

Research Article

Effects of leguminous green manure–crop rotation on soil enzyme activity and stoichiometry

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Abstract

As a crucial strategy for sustainable agricultural production, green manure–crop rotation can regulate soil nutrient cycling and decrease the reliance on nitrogen fertilizers. However, we still lack a comprehensive understanding of the changes in soil eco-enzyme activities, microbial metabolism and nutrient limitations caused by leguminous green manure–crop rotation. Here, we conducted field experiments of leguminous green manure–crop rotation across China to analyze soil extracellular enzyme activities, specifically β -glucosidase (BG), *N*-acetyl- β -D-glucosaminidase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (AP). The study revealed that long-term green manure–crop rotation increased carbon and nitrogen accumulation in farmland, with a significant average increase of 20.1% and 36.4% in BG, AP enzyme activities in topsoil, while showing a decrease in $\ln(\text{NAG} + \text{LAP}) : \ln(\text{AP})$ ratios. The ratios of $\ln(\text{BG}) : \ln(\text{NAG} + \text{LAP})$ and $\ln(\text{NAG} + \text{LAP}) : \ln(\text{AP})$ in soil across various regions were typically below 1:1, indicating that soil microbial activity is more constrained by nitrogen and phosphorus nutrients rather than by carbon. Precipitation, temperature, soil total carbon (TC) and total nitrogen (TN) were identified as key environmental factors for extracellular enzyme activities and stoichiometric ratios. Our study highlights that the green manure–crop rotation alleviates nitrogen limitation while enhancing phosphorus limitation, and is closely related to the accumulation of TC and TN in the soil.

Keywords: green manure, crop rotation, soil extracellular enzyme activity, stoichiometric ratio, nutrient

豆科绿肥-作物轮作对土壤酶活性及其化学计量比的影响

摘要：绿肥-作物轮作作为可持续农业的重要策略，有助于调节土壤养分循环，减少氮肥依赖。然而，目前仍不清楚豆科绿肥作物轮作如何影响土壤生态酶活性、土壤微生物代谢以及土壤养分限制。为此，我们在中国不同地区开展了豆科绿肥-作物轮作的田间试验，以分析土壤胞外酶活性的变化，特别是 β -葡萄糖苷酶(BG)、*N*-乙酰- β -D-氨基葡萄糖苷酶(NAG)、亮氨酸氨肽酶(LAP)和酸性

磷酸酶(AP)。研究发现，长期绿肥-作物轮作增加了农田土壤碳和氮的积累，表层土壤BG、AP酶活性平均显著增加了20.1%和36.4%，而 $\ln(\text{NAG} + \text{LAP}):\ln(\text{AP})$ 比值显著降低。此外，不同地区土壤中 $\ln(\text{BG}):\ln(\text{NAG} + \text{LAP})$ 和 $\ln(\text{NAG} + \text{LAP}):\ln(\text{AP})$ 的比值整体低于1:1，这表明土壤微生物活性更多受土壤中氮和磷养分的限制而非碳制约。降水、温度、土壤总碳和总氮是影响土壤胞外酶活性及其化学计量比变化的关键环境因素。上述研究结果表明，绿肥-作物轮作在减轻土壤氮限制的同时增强了土壤磷限制，这与土壤中总碳和总氮的积累密切相关。

关键词：绿肥，轮作，土壤胞外酶，化学计量比，养分

INTRODUCTION

The application of chemical fertilizers plays a crucial role in ensuring crop yield and quality (Zhang *et al.* 2022a). However, the excessive use of chemical fertilizers, often surpassing the recommended dosage, can lead to various negative effects such as low fertilizer utilization rates, soil quality degradation, a significant decrease in microbial diversity and water body eutrophication (Qiang *et al.* 2022). Consequently, reducing the input of chemical fertilizers at the source is an essential strategy for mitigating soil ecological environmental pollution (Wang *et al.* 2022b).

Utilizing the advantages of crops to promote sustainable development in agricultural production has become the focus of current research. Green manure-crop rotation has emerged as a crucial means for increasing food production and mitigating land degradation by regulating soil nutrient cycling and reducing the need for nitrogen fertilizers (Borase *et al.* 2020). Green manures vary in nutrient composition and carbon (C) to nitrogen (N) ratio, leading to diverse effects on soil improvement (Liang *et al.* 2022). Leguminous green manures facilitate biological nitrogen fixation through interactions with rhizosphere microorganisms, thus providing an additional N source for subsequent crops (Liang *et al.* 2023). Given the essential link between the C and N cycles in terrestrial ecosystems, enhancing N efficiency is crucial for the formation and decomposition of soil organic matter. Therefore, the cultivation of green manures is a promising key measure for enhancing farmland quality and ensuring food production stability in the future.

Soil extracellular enzyme activity is considered a crucial indicator for evaluating the soil health status, reflecting key processes of biogeochemical cycling and dynamics change of soil organic matter (Cui *et al.* 2018; Moorhead *et al.* 2016; Sinsabaugh *et al.* 2010). Previous studies have shown that soil

enzyme activities exhibit differences in response to plant type and other environmental factors (Curtright and Tiemann 2021), being particularly sensitive to changes in N and phosphorus (P) deposition, compared with changes in temperature and precipitation (Xiao *et al.* 2018). Alterations in soil nutrients and environmental conditions induced by legume crop rotation significantly impact soil enzyme activities and composition (Wang *et al.* 2022a). Furthermore, the duration of legume crop rotation has been linked to higher enzyme activities and increased soil N content (Tiemann *et al.* 2015). Over the years, researchers have expanded into the response of soil enzyme activities to soil nutrients, revealing diverse trends and levels of variability (Marklein and Houlton 2012; Yokoyama *et al.* 2017).

Meanwhile, stoichiometry influenced by soil enzyme activities has become a focal point in ecological research for visualizing nutrient limitation in soil microbial communities by mapping soil extracellular enzyme stoichiometric ratio associated with C, N and P acquisition (Ma *et al.* 2021b; Sinsabaugh *et al.* 2009). This stoichiometric ratio can be converted to vector length (VL) and vector angle (VA) to demonstrate the synchronized relativity of microbial communities to resource requirements (Moorhead *et al.* 2016). Similar to soil enzyme activities, their stoichiometric ratios are largely regulated by crop type and soil physicochemical properties (Cui *et al.* 2018; Sinsabaugh *et al.* 2010). However, chemometrics currently focuses on studies at regional and ecological scales, aiming to reveal mechanisms such as nutrient cycling and elemental limitation. For instance, Ma *et al.* discovered that microbial growth in the Loess Plateau region was mainly limited by soil P nutrients through enzyme stoichiometric ratios analysis (Ma *et al.* 2021b). While some investigations have explored the impact of soil enzyme activities in inter-root soils during green manure-crop rotations, there remains a notable gap in the stoichiometric characterization of these processes.

