

Article

Alleviating Microbial Carbon Limitation in *Pinus armandii* Forests Through *Panax notoginseng* Cultivation

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Abstract: The cultivation of *Panax notoginseng* (Sanqi) within the *Pinus armandii* forest understory has been widely promoted in Yunnan, China. However, relatively little is known about how Sanqi cultivation influences microbial metabolic limitations and their driving factors in *P. armandii* ecosystems in terms of soil extracellular enzyme activity (EEA) and stoichiometry (EES). In this study, we established monoculture *P. armandii* (MPA) and Sanqi–*P. armandii* agroforestry (SPA) systems to investigate microbial resource limitations in *P. armandii* soils over 12 months (semi-monthly sampling). Sanqi cultivation decreased EEAs in *P. armandii* soils in the SPA system. Moreover, the vector length in both the bulk and rhizosphere soils of *P. armandii* decreased significantly from 1.31 to 1.12 and 1.29 to 1.21, respectively, indicating a decrease in the microbial C limitation of *P. armandii* soils. A vector angle < 45° in both systems revealed that N, rather than P, predominantly restricted microbial metabolism. The most influential factors affecting vector length and angle were Sanqi cultivation and seasonal dynamics. Structural equation modelling (SEM) revealed that fungi-to-bacteria ratios and soil chemical properties were direct factors positively affecting vector length. Overall, our findings suggest that Sanqi cultivation benefited soil microorganisms in *P. armandii* soils and should be encouraged to supply N to promote the sustainable development of *P. armandii*.

Keywords: *Pinus armandii*; Sanqi cultivation; enzymatic stoichiometry; fungi-to-bacteria ratios; metabolism; microbial limitation



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1. Introduction

Soil extracellular enzymes secreted by microbes catalyse the decomposition of soil organic matter (SOM), releasing energy and mineral nutrients for plant and microorganismal growth [1]. This process signifies an equilibrium between nutrient and energy supply and demand [2], with extracellular enzyme activity (EEA) serving as a robust indicator of microbial metabolic activity and soil quality [3]. C-acquiring enzymes, including xylosidase (XYL), β -1,4 glucosidase (BG), α -glucosidase (AG), and cellulosic disaccharide hydrolase (CBH), break down complex carbohydrates into simpler sugar molecules, thereby enhancing the uptake and utilisation of carbon by plants. Leucine aminopeptidase (LAP) and β -1,4-N-acetylaminoglucosidase (NAG) primarily degrade proteins and polysaccharides, providing a nitrogen source for microorganisms by releasing amino acids and

N-acetylglucosamine. Acid phosphatase (AP) is chiefly involved in the phosphorus cycle, catalysing the hydrolysis of organic phosphoric acid compounds to release phosphorus [4]. Thus, the ratios of soil hydrolytic enzyme activities can serve as quantitative indicators of microbial resource allocation to carbon, nitrogen, and phosphorus acquisition, as well as soil organic carbon stability [5]. Soil ecoenzymatic stoichiometry (EES) reflects the restriction of soil microbial energy and nutrient resources compared with soil extracellular enzyme chemometrics on a global scale (C:N:P = 1:1:1) [6] or vector length and angle values [7–9]. Numerous factors, including introducing plants [10], seasonal dynamics [11], and soil compartments [12] modulate biological and abiotic factors, thereby affecting soil EEA and EES. Therefore, understanding the relationships among environmental (abiotic) factors, biological factors, EEA, and EES is pivotal for elucidating the investment priority of resource acquisition [13], and providing a theoretical basis for production practice [14], plant growth [15], productivity [16], soil characteristics [17], and responses to climate change in terrestrial ecosystems.

Sustainable development land, agroforestry systems that organically combine agricultural plants (introduced) with forestry plants (in situ), have a strong influence on soil EEAs [18–20]. Previous studies have demonstrated that plant species introductions either have positive, negative, or no effects on EEA related to C, N, and P in plants in situ. For example, after introducing plants, the soil EEA of in situ plants such as kiwifruit (kiwifruit/alfalfa) (*Actinidia chinensis*/*Medicago sativa* L.) [21], apple (apple/corn) [22], hickory (hickory/peony or kula clover) (*Carya cathayensis* Sarg./*Paenonia* L. or *Trifolium* L.) [23,24], and peach (peach/morel) (*Amygdalus persica* Linn./*Morchella esculenta* L.) [25] increased significantly, but the degree of EEA increase involved in C, N, and P was inconsistent. In contrast, C-, N-, and P-acquiring enzyme activities in alfalfa (alfalfa/poplar) (*Medicago sativa* L./*Populus tomentosa* Carr.), coffee (*Coffea arabica* Linn.) (coffee/thorn), and pear (pear/konjac) (*Pyrus* L./*Amorphophallus konjac* K. Koch) soils were significantly reduced or unchanged by the introduced plants [20,26,27]. In addition, changes in enzyme activities related to C, N, and P vary (increases, decreases, or no changes) in soils with different plants [28–30]. This variability can be attributed to differences in the soil extracellular enzyme species [31], introduced and in situ plant species [24], and environmental factors [20]. In summary, a deep understanding of how introduced plants cause changes in the soil EEA of in situ plants is crucial for evaluating the sustainable development of agroforestry systems.

