{ST} (Fig. 5). Low CUE_{ST}, in turn, suggests that microbes decompose more C through respiration than growth, and that SOM decomposes more C from sources used for synthetic biomass (its own growth), to the detriment of carbon stability (Manzoni, 2017).

5. Conclusions

The impact of N fertilizer application on microbial C, N/P limitations and CUE_{ST} are largely driven by the effect of N fertilizer on root exudation rates. Plants compete with microbes for nutrients by increasing the rates of root exudation, thereby increasing microbial nutrient limitations. At the same time, roots release labile soluble molecules that are utilized by microbes to promote microbial extracellular enzyme production, which stimulates the decomposition of SOM. Consequently, more energy is allocated by microbes to resource acquisition rather than to growth, thus decreasing the CUE_{ST}. These findings suggest that plant-mediated alterations in subterranean carbon allocation, induced by nitrogen fertilization, modulate microbial metabolic limitations in the rhizosphere soils. These findings significantly advance our fundamental understanding of the potential significance of the rhizosphere in the global carbon balance and plant-microbiome interactions aiming to enhance soil protection and sustainable intensive agriculture production in the future.

Credit authorship contribution statement

Jinshan Lian: Writing – original draft, Methodology, Investigation, Conceptualization. **Guihua Li:** Writing – review & editing, Supervision, Conceptualization. **Jianfeng Zhang:** Resources, Project administration, Funding acquisition, Conceptualization. **Sébastien Massart:** Writing – review & editing, Supervision.

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 and materials availability

All data are available in the main text or the supplementary materials. Additional data related to this paper may be requested from the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174933.

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