Soil extracellular enzyme activity and its stoichiometry play a crucial role in organic matter decomposition and nutrient cycling in agroecosystems (Fatemi *et al.* 2016). When soil nutrient supply is insufficient, soil microorganisms degrade organic compounds mainly by inducing the production of extracellular enzymes (Sinsabaugh and Follstad Shah 2012). For instance, adding N or ensuring adequate N supply directly stimulates the activities of enzymes involved in the C and P cycles, resulting in increased nutrient release (Chen *et al.* 2018; Tiemann *et al.* 2015; Xiao *et al.* 2021). The above process is helpful to alleviate the limitation of soil C and P nutrients and ultimately promote the balance of soil nutrient availability (Xiao *et al.* 2018). It is worth noting that the phosphatases responsible for P release are rich in N, and it needs the biological sacrifice of N to obtain P (Houlton *et al.* 2008; Marklein and Houlton 2012). Moreover, soil extracellular enzyme activity and its stoichiometry are influenced by factors such as soil pH, temperature, humidity and vegetation type (Guangming *et al.* 2017; Jia *et al.* 2020; Sun *et al.* 2013). Research has demonstrated that changes in soil extracellular enzyme activities can impact the utilization and cycling of nutrients such as soil C, N and P, and then affect plant growth and ecosystem function (Sinsabaugh and Follstad Shah 2012). Therefore, a comprehensive investigation of soil extracellular enzyme activity and its stoichiometry in green manure–crop rotation farmland ecosystems can enhance our understanding of the nutrient demands and utilization strategies of soil microbial communities, establishing a scientific foundation for agricultural production and soil management.

Leguminous crops are frequently integrated into crop rotation systems, offering an important advantage in soil fertility regulation. In previous studies, the comparative evaluation of crop rotation and continuous monoculture has a good record in terms of crop productivity and resource utilization efficiency. Nevertheless, there is little information about the chemometrics of ecological enzymes involved in C, N and P cycles and the nutritional limitations of soil microorganisms in different regions. To solve the above problems, we carried out three groups of control experiments in northeast (NC), central (CC) and southwest China (SC), respectively, to explore the role of enzyme activities and stoichiometric ratios in improving the availability of soil N and P in leguminous green manure–crops rotation planting mode (soybean–maize–soybean, oil seed rape–wheat–oil seed rape and pea–cabbage–pea)

compared with single crops planting mode (maize, wheat and cabbage). Our study hypothesized that: (i) the two planting modes (leguminous green manure–crops rotation planting mode and single crop planting mode) have a great influence on the enzyme activities and their stoichiometry; (ii) soil microorganisms are restricted by N or P instead of C in terms of nutrient acquisition. Furthermore, our results revealed the environmental factors influencing the eco-enzyme stoichiometry and microbial nutrient limitations. Our study provides a theoretical basis for explaining the mechanism of leguminous green manure–crop rotation to reduce the harm of continuous cropping. It is very important to understand the soil eco-enzyme stoichiometry and nutrient turnover mode, as well as the circulation and availability of soil nutrients in ecological critical zones.

MATERIALS AND METHODS

Our experiment was conducted in NC, CC and SC. The specific characteristics of each plot are described as follows. One experimental plot selected in NC has a temperate continental monsoon climate, featuring a short warm summer and long cold winter, with an average temperature of 2–6 °C. The annual average precipitation, influenced by the Pacific monsoon, ranges from 400 to 650 mm, with about 75% of the precipitation occurring from May to September. One experimental plot chosen in CC lies within the temperate and subtropical monsoon climate zones. The average temperature ranges from 17 to 19 °C, and the average precipitation is 900–1000 mm. This region experiences abundant heat and precipitation, with distinct seasons throughout the year. One experimental plot situated in SC falls within the subtropical monsoon climate, with an average temperature of 15–17 °C and an average precipitation of 1300–1600 mm. The regional microclimate has obvious characteristics. The rainfall and daily average temperatures are shown in Fig. 1 during the crop growing season from May to October in different regions in 2019.

Experimental design

In each region (NC, CC and SC), we selected two sampling sites with similar altitudes, aspects and slopes and established manure–crop rotation treatment and control treatment at each site. The descriptions of each sampling site are shown in Table 1. Six 100 m × 100 m plots were established at each sampling site in May 2013. The control

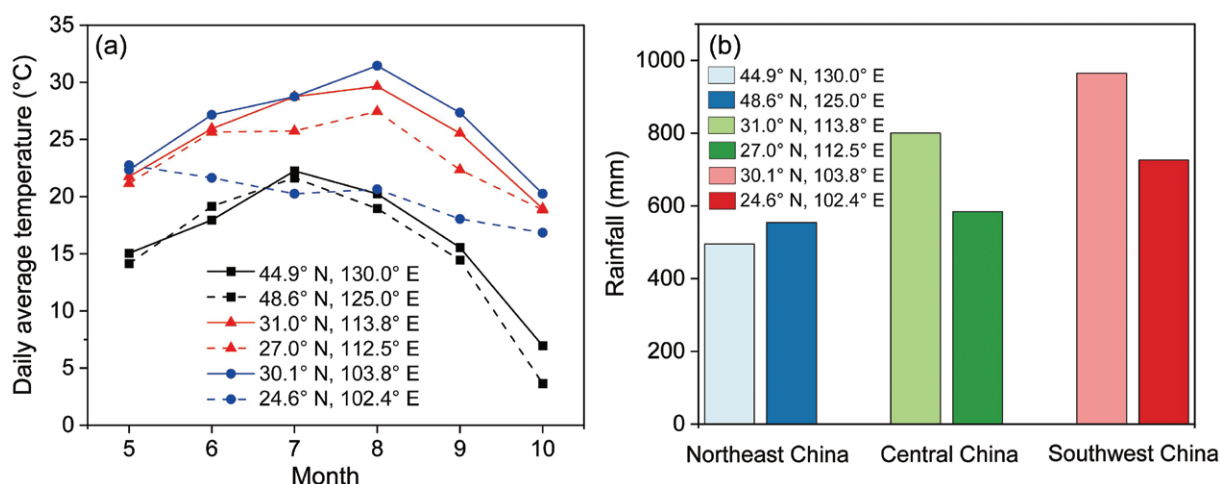


Figure 1: Daily average temperature (a) and monthly rainfall (b) during the crop growing season in different regions in 2019.

Table 1: Main information of national sampling points

Region	Longitude and latitude	Planting system	Total fertilizer application (kg hm ⁻²)		
			Urea (N 46.7%)	Calcium superphosphate (P ₂ O ₅ 16%)	Potassium chloride (KCl 60%)
Northeast China	44.9° N, 130.0° E	maize	225	150	75
	48.6° N, 125.0° E	soybean–maize	225	150	75
Central China	31.0° N, 113.8° E	wheat	240	150	75
	27.0° N, 112.5° E	oil seed rape–wheat	240	150	75
Southwest China	30.1° N, 103.8° E	cabbage	180	105	50
	24.6° N, 102.4° E	pea–cabbage	180	105	50

treatment used a single crop planting mode (such as corn in NC, wheat in CC and cabbage in SC), while the experimental treatment used a legume green manure–crop rotation mode (such as soybean–corn–soybean in NC, rapeseed–wheat–rapeseed in CC and pea–cabbage–pea in SC). In our experiment, urea (N 46.7%) was used as nitrogen fertilizer, calcium superphosphate (P₂O₅ 16%) as phosphorus fertilizer and potassium chloride (KCl 60%) as potassium fertilizer, all applied manually before plowing. The experimental treatments and nutrient amounts are shown in Table 1.