Seasonal variations (e.g., temperature and precipitation) significantly influence biotic and abiotic factors [32–35], which further lead to alterations in soil EEA and EES [36,37]. Seasonal changes can alter soil microbial biomass and the quality and quantity of soil input by plants, thereby affecting EEA and EES. For example, EEAs are usually higher in summer than in other seasons because of the higher microbial biomass [11,34,36]. Moreover, changes in the quality and quantity of soil input by plants (e.g., lignin-to-nitrogen ratio) affect microbial nutrient utilisation efficiency, leading to changes in EEA and EES [2,13]. In addition, seasonal variations in temperature and rainfall cause variations in edaphic factors, microbial growth, and available soil nutrients, which can directly or indirectly affect EEA and EES [35,37–39]. Although distinguishing the individual effects of seasonal dynamics, plants, and the soil environment on EEA is challenging, a deeper understanding of the relationship between EEA/EES and biological/abiotic factors under different seasonal conditions [4,35] is beneficial for elucidating microbial responses to climate change [40].

Different soil compartments (rhizosphere and bulk) significantly affect soil EEAs and EES [12,41], and rhizosphere rather than bulk soils, had higher soil enzyme activities [12,42]. On the one hand, rhizosphere soil exhibits higher microbial activity and more frequent nutrient flow [12]. In contrast, soil physicochemical properties are influenced by plant

root growth, further altering the rhizosphere soil microbial activity [8]. Furthermore, root-derived carbon compounds [43], mainly derived from root secretions, stimulate microbial activity, leading to higher rhizosphere soil enzyme activities [41]. Plant root biomass also increases rhizosphere soil enzyme activities (proteins and gibberellins) to address N limitations [44]. As a physiological hub of the arable crops in agroforestry systems, the rhizosphere significantly affects soil microorganisms, root exudates, soil physicochemical properties, and plant root biomass [41,44,45]. Nevertheless, the relationship between the trees and arable crops in agroforestry systems is more intricate [18], with nutrient competition among different plants [46], the mutual transmission of soil microorganisms [47], and the diffusion of root exudates [48] significantly impacting EEA. Therefore, an in-depth analysis of the changes in EEA/EES in different soil compartments can reveal the nutrient requirements, acquisition strategies, and metabolic models of microbes in various soils [8,10,12], providing a basis for establishing a rational agroforestry system.

The long-term monoculture management of pine forests can trigger ecological issues such as soil degradation, biodiversity loss, and environmental disturbance [49,50]. Conversely, the SPA system, as sustainable land-use management [47], has been widely adopted in the Yunnan province, covering approximately 666.67 ha [51]. Sanqi is a rare herbal medicine, which belongs to the family Araliaceae [52,53]. Simulative habitat cultivation involves simulating the original habitat and site environments of Chinese medicinal plants to preserve their quality [54]. The simulative cultivation of organic Sanqi in *P. armandii* forests has been shown to improve Sanqi quality, prevent obstacles to continuous cropping, and increase the economic benefits for producers [51–53]. However, attention should be given not only to Sanqi but also to pine trees in the SPA system. This is because pine forests have been deficient in carbon, and the organically managed Sanqi with no application of pesticides and fertilisers potentially affects the carbon demand in the SPA agroforestry system [51]. Reducing microbial carbon restriction can enhance soil organic carbon accumulation [35], bolster ecosystem stability and resilience, improve soil fertility and structure, mitigate climate change [13], and foster the sustainable development of agricultural systems. Consequently, investigating the impact of Sanqi cultivation on microbial carbon restriction in pine forests holds not only practical application value for local regions but also offers theoretical significance and practical guidance for other agroforestry ecosystems globally. By illustrating how to optimize ecosystem functions through plant–microbial interactions, it presents innovative concepts for the design and sustainable advancement of agroforestry ecosystems on a worldwide scale.

Previous studies have primarily focused on the planting methods [51], quality analysis [55], and pest control [51] of Sanqi. However, less attention has been paid to the effects of Sanqi planting on Pinus soil quality, particularly on the soil EEA. Therefore, we designed monoculture *P. armandii* (MPA) and Sanqi–*P. armandii* agroforestry (SPA) systems to compare and analyse the changes in EEA (CBH, AG, XYL, BG, NAG, LAP, and AP) and EES in *P. armandii* soils. Furthermore, we assessed the interactive effects of season, plant introduction, and soil compartments on EEA and EES in *P. armandii* soils. We also conducted a one-year multi-frequency (twice per month) sampling to accurately analyse the nutrient limitations of soil microorganisms in different *P. armandii* systems. We tested the following hypotheses: (1) Sanqi planting significantly reduces the coenzymatic C:N and C:P and vector length and alleviates the C limitations of microbial resources in *P. armandii* soils; and (2) plant introduction, seasons, and soil compartments (rhizosphere/bulk) exert differential effects on EEA and EES in *P. armandii* soils.

2. Materials and Methods

2.1. Study Area

The base of Sanqi cultivation is mainly located in Xundian Hui and Yi autonomous county, Kunming city, Yunnan province (103.25° E, 25.55° N, 2247.78 m a.s.l.), with an average annual temperature of 14.7 °C and precipitation of 1624.0 mm. This region experiences a subtropical plateau monsoon climate with distinct dry (November–April) and rainy seasons (May–October). The forest soil was mountainous red soil, and the vegetation was dominated by *P. armandii*, with an average diameter at a breast height of 18 cm and an average tree height of 9.5 m.