Soil sample collection

In August 2019, three soil samples (0–20 and 20–50 cm) were randomly collected from each of the selected 36 plots. Visible roots and gravel were

removed, and the treated soil samples were mixed. The soil samples were stored at 4 °C and transported back to the laboratory. Each sample was sieved through a 2-mm sieve and then divided into two subsamples. One subsample was stored at 4 °C for the determination of soil extracellular enzyme activities, while the other subsample was naturally air-dried and sieved through a 100-mesh sieve for the determination of soil chemical properties such as soil pH, soil total carbon (TC), total nitrogen (TN) and total phosphorus (TP).

Determination of soil chemical properties

The soil pH was measured using a pH meter after adding deionized water following a water–soil ratio of 5:1. Soil TC was analyzed using an elemental analyzer (Vario Micro cube, Elementar, Hanau, Germany). Soil

TN was determined using the semi-micro Kjeldahl method. Soil TP was determined through molybdenum, antimony and scandium colorimetry. For detailed methods, please refer to Bao Shidan's soil analysis and determination method (Bao 2000).

Determination of soil extracellular enzyme activities

According to the method described by Zhou *et al.* (2020), we measured the activities of four extracellular enzymes, namely β -glucosidase (BG), *N*-acetyl- β -D-glucosaminidase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (AP), which are involved in soil carbon, nitrogen and phosphorus cycles. The methods were as follows.

Substrate configuration

Weigh 0.0169 g BG, 0.0190 g NAG, 0.0162 g LAP and 0.0128 g phosphatase and put them into a 25-mL brown volumetric flask for constant volume ($c = 2000 \mu\text{mol L}^{-1}$).

Marking preparation

Accurately weigh 0.0088 g of MUB (4-methylumbelliferone) and add it into a 25-mL brown volumetric flask to a constant volume ($c = 2000 \mu\text{mol L}^{-1}$ MUB standard solution). Then, remove 10 mL of the standard solution to 100 mL to obtain 200 $\mu\text{mol L}^{-1}$ MUB standard solution. Remove 0, 1.25, 2.5, 5, 12.5, 25 and 37.5 mL to 50 mL, respectively, to obtain standard gradient solutions with MUB concentrations of 0, 5, 10, 20, 50, 100, 150 and 200 $\mu\text{mol L}^{-1}$.

Sample determination

Weigh 2.75 g of soil and add 91 mL of acetic acid buffer. Shake the mixture for 30 min to obtain a soil suspension. Remove 50 μL of the soil slurry and add it to a 96-well microplate, then add 200 μL of a 200 $\mu\text{mol L}^{-1}$ fluorescent substrate specific for each enzyme to each well, with eight replicates for each sample. Then, line mark inoculate 50 μL of MUB standard solution with different concentration gradients to the deep well plate. Incubate the prepared 96-well plate in the dark at 25 °C for 4 h. After incubation, 50 μL of 0.5 M NaOH is added to each well to stop the reaction. Measure the fluorescence using an enzyme-labeled instrument (SpectraMax Gemini, Molecular Devices, CA, USA) at excitation and emission wavelengths of 365 and 450 nm, respectively. Calculate the enzyme activity as nmol per gram of dry soil per hour (nmol activity g^{-1} dry soil h^{-1}).

Statistical analyses

We conducted a two-way analysis of variance (ANOVA) to assess the impacts of treatments, regions and their interactions on soil chemical properties, soil extracellular enzyme activities and stoichiometric ratios. Additionally, we utilized one-way ANOVA to investigate the effects of different cropping modes (single crop continuous cropping and leguminous green manure–crops rotation) on soil chemical properties, enzyme activities, VLs and VAs. Furthermore, we employed principal component analysis (PCA) after standardizing the data for environmental factors, soil enzyme activities and stoichiometric ratios to elucidate the impact of cropping modes. Linear regressions were conducted between VL and VA using the R software. Random forest analysis was carried out with the randomForest package in R. To explore the relationship between soil enzyme activities and soil chemical properties, we conducted Pearson's correlation analysis. Bar graphs, box plots and point plots were generated using Origin 2023b. Statistical significance was considered at the $P < 0.05$ and $P < 0.01$ levels.

RESULTS

Effect of manure–crop rotation on soil chemical properties

The soil chemical properties in various regions are presented in Fig. 2 and Table 2. A two-way ANOVA revealed that different treatments were the primary factor affecting changes in soil chemical properties. In our analysis, the sites and the interactions between sites and treatment did not significantly impact overall soil chemical properties. Hence, our focus remains on the effects of manure–crop rotation on soil chemical properties, disregarding interference from other factors such as sites. Notably, the content of TC, TN and TP significantly increased under the green manure–crops rotation in NC, CC and SC ($P < 0.05$, Fig. 2). In CC and northwest China, the soil pH under the green manure–crops rotation was significantly higher than that under the control treatment ($P < 0.05$), but there was no significant difference in soil pH in NC ($P > 0.05$, Fig. 2).

Effect of manure–crop rotation on soil enzyme activities and stoichiometric ratios

Before analyzing the changes in soil enzyme activities and stoichiometric ratios, we examined the effects of

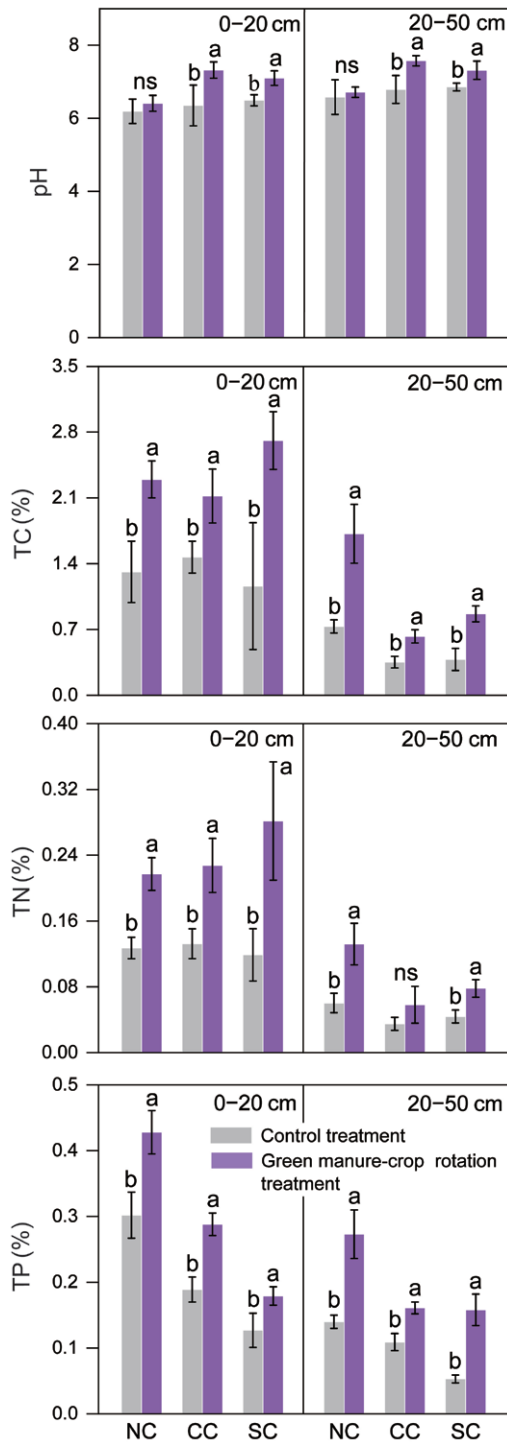


Figure 2: Effects of different treatments on soil chemical properties. (a) The effects of different treatments on pH, (b) the effects of different treatments on soil TC, (c) the effects of different treatments on soil TN, and (d) the effects of different treatments on soil TP. Different lowercase letters (a, b) indicate significant differences between treatments, while 'ns' indicates no significant differences between treatments.

different treatments, sites and their interactions on soil enzyme activities and stoichiometric ratios (Table 2).

Based on the results, we reconfirmed and found that crop rotation was the main factor affecting the changes in soil enzyme activities and stoichiometric ratios. The interactions between different sites and between sites and treatments did not significantly impact the overall changes in soil enzyme activities and stoichiometric ratios, except for a few significant cases. Therefore, in our next analysis, we only pay attention to the effects brought by different treatments, while ignoring other factors such as site interference.

PCA was used to identify the differences among different treatments, as it effectively reflects the structural characteristics of soil enzyme communities. In PCA, the closer the samples were the smaller the difference in the metabolic function of the soil enzyme community. We selected the first two principal components in NC, CC and SC for analysis: principal component 1 (PCA1) and principal component 2 (PCA2) (Fig. 3). The variance contribution rate of these components was 52.4%, 26.4%, 59.2%, 22.9%, 45.1% and 36.5%, respectively, with a cumulative variance contribution rate of 78.8%, 82.1% and 81.6%. The results showed that PCA1 and PCA2 explained most of the differences in soil enzyme activities and nutrient metabolism and utilization under different treatments.

We compared soil enzyme activities among different treatments (Fig. 4). In general, leguminous green manure-crops rotation planting mode increased the enzyme activities involved in C, N and P nutrient cycles in the 0–20 cm. The activities of BG and AP enzymes increased by 20.1% and 36.4% on average, respectively, with significant differences ($P < 0.05$). While the soil NAG + LAP enzyme activity was improved by the green manure-crops rotation in all regions, a significant difference was observed only in NC. Additionally, only the AP enzyme showed a significant difference between treatments in the 20–50 cm (Fig. 4), with no significant differences in BG and NAG + LAP enzyme activities. The enzyme activities in the 0–20 cm soil layer were significantly higher than in the 20–50 cm layer, both in the control treatment and the green manure-crop rotation mode.

Soil enzyme stoichiometric ratios revealed significant differences between the control treatment and green manure-crop rotation (Fig. 5). The $\ln(\text{BG}):\ln(\text{NAG} + \text{LAP})$ were consistently less than 1:1 in all regions, indicating that soil microorganisms are primarily limited by N or P rather than C for nutrient acquisition. Further research showed that the ratios

Table 2: Results of repeated measures ANOVA on the effects of different treatments, sites and their interactions on soil chemical properties, soil extracellular enzyme activities and stoichiometric ratios

Source of variance	Soil chemical properties						Soil extracellular enzyme activity						Stoichiometric ratio			
	pH	TC	TN	TP	BG	NAG + LAP	AP	E _{C/N}	E _{C/P}	E _{N/P}						
Northeast China																
0–20 cm																
T	0.235	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	0.542	<0.01**	<0.01**	<0.01**
S	0.484	0.233	0.126	0.142	0.620	0.774	0.086	0.627	0.116	0.072	0.131	0.118	0.131	0.072	0.131	0.131
T*S	0.472	0.705	0.954	0.593	0.781	0.704	0.139	0.806	0.118	0.131	0.131	0.131	0.118	0.131	0.131	0.131
20–50 cm																
T	0.562	<0.01**	<0.01**	0.01**	<0.01**	<0.05*	<0.01**	<0.01**	<0.05*	<0.01**	<0.01**	<0.01**	<0.05*	<0.01**	<0.01**	<0.01**
S	0.713	0.189	0.091	0.05*	0.449	0.938	<0.05*	0.371	0.078	<0.05*	<0.05*	0.078	0.078	<0.05*	<0.05*	<0.05*
T*S	0.879	0.160	<0.01**	0.05*	0.383	0.270	<0.05*	0.505	0.160	0.094	0.094	0.160	0.160	0.094	0.094	0.094
Central China																
0–20 cm																
T	<0.01**	<0.01**	<0.01**	0.01**	<0.05*	<0.01**	<0.01**	0.161	0.196	<0.01**	<0.01**	<0.01**	0.196	<0.01**	<0.01**	<0.01**
S	0.187	0.889	0.402	0.362	0.384	<0.05*	0.879	0.141	0.636	0.272	0.272	0.272	0.636	0.272	0.272	0.272
T*S	0.05*	0.764	0.508	0.334	0.680	<0.05*	0.617	0.246	0.554	0.709	0.709	0.709	0.554	0.709	0.709	0.709
20–50 cm																
T	<0.01**	<0.01**	<0.05*	0.01**	0.161	0.207	<0.01**	<0.05*	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**
S	0.332	0.761	0.059	0.531	0.374	0.988	0.428	0.615	0.566	0.190	0.190	0.190	0.566	0.190	0.190	0.190
T*S	0.059	0.530	0.231	0.240	0.799	0.694	0.136	0.772	0.127	0.084	0.084	0.084	0.127	0.084	0.084	0.084
Southwest China																
0–20 cm																
T	<0.01**	<0.01**	<0.01**	0.01**	<0.01**	<0.01**	<0.01**	<0.01**	0.288	<0.01**	<0.01**	<0.01**	0.288	<0.01**	<0.01**	<0.01**
S	0.975	0.247	0.067	0.627	0.974	0.839	0.819	0.974	0.939	0.943	0.943	0.943	0.939	0.943	0.943	0.943
T*S	0.178	0.596	0.363	0.622	0.822	0.408	0.475	0.967	0.912	0.859	0.859	0.859	0.912	0.859	0.859	0.859
20–50 cm																
T	<0.01**	<0.01**	<0.01**	<0.01**	0.097	0.072	<0.05*	<0.01**	0.189	<0.05*	<0.05*	<0.05*	0.189	<0.05*	<0.05*	<0.05*
S	0.197	0.913	0.919	0.587	0.060	0.829	<0.05*	0.059	<0.05*	0.074	0.074	0.074	<0.05*	0.074	0.074	0.074
T*S	0.197	0.260	0.749	0.600	0.157	0.300	<0.05*	<0.05*	<0.05*	0.128	0.128	0.128	<0.05*	0.128	0.128	0.128

Abbreviations: E_{C/N} = ln(BG):ln(NAG + LAP), E_{C/P} = ln(BG):ln(AP), E_{N/P} = ln(NAG + LAP):ln(AP), S = site, T = treatment, T*S = treatment*site. ** means that there is a very significant interaction between them (P < 0.01); * means that there is a significant interaction between them (P < 0.05).