2.2. Experimental Design and Sampling

The MPA and SPA systems were established under similar conditions, including vegetation, soil types, aspects, and angles. The planting of Sanqi involved several steps. First, weeds and shrubs in the forest understory were removed and *P. armandii* branches were trimmed up to a height of ~3 m. Second, the soils (0–20 cm) were ploughed deeply 2–3 times using a small rotary tiller. Moreover, a soil bed was set up along the contour line of the *P. armandii* forest (10–20 m, 1–1.5 m, and 0.3–0.4 m in length, width, and height), and a drainage outlet was left between two adjacent plots (1.5–2.0 m). Subsequently, 1 year old Sanqi seedlings were transplanted into the soil bed with intra- and inter-row spacings of 10 and 15 cm, respectively, and covered with *P. armandii* needles (3–5 cm). In addition, a circular arched rain shelter was built to prevent water accumulation during the rainy season, drip pipes were installed for irrigation, and no pesticides, fertilisers, or artificial rodent control measures were applied (Figure 1).



Figure 1. Monoculture *P. armandii* (MPA) and Sanqi–*P. armandii* agroforestry (SPA) systems.

Six plots of 10 m × 10 m were randomly set up in both the MPA and SPA system. Sanqi was planted in January 2020, but due to the influence of COVID-19, 288 soil samples were collected from 10 September 2020 to 20 August 2021, including two soil compartments (rhizosphere and bulk) × 2 systems × 12 months × 2 times/month × 3 replicates. Semi-monthly sampling was applied to accurately analyse the seasonal dynamic of EEA and EES. The soil sampling method used the 5-point method, in which three soil samples were collected at each point and mixed as a composite sample. Soils attached to the root surface at a thickness of 0–2 mm and 20 cm away from the *P. armandii* tree (depth of 0–20 cm) were considered as the rhizosphere and bulk soil, respectively. Soil samples were stored in self-sealing bags and returned to the laboratory in liquid nitrogen. After removing

litter and impurities from the soil sample, the soil was sieved through a 4 mm sieve. The processed soil samples were separated into two subsamples and stored at 4 °C and −80 °C for subsequent analyses of soil EEA, edaphic factors, and microbes, respectively.

2.3. Analysis of Edaphic Factors and Soil EEA

Fresh soils underwent oven-drying at 100 °C for 24 h to determine the water content (WC) by measuring the weight variation. A pH meter was used to determine the soil pH (soil/water = 1:5). After the soils were treated (H_2SO_4 digestion and KCl extraction), the total nitrogen (TN), total phosphorus (TP), nitrate nitrogen ($\text{NO}_3^- - \text{N}$), and ammonium nitrogen ($\text{NH}_4^+ - \text{N}$) were determined using a flow analyser (SEAL Analytical AA3, Mequon, WI, USA). Soil organic carbon (SOC) was measured using the $\text{K}_2\text{Cr}_2\text{O}_7$ oxidation method. The total potassium (TK) was measured using atomic emission spectrometry and an AA-6300C flame photometer (Shimadzu Corporation, Kyoto, Japan).

Four C-acquiring enzymes (xylosidase, XYL; β -1,4 glucosidase, BG; α -glucosidase, AG; cellulosic disaccharide hydrolase, CBH), two N-acquiring enzymes (leucine aminopeptidase, LAP; β -1,4-N-acetylaminoglucosidase, NAG), and one P-acquiring enzyme (acid phosphatase, AP) were assayed using fluorometric protocols. Briefly, 4-methylumbelliferone (MUB) and 7-amino-4-methylcoumarin (MUC) solutions with varying concentrations (0, 2.5, 5, 10, 25, 50, and 100 μM , 150 μL of each concentration) were prepared and added to enzyme-labelled plates. A standard curve was generated after incubating the standard solutions in the dark at 25 °C for 3 h. Furthermore, 1 g of fresh soil was mixed with 125 mL of acetate buffer (50 mM, pH 5.0) in a 250 mL conical flask and stirred thoroughly with a magnetic stirrer. Subsequently, 150 μL of the suspension was pipetted to a black polystyrene 96-well microtiter plate, to which an enzyme–substrate solution (50 μL) was added. After incubation, the catalytic reaction was terminated by adding 10 μL of NaOH solution (1 M, termination solution) to each well. Finally, fluorescence intensity was determined using a microplate reader (Tecan Infinite M200, Salzburg, Austria) at excitation and emission wavelengths of 365 and 450 nm, respectively. Eight replicates were analysed from each soil sample.

2.4. DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR) Assays

Total DNA was extracted from soil samples (0.5 g) according to the instructions of the Qiagen DNeasy PowerSoil Kit (MP Biomedicals, Solon, CA, USA), and DNA quality and concentration were assessed using agarose gel electrophoresis and a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). Absolute quantification of bacterial and fungal genes was carried out on a LightCycler 480 II system (Roche, Basel, Switzerland), with the amplification primers (338F/806R and ITS1F/ITS2R) shown in Table S1 [47,51]. The PCR reaction system totalled 20 μL and consisted of 1 μL of DNA, 0.5 μL of forward and reverse primers, 10 μL of TB Green Premix Ex Taq (TaKaRa, Kyoto, Japan), and 8 μL of ddH_2O . Standard curves were generated after PCR reaction using a tenfold gradient dilution of plasmid DNA concentration (104.1 ng/ μL for bacteria and 130.6 ng/ μL for fungi). The amplification efficiencies and slopes for bacterial and fungal genes were 93.43%/95.30% ($R^2 > 0.991$) and $-3.49/-3.44$, respectively. All samples were analysed in triplicate for qPCR.