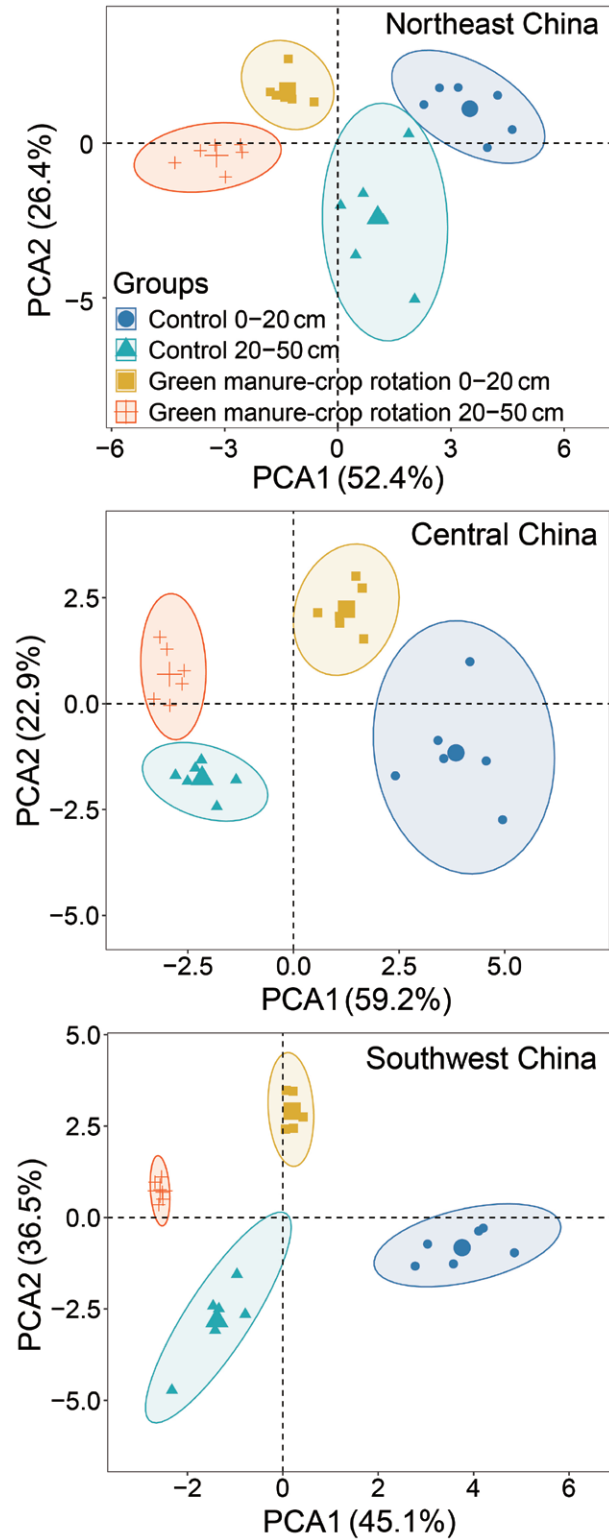


Figure 3: PCA for soil extracellular enzyme activities. (a) NC, (b) CC and (c) SC.

of $\ln(\text{NAG} + \text{LAP}) : \ln(\text{AP})$ under the green manure-crop rotation decreased compared with the control treatment. The results showed that the adoption of green manure-crop rotation effectively reduces the

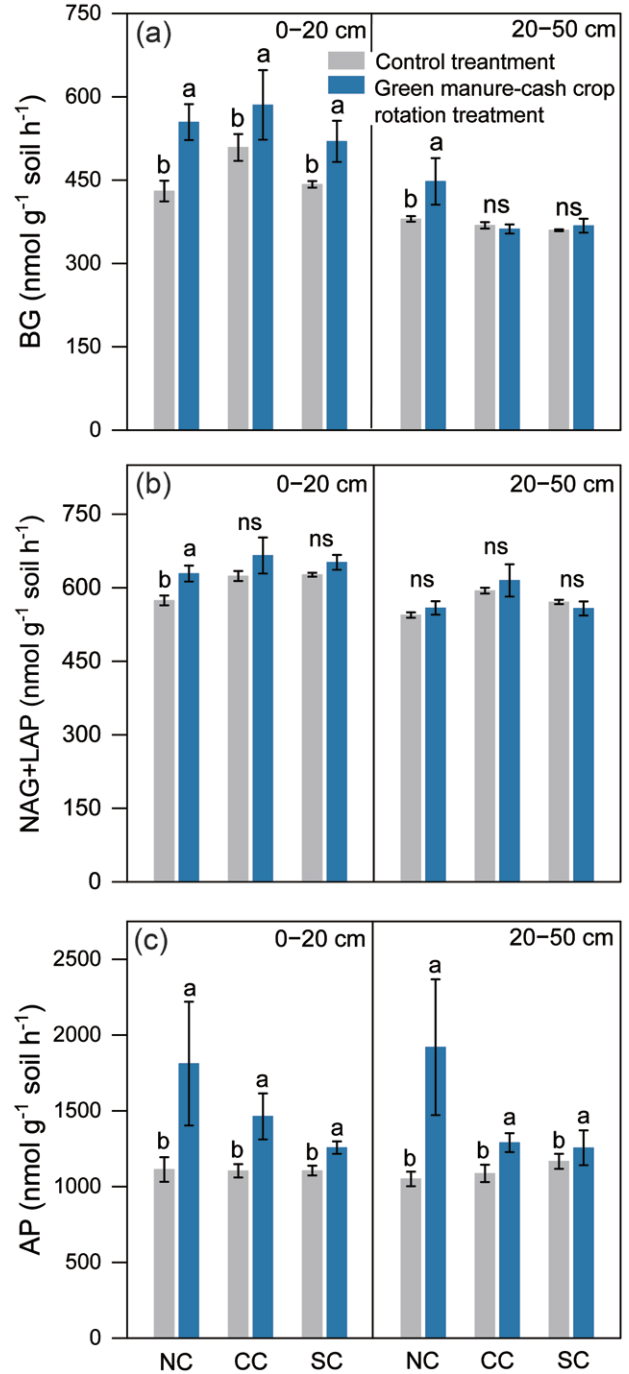


Figure 4: Soil extracellular enzyme activities in soil between different treatments. (a) BG, (b) NAG and (c) LAP. Different lowercase letters (a, b) indicate significant differences between treatments, while 'ns' indicates no significant differences between treatments.

N limitation and increases the P limitation in soil across all regions.

Subsequently, we analyzed the VL and VA of soil extracellular enzyme activities (Fig. 6). There was no significant difference in VL between treatments

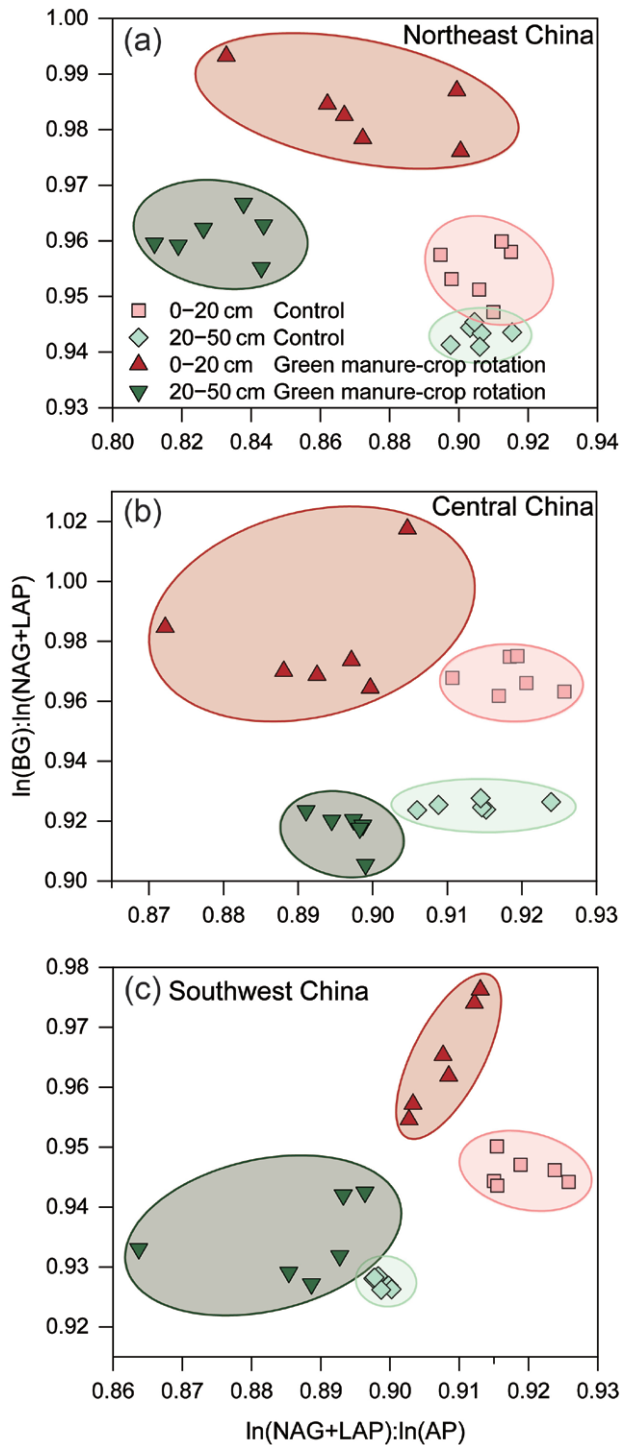


Figure 5: Effects of different treatments on stoichiometric ratio of soil extracellular enzymes. (a) NC, (b) CC and (c) SC.

in the three regions ($P > 0.05$), indicating that the green manure–crop rotation did not lead to competition among soil microorganisms for C nutrients. Conversely, the VA of soil under the green manure–crop rotation was significantly higher than that of the control treatment in all regions. Our

result supports the conclusion that crop rotation can effectively soil alleviate N limitation. Random forest analysis identified key factors influencing VL and VA. For VL, $E_{C:P}$ was the most crucial factor, followed by BG, $E_{C:N}$, AP, TN, $E_{N:P}$, TC and NAG + LAP. For VA, the most crucial factors were $E_{N:P}$, AP, $E_{C:P}$ and TP ($P < 0.05$, Fig. 6d and e).

Relationship between environmental factors, soil enzyme activities and stoichiometric ratios

To elucidate the correlation between soil extracellular enzyme activities, stoichiometric ratio and soil environmental factors under different treatments, we conducted Pearson correlation analysis (Fig. 7). BG enzyme activity was positively correlated with TC, TN, C:P and N:P. NAG + LAP activity was positively correlated with Mean Annual Temperature (MAT), Mean Annual Precipitation (MAP), TC, TN and N:P, however, negatively correlated with C:N. AP activity was positively correlated with MAT, MAP, TC and TN. Additionally, $E_{C:N}$ was positively correlated with TC, TN and C:P, and negatively correlated with MAT, MAP and pH. $E_{C:P}$ was positively correlated with TC and TN, while negatively correlated with C:N. $E_{N:P}$ was positively correlated with MAT and MAP, and negatively correlated with TC and C:N. These results demonstrate significant correlations between enzyme activities, stoichiometric ratios and environmental factors, reflecting soil nutrient fertility levels.

DISCUSSION

Green manure–crop rotation has become a crucial measure to support the sustainable development of agriculture, with a positive impact on soil nutrients, microbial diversity and abundance and crop yield (Song *et al.* 2021). Our study compared two planting modes—single crop planting and leguminous green manure–crop rotation planting—to evaluate the effects of green manure–crop rotation on soil chemical properties, soil extracellular enzyme activities and stoichiometric ratios, confirming the role of soil enzymes in regulating soil nutrient balance and promoting nutrient release.

Effect of leguminous green manure–crops rotation on soil chemical properties

Soil pH is a critical indicator of soil quality, affecting soil nutrient form, transformation, availability and crop nutrient absorption (Puissant *et al.* 2019; Wang *et al.* 2017). In addition, we noticed that the