2.5. Determination of Soil EEA

$\text{Ln}(\text{CBH}+\text{BG}+\text{XYL}+\text{AG}):\text{Ln}(\text{NAG}+\text{LAP})$, $\text{Ln}(\text{CBH}+\text{BG}+\text{XYL}+\text{AG}):\text{Ln}(\text{AP})$, and $\text{Ln}(\text{NAG}+\text{LAP}):\text{Ln}(\text{AP})$ denote the ecoenzymatic C:N, C:P, and N:P ratios, respectively. Moreover, the vector length and angle of the soil EEA quantify nutrient limitations for soil microorganisms in ecosystems [44]. Specifically, the vector length signifies the carbon limitation of microbial metabolism, with a longer length indicating stronger carbon limitation [56].

The vector angle represents the nitrogen or phosphorus limitation of microbial metabolism, with a vector angle greater or less than 45° indicating significant P and N limitations, respectively. The vector length and angle were calculated as follows:

$$\text{Vector length (unitless)} = \text{SQRT}(x^2 + y^2) \quad (1)$$

$$\text{Vector angle (degrees)} = \text{Degrees} [\text{Atan2}(x,y)] \quad (2)$$

where x denotes the EES related to C, N ($\text{Ln}(\text{CBH} + \text{BG} + \text{XYL} + \text{AG}) : \text{Ln}(\text{AP})$), y denotes the EES related to P ($\text{Ln}(\text{CBH} + \text{BG} + \text{XYL} + \text{AG}) : \text{Ln}(\text{NAG} + \text{LAP})$), and Atan2 denotes the four-quadrant inverse tangent ($\tan^{-1}(y/x)$), which serves as the reference angle for the positive x-axis of all plots.

2.6. Statistical Analysis

One- and two-way ANOVA, correlation analysis and principal effects analysis were performed using SPSS 25.0. Each ANOVA used LSD and Duncan for multiple post-hoc testing. Graphing and redundancy analyses (RDA) were performed using GraphPad Prism 10 and Canoco 5, respectively. In addition, structural equation modelling (SEM) analysis was carried out using IBM SPSS Amos v28.0.0 to determine the direct and indirect impacts of edaphic factors, fungal gene abundance, bacterial gene abundance, and fungi-to-bacteria (F:B) ratios on microbial metabolic restriction. Each structural model for the overall goodness of fit analysis was assessed using the chi-square (χ^2) test, distribution, degrees of freedom (df), p -values, approximate root mean square error (RMSEA), and goodness of fit index (GFI).

3. Results

3.1. Soil Physicochemical Properties

Sanqi cultivation dramatically affected various *P. armandii* soil properties. Specifically, fungal gene abundance and SOC, TN, TP, and TK, and soil C:P and N:P values decreased significantly (ANOVA, $p < 0.05$) in both the rhizosphere and bulk soils of *P. armandii*. Conversely, the NO_3^- –N contents, pH, WC, and soil C:N increased, whereas bacterial gene abundance, ST, and F:B content did not show significant differences. In the monoculture *P. armandii* system, rhizosphere soils had a higher SOC, whereas they had a lower NH_4^+ –N content and WC compared to bulk soil. In the Sanqi–*P. armandii* agroforestry system, the SOC, NH_4^+ –N, NO_3^- –N, and TN contents and soil C:N and C:P ratios in rhizospheric soil were higher than those in bulk soil, whereas the WC and pH values in rhizospheric soil were lower than those in bulk soil (Table S2 and Figure S1).

3.2. Soil EEA in the MPA and SPA Systems

The planting of Sanqi caused a decrease in EEA (NAG, BG, CBH, AP, XYL, and AG) in *P. armandii* soil in the SPA system compared to the MPA system, while LAP activity remained unchanged. Moreover, except for the highest LAP activity that was observed in autumn, all EEAs were higher in spring and summer than in autumn and winter. In addition, there was no difference in extracellular enzyme activity between *P. armandii* soils in the MPA and SPA systems for either the rhizosphere or bulk soils (Figures 2 and S2).

3.3. Variation in EES and EEA in the MPA and SPA Systems

Compared to the monoculture *P. armandii* system, the ecoenzymatic C:N and C:P ratios decreased significantly in the Sanqi–*P. armandii* system, whereas the ecoenzymatic N:P ratio remained unchanged. Moreover, the ecoenzymatic C:N ratio was consistently lower in autumn across all treatments than in the other seasons, whereas the enzymatic C:P

ratio varied seasonally. The eco-enzymatic N:P ratio was lowest in summer and highest in autumn. In addition, the cultivation of Sanqi resulted in a significant decrease in the C:N and C:P ratios in the bulk soils rather than in the rhizosphere soils of *P. armandii*, whereas the N:P ratio increased dramatically in the bulk soil of *P. armandii* (Figure 3). Notably, all treatments exhibited C:N and C:P ratios of less than one, whereas the N:P ratios were greater than one (Figure 3). The C:N:P ratios of rhizospheric and bulk soils in the MPA system were 1:1.10:1.06 and 1:1.11:1.09, respectively, whereas those in the SPA system were 1:1.30:1.24 and 1:1.17:1.17, respectively (Table S3). Compared to the monoculture *P. armandii* system, the vector length decreased in the Sanqi–*P. armandii* system, whereas the vector angle remained unchanged, and both the vector length and angle were lowest in autumn. Moreover, there were no differences between the vector lengths and angles of the rhizosphere and bulk soils in the MPA system, whereas planting in Sanqi led to a significant decrease in the vector lengths and angles of the rhizosphere and bulk soils in autumn (Figure 3). These results indicate that the planting of Sanqi can alleviate carbon limitation, with the strongest effect observed in autumn. In the different systems, P was significantly restricted in summer, whereas N was significantly restricted in spring, autumn, and winter, with the strongest restriction observed in autumn (Table S4).

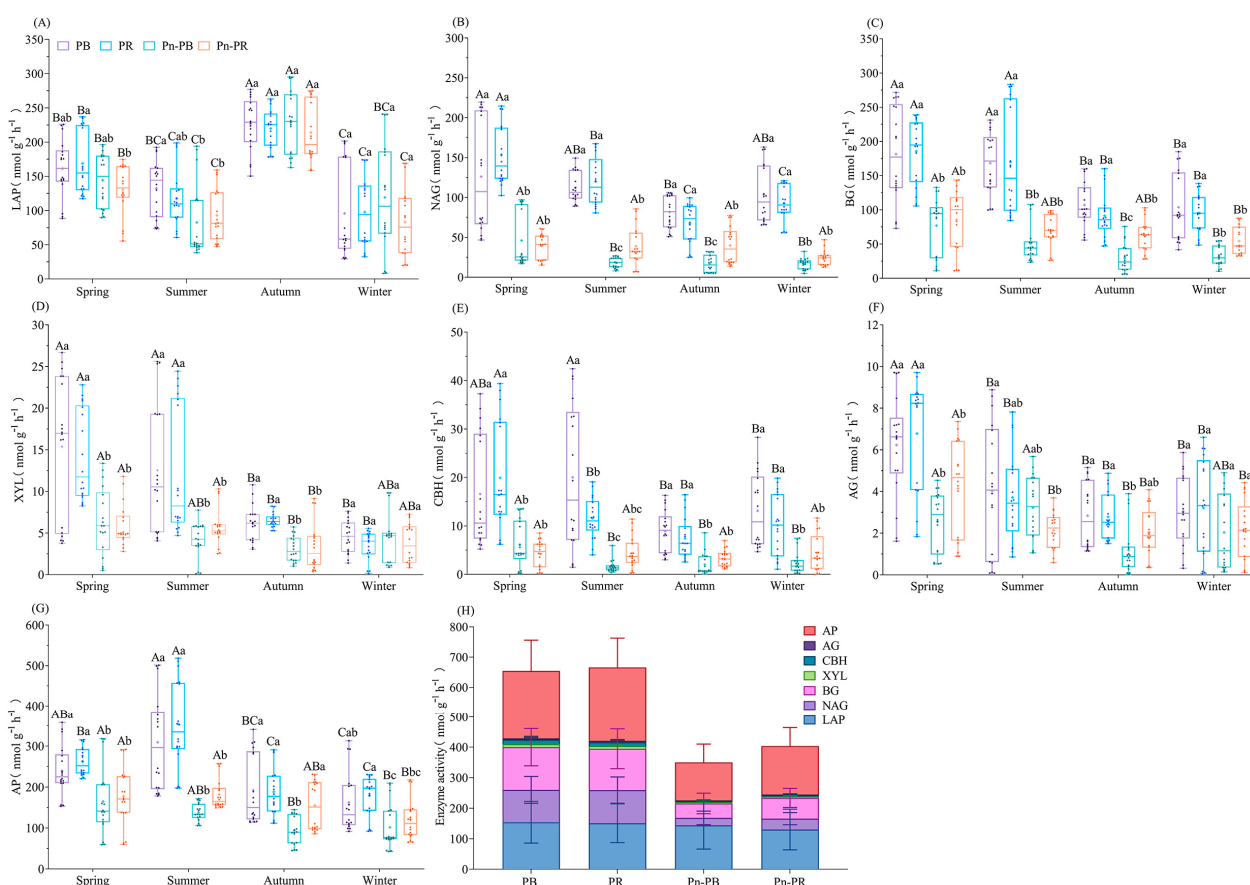


Figure 2. Seasonal dynamics of soil EEAs under different *P. armandii* systems. PB and PR: the bulk and rhizosphere soils of *P. armandii* in the MPA system; Pn-PB and Pn-PR: the bulk and rhizosphere soils of *P. armandii* in the SPA system. Different upper- and lower-case letters denote significant differences at $p < 0.05$. (A–G) the activity of leucine aminopeptidase (LAP), β -1,4-N-acetylaminoglucosidase (NAG), β -1,4 glucosidase (BG), xylosidase (XYL), cellulosic disaccharide hydrolase (CBH), α -glucosidase (AG), acid phosphatase (AP) under different seasons, respectively. (H) the activity of seven extracellular enzyme under different treatments.

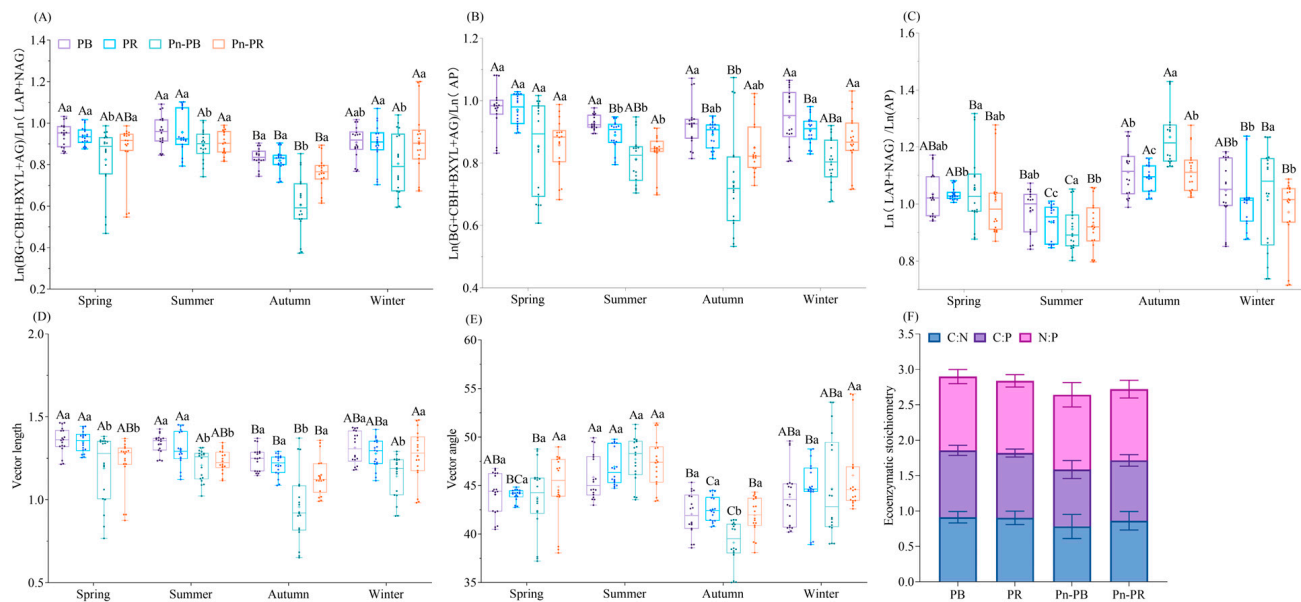


Figure 3. Seasonal dynamics of enzymatic ratios and vector length/angle in the *P. armandii* soils under the MPA and SPA systems. Different upper- and lower-case letters denote significant differences at $p < 0.05$. (A) extracellular enzyme C (XYL+BG+AG+CBH)/N (LAP+NAG). (B) extracellular enzyme C (XYL+BG+AG+CBH)/P (AP). (C) extracellular enzyme N (LAP+NAG)/P (AP). (D,E) vector length and vector angle of extracellular enzyme under different seasons. (F) eco-enzymatic stoichiometry under different treatments.

3.4. Influence of Environmental Factors on EEA and EES

Correlation analysis revealed significant positive correlations between SOC, TN, TP, TK, and fungal abundance and the activities of seven soil EEAs, as well as enzymatic C:N and C:P ratios. Moreover, $\text{NO}_3^- - \text{N}$, and ST exhibited significant negative and positive correlations with the economic N:P ratio, respectively. In addition, vector length displayed a notable positive correlation with SOC, TN, TK, and fungal gene abundance but a negative correlation with $\text{NO}_3^- - \text{N}$, WC, and pH. Vector angle exhibited a dramatically positive correlation with ST and a negative correlation with $\text{NH}_4^+ - \text{N}$ and TK (Table S5).

The RDA results show that the factors influencing soil EEA and EES varied among the different *P. armandii* systems. ST, TK, pH, WC, and soil C:P ratio were the most significant factors in the MPA system, whereas $\text{NO}_3^- - \text{N}$, ST, F:B, $\text{NH}_4^+ - \text{N}$, and soil C:P were the most crucial factors in the agroforestry system (Figure 4, Table S6).

3.5. The Main Effect Analysis of EES

The main effect analysis evaluated the effects of plant introduction (planting), seasons, and soil compartments (rhizosphere and bulk) on soil EES (Figure 5, Table S7). We found that plant introduction, seasons, and soil compartments were important factors affecting soil EES, vector length, and vector angle. However, the degree of influence of these factors on EES was inconsistent. Notably, the season emerged as the strongest factor affecting C:N and N:P ratios ($\eta^2 = 0.296$, $p < 0.001$; $\eta^2 = 0.35$, $p < 0.001$, respectively), while plant introduction exerted the greatest impact on C:P ratio ($\eta^2 = 0.275$, $p < 0.001$). In addition, the factors affecting the vector length ranked in the following order: plant introduction ($\eta^2 = 0.248$, $p < 0.001$) > season ($\eta^2 = 0.218$, $p < 0.001$) > soil compartment ($\eta^2 = 0.017$, $p < 0.05$). Those affecting the vector angle ranked in the following order: season ($\eta^2 = 0.343$, $p < 0.001$) > soil compartment ($\eta^2 = 0.027$, $p < 0.01$) > plant introduction ($\eta^2 = 0.001$, $p > 0.05$) (Figure 5).

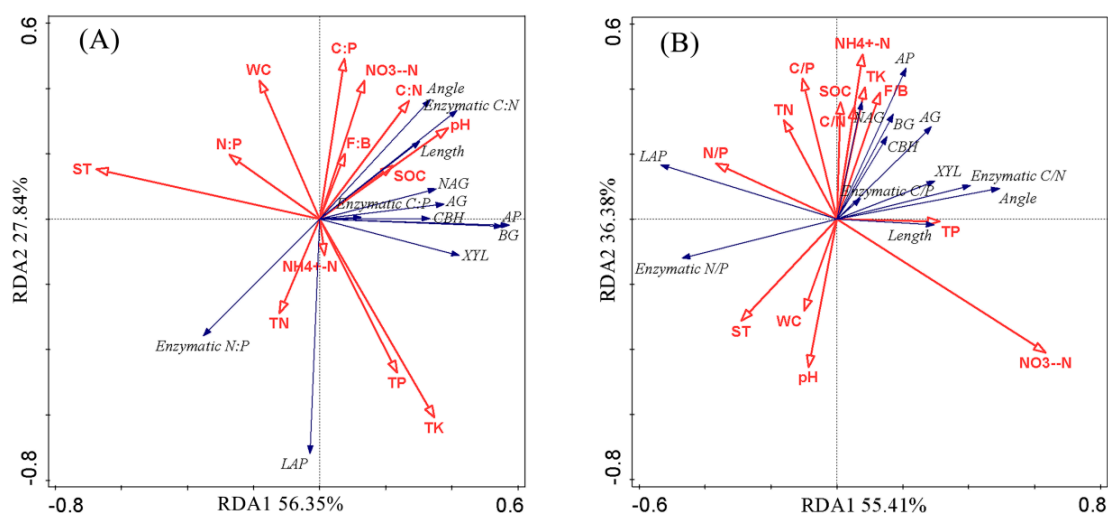


Figure 4. Redundancy analysis for the relationship between soil EEAs/EES and edaphic factors under the MPA (A) and SPA (B) systems.

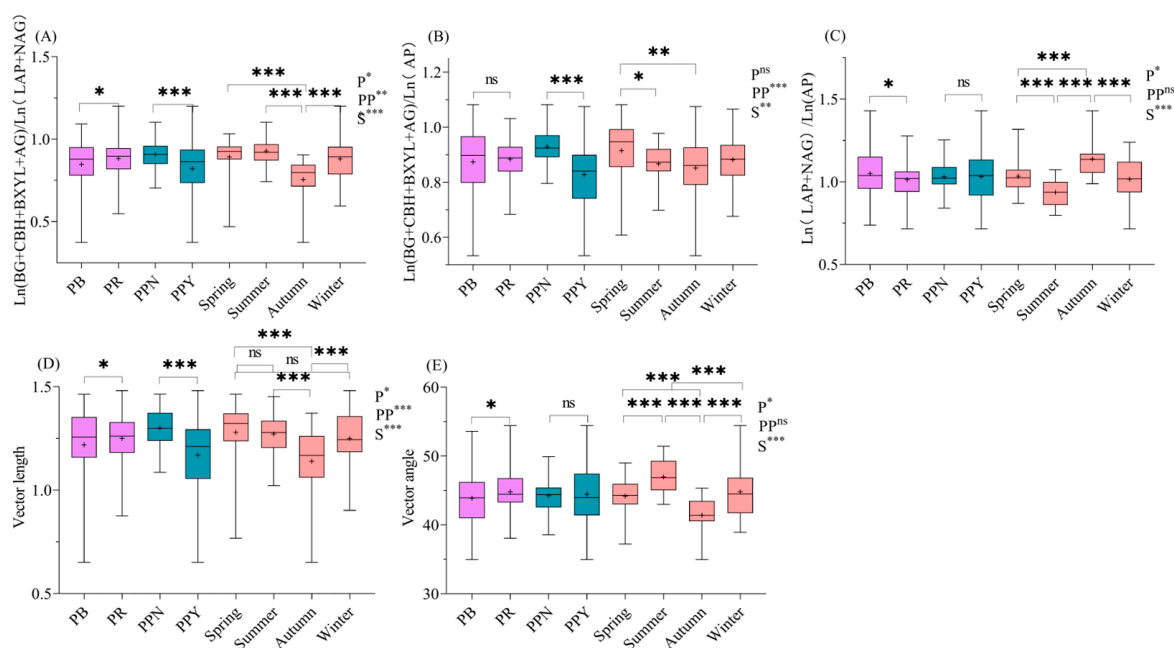


Figure 5. Main effect analysis of soil EES (A–C), vector length (D), and angle (E). P, PP, and S represent soil compartments factors, planting factors, and seasonal factors, respectively. PB and PR: *P. armandii* bulk or rhizosphere soils, respectively; PPY and PPN: *P. armandii* soils with or without Sanqi cultivation, respectively. “*”, “**”, and “***” indicate statistically significant differences at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively, and “ns” denotes no statistical difference.

3.6. Structural Equation Modelling (SEM) Analysis in the MPA and SPA Systems

SEM explained the direct and indirect effects of the soil microenvironment, soil chemical properties, fungal and bacterial gene abundance, and F:B ratios on vector length and angle (Figure 6; Table S8). In both the monoculture *P. armandii* and Sanqi-*P. armandii* systems, F:B ratios and soil chemical properties as direct factors positively affected vector length, whereas bacterial and fungal abundances served as indirect factors that significantly affected vector length. Moreover, vector length, and soil microenvironment as direct factors had positive and negative effects on the vector angle, respectively. In addition, the chemical properties of SPA, rather than the MPA system, displayed a negative effect on the vector angle as a direct factor.

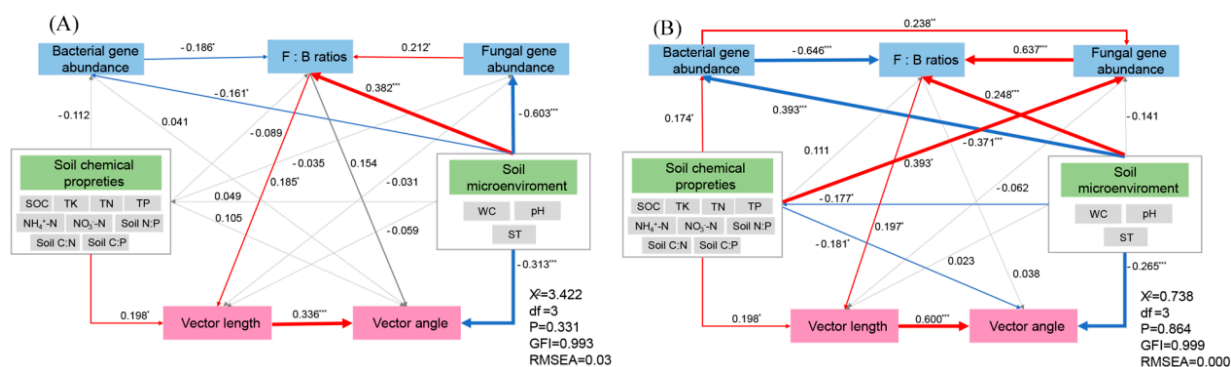


Figure 6. Analysis of the SEM paths under the MPA (A) and SPA (B) systems. The colour and width of the arrows denote the negative (blue) and positive (red) relationships and the coefficient strength, respectively. *, **, and *** indicate $p < 0.05$, 0.01 , and 0.001 levels, respectively.

4. Discussion

The effects of introduced plant species in agroforestry systems on the activities of extracellular enzymes involved in C, N, and P in plant soils in situ have been inconsistent [20,21,27]. In the Sanqi–*P. armandii* agroforestry system, the cultivation of Sanqi resulted in a decrease in the EEA related to C, N, and P in *P. armandii* soils. Our findings diverged from those observed in the kiwi/alfalfa and apple/corn agroforestry systems [21,22] yet aligned with the outcomes in the alfalfa/poplar and coffee/bramble systems [20,26]. Additionally, the C:N and C:P ratios decreased, whereas the N:P ratio increased in bulk soils compared to rhizosphere soils. Furthermore, the EES (C:N, C:P, N:P, and C:N:P) in both the rhizosphere and bulk soils did not deviate from global scale EES benchmarks (C:N = 1.41; C:P = 0.62; N:P = 0.44; C:N:P = 1:1:1) [57]. In addition, vector lengths were significantly reduced in the SPA system, indicating that planting Sanqi could alleviate C limitation in *P. armandii* soils. This evidence supports our first hypothesis. Notably, the vector angles across the different systems were all less than 45° , suggesting an overall N limitation. Previous studies have demonstrated that a reduction in C limitation could potentially exacerbate N or P limitation in various terrestrial systems [13,58], owing to variations in climate and land-use conversion types, although this was not observed in our study. However, in agroforestry systems, plant introduction [10], seasonal changes [11], and soil compartments [12] can alter both biotic and abiotic factors such as soil microbes [25,58,59] and soil physicochemical properties [10,20,24,54], thereby influencing EEA and EES. These factors support our second hypothesis and significantly affect the C limitations in *P. armandii* soils within agroforestry systems.

Introducing plants has been identified as a significant factor influencing the EES [60], largely because of their ability to alter soil physicochemical properties [10,20,54] and soil microbial community structures [60]. In our study, Sanqi planting significantly reduced vector length rather than vector angle, implying a reduction in C limitation rather than constraints related to N and P. Correlation analyses showed that SOC, TN, TK, and fungal abundance were positively correlated with EEA vector length, all of which were significantly lower in the *P. armandii* soils of the SPA system. Therefore, we posit that introducing Sanqi-induced alterations in soil physicochemical properties and microbial structure serves as the primary driver mitigating C limitation in *P. armandii* soils.

Previous studies have consistently demonstrated positive correlations among SOC, TN, TK, and EEA/EES [10,11], which is consistent with our findings. The reduction in SOC, TN, and TK levels in soils could be attributed to nutrient competition between Sanqi and *P. armandii*, thereby diminishing C restrictions. However, soil tillage during Sanqi cultivation can influence soil physicochemical properties, reducing soil EES and vector

length [61,62], which may be the main reason for the alleviation of C restriction in the SPA system. Furthermore, pine soil in both the MP and SPA systems is limited by N (vector angle less than 45°), although the cultivation of Sanqi can marginally alleviate this N limitation in the SPA system (with no significant difference). On one hand, Sanqi has a substantial nitrogen requirement and competes with pine trees for this nutrient [63], thereby reducing the soil's nitrogen content. The application of organic fertiliser during Sanqi cultivation can partially mitigate nitrogen deficiency, which explains the slight reduction in nitrogen limitation in the SPA system's pine soil. On the other hand, subtropical pine forests are inherently nitrogen-limited (Luo et al., 2020), and the organic management of Sanqi without pesticides and fertilisers [64] can intensify the nitrogen demand within the SPA system. The decline in nitrogen typically elevates the C/N ratio and accelerates organic matter decomposition, while carbon decomposition proceeds more slowly, potentially leading to rapid nitrogen depletion in the soil [65]. Our findings also indicate that pine soils in both the MP and SPA systems are not restricted by P (vector angle less than 45°), likely due to Sanqi's relatively low P demand and the high background P content in pine forest soil, which suffices for both Sanqi and pine requirements. Consequently, we recommend increasing the input of organic fertiliser during Sanqi cultivation to mitigate microbial N restriction. In addition, fungal diversity is more susceptible to the influence of EES than bacterial diversity [59]. Introducing Sanqi alters the fungal community structure in *P. armandii* soil significantly, potentially transmitting certain fungi to pine-associated soil microorganisms