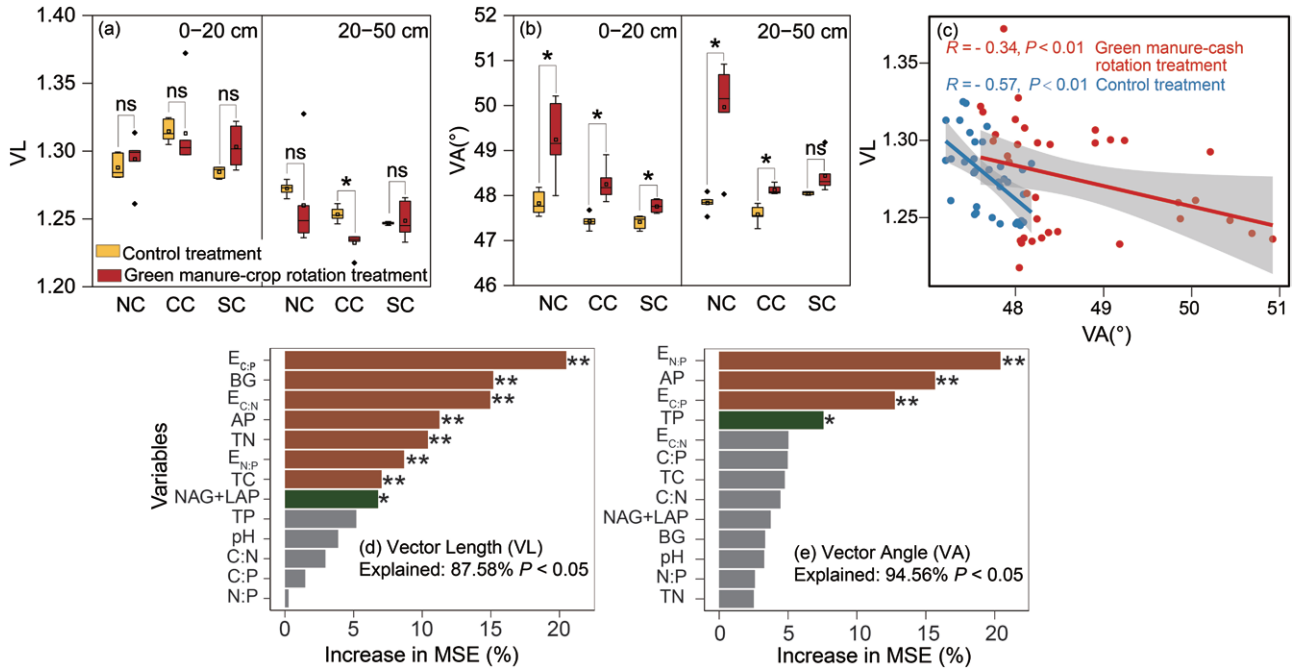


Figure 6: (a) and (b) show the effects of different treatments on the VL and VA of soil extracellular enzymes; (c) shows the linear correlation between VA and VL; (d) and (e) importance analysis of impact factors of soil extracellular enzyme activities VL and VA (random forest analysis). ‘***’ means that there is a significant difference between treatments ($P < 0.01$); ‘*’ means that there are significant differences between treatments ($P < 0.05$); while ‘ns’ indicates no significant differences between treatments ($P > 0.05$).

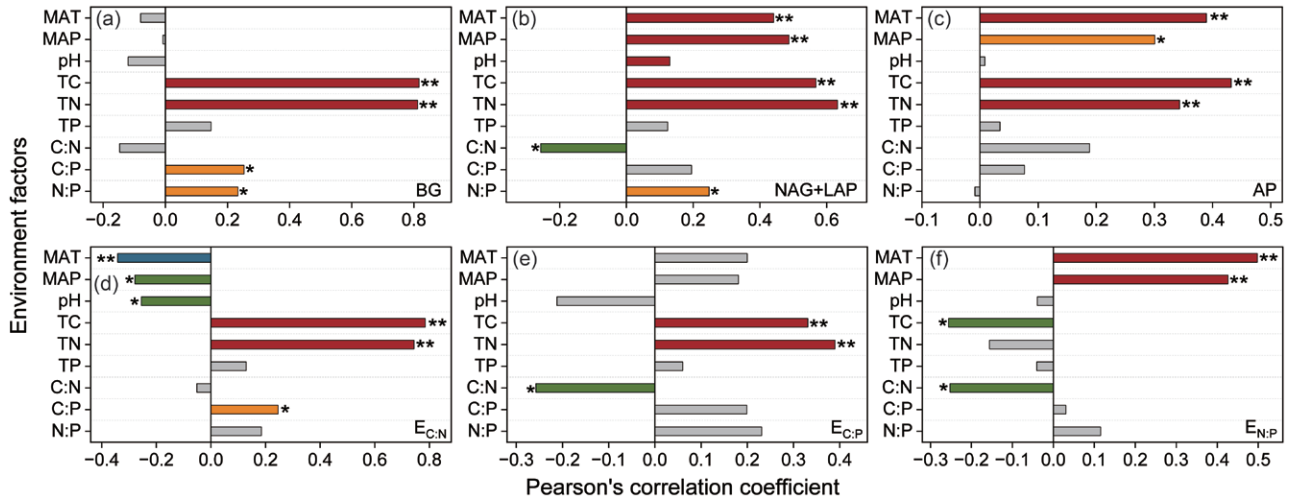


Figure 7: The Pearson correlation analysis with environment factors, enzyme activities and stoichiometric ratios. (a) BG, (b) NAG+LAP, (c) AP, (d) $\ln(BG):\ln(NAG + LAP)$ ($E_{C:N}$), (e) $\ln(BG):\ln(AP)$ ($E_{C:P}$) and (f) $\ln(NAG + LAP):\ln(AP)$ ($E_{N:P}$). ‘***’ means that there is a very significant interaction between them ($P < 0.01$); ‘**’ means that there is a significant interaction between them ($P < 0.05$).

rotation planting mode of green manure and crops significantly improved the pH value of soil in CC and northwest China. Studies show that one of the main effects of leguminous plants is to increase the soil pH value compared with chemical fertilizers and untreated plots (Amede *et al.* 2021). However,

our findings demonstrate that the soil pH value in NC did not change. The analysis may be due to seasonal changes, meteorological conditions and other environmental factors that masked the effects of green manure crops. In summary, our results confirm the role of leguminous green manure in

enhancing soil nutrients, reducing soil acidification and promoting soil organic carbon sequestration (Tao *et al.* 2017), highlighting its importance for sustainable agriculture.

The effect of leguminous green manure crops on the nutrient content of farmland soil has become a key research focus. Through the analysis of the research results, we found that the leguminous green manure–crop rotation significantly increased the contents of TC, TN and TP ($P < 0.05$). It may be due to the N fixation of leguminous green manure crops and the decomposition of litter during the growth process, leading to increased input of C, N and P nutrients in the soil (Rose *et al.* 2019; Tao *et al.* 2017). Our result is supported by previous research results, and green manure crops can effectively increase soil organic matter content, with varying effects based on soil depth and green manure type (Ma *et al.* 2021a). Compared with conventional crops, green manure crops reduce soil N loss by preventing the downward infiltration of soil runoff and rainwater (Rose *et al.* 2019). Additionally, leguminous green manure fixes N from the air through its roots, enriching the rhizosphere with N and increasing soil N content (Couëdel *et al.* 2018; Liang *et al.* 2022). The dual benefit makes green manure a sustainable agricultural practice for soil protection and nutrient management. Soil P, particularly available P, is one of the most easily fixed chemical nutrients in the soil, and its availability is an important factor restricting the grain yield of farmland crops (Hou *et al.* 2015; Marklein and Houlton 2012). Recent studies have shown that the rhizosphere effect of green manure crops helps promote the transformation of ineffective P into available P, enhancing soil P availability (Zhang *et al.* 2022b). Our research also indicates a significant increase in soil C, N and P contents under leguminous green manure influence, suggesting the potential of green manure–crop rotation to reduce chemical fertilizer use in crop growth.

Response of soil enzyme activities under green manure–crops rotation

Soil extracellular enzyme activity is a critical indicator for evaluating soil quality and plays a significant role in soil organic matter transformation and nutrient cycling (Liu *et al.* 2017). We focused on the impact of leguminous green manure–crop rotation on soil enzyme activities. Compared with the control treatment, leguminous green manure–crop rotation significantly increased the activity

of extracellular enzymes involved in C, N and P cycles (0–20 cm). Specifically, the leguminous green manure–crop rotation increased the activities of BG and AP in farmland soil by an average of 20.1% and 36.4%, respectively, showing significant differences compared with the single crop planting mode. The increase in our results may be due to the release of various organic substances, such as rhizosphere exudates, from leguminous green manure crops through their developed roots, which are rich in carbon sources, significantly affecting the composition of microbial communities in soil and the activities of related extracellular enzymes (Li *et al.* 2019). In turn, it stimulates the secretion of specific enzymes in the soil, promoting the degradation of organic matter and the release of nutrients (Piotrowska and Wilczewski 2012). Additionally, the humus–protein complex formed by the combination of soil organic matter and enzymes interacts well with soil enzymes (Allison and Jastrow 2006). Pearson correlation analysis demonstrated a positive correlation between enzyme activities and soil TC and TN (Fig. 7). These results suggest that chemical properties, especially soil C and N content, influence the change in soil enzyme activities in the soil of green manure–crop rotation, which is a crucial factor affecting farmland productivity (Amede *et al.* 2021; Li *et al.* 2023). The calculation revealed a close relationship between MAP and MAT with the enzyme activities involved in soil nitrogen and phosphorus cycles.

The nitrogen-to-phosphorus ratio of soil microbes and their nutrient requirements play a crucial role in influencing the health of soil ecosystems and nutrient cycling. It has been observed that the collaboration between leguminous plants and N-fixing bacteria in symbiotic root nodules can convert atmospheric N into organic N in the soil, thereby increasing soil N content. However, the increase in soil N may affect the extracellular enzyme activities involved in N cycling (Tang *et al.* 2014). Our conclusion seems to verify the view: compared with the control treatment, green manure–crops rotation can improve the activity of NAG + LAP in 0–20 cm soil layer across all regions, with significant differences observed only in NC (Fig. 4). These differences may be attributed to the specific soil conditions influencing the nutrient requirements of soil microbes. Our study highlights that soil microbial metabolic activity increases the production of relevant enzymes under nutrient limitations. Furthermore, the input of soil nutrients stimulates the production of soil extracellular enzymes, indicating the response of soil microbes

to external nutrient sources (Ramirez *et al.* 2012). Consistent with previous studies, we found that N enrichment may inhibit enzyme activity associated with soil microbial N cycling while increasing soil phosphatase activity, underscoring the regulatory role of N elements in soil microbial metabolism (Marklein and Houlton 2012). Therefore, in the green manure–crops rotation mode, careful consideration of P fertilizer application, in addition to N fertilization, is essential for promoting nitrogen–phosphorus balance in the soil and maintaining soil health and ecosystem equilibrium. The guidance is vital for sustainable soil management and agricultural production.

In the 0–20 cm soil layer, the activities of enzymes involved in C, N and P cycling were higher compared with the 20–50 cm soil layer. There was no significant difference in enzyme activities between BG and NAG + LAP in the 20–50 cm soil layer (Fig. 4). The reason for this may be that the roots and exudates of green manure crops are mainly distributed in the surface soil, while the soil nutrient migration is slow and the infiltration capacity of deep soil is weak. As a result, enzyme activities in the soil with a depth of 20–50 cm have no discernable change. Generally speaking, the effect of leguminous green manure on soil extracellular enzyme activity requires further attention.

Response of stoichiometric ratios under green manure–crops rotation

The stoichiometric ratio of soil extracellular enzymes is an effective means for evaluating the function and dynamic changes of the soil ecosystem. By monitoring the dynamic changes in the ratio, we can better understand changes in biochemical processes in soil and formulate more effective soil management strategies (Cui *et al.* 2018; Sinsabaugh *et al.* 2009, 2010). By considering the stoichiometric ratio of soil extracellular enzymes, we observed that farmland planted with green manure crops in our study effectively reduces the limitation of soil microorganisms on N and increases the limitation on P (Fig. 5). We then analyzed the VL and VA of soil extracellular enzymes, which supported the above conclusion (Fig. 6). The response may be attributed to changes in soil properties induced by leguminous green manure crops. Previous studies have demonstrated the significant influence of crop species and litter quality on soil enzyme activities and microbial community structure in farmland ecosystems (Yin *et al.* 2014).

Leguminous green manure crops introduce a substantial amount of exogenous N and organic matter into the soil through N fixation with rhizobia, decreasing the N demand of soil microorganisms and suppressing the secretion of N-cycling enzymes in the soil (Shi *et al.* 2021). It stimulated the demand of soil microorganisms for P, enhancing the activity of soil extracellular enzymes, particularly phosphatase (Johnson *et al.* 2010). The research suggests that cultivating leguminous crops enhances the soil N environment, stimulating the microbial demand for phosphorus and accelerating the mineralization of phosphorus-related organic matter. While it is expected to alleviate phosphorus limitations in the ecosystem, providing more available resources for soil phosphorus cycling, it is important to note that accelerated phosphorus mineralization may lead to the gradual depletion of soil P reserves in the long term (Zheng *et al.* 2023). Therefore, when evaluating the interaction of N and P elements in soil microbial activities, it is essential to comprehensively consider their long-term effects on soil nutrient cycling and ecosystem health. Such studies contribute to a more comprehensive understanding of nutrient dynamics, providing a scientific basis for sustainable soil management and ecosystem conservation.

Studies have shown that soil enzyme dynamics are primarily controlled by soil chemical properties rather than microbial abundance or potential activity (Tan *et al.* 2021). We confirmed the above hypothesis through correlation analysis. Soil $E_{C:N}$, $E_{C:P}$ and $E_{N:P}$ show significant interactions with soil TC and TN (Fig. 7), while soil VA and VL exhibit significant interactions with soil pH, TP, C:N and C:P. Our research also confirmed the stoichiometric relationship between soil nutrient content and microbial nutrient demand, directly impacting microbial community structure, enzyme activities and nutrient release patterns during organic matter decomposition (Xu *et al.* 2023). Experimental results indicate that soil extracellular enzyme activities play a significant role in regulating soil organic matter degradation and nutrient utilization efficiency. The stoichiometric relationship of soil extracellular enzymes is crucial for predicting dynamic processes of organic matter decomposition in soil ecosystems. Our research provides valuable insights into the interactions between soil microorganisms and organic matter, serving as a scientific basis for optimizing soil nutrient management and ecosystem maintenance in the green manure–crop rotation mode.

CONCLUSIONS

Our study emphasizes the importance of considering the stoichiometric relationship between soil nutrients and microorganisms in sustainable soil management. We have drawn two significant conclusions. First, the long-term green manure–crop rotation promoted the accumulation of C and N in farmland, leading to a 20.1% increase in BG activity and a 36.4% increase in AP activity on average. Second, in the farmland ecosystem of China, the soil microbial communities were found to be co-limited by N and P rather than C in terms of nutrient competition. The soil eco-enzyme stoichiometry plays a key role in regulating the N and P limitations of microbial communities, indicating that the long-term rotation has alleviated soil N limitations while increasing soil P limitations. Therefore, we suggest increasing the application of phosphate fertilizer during the growth period, although the specific dosage requires further investigation. In summary, the green manure–crops rotation shows great potential in alleviating soil acidification, enhancing the accumulation of soil organic C and N, regulating soil enzyme activities and maintaining soil nutrient balance.

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Conflict of interest statement. The authors declare that they have no conflict of interest.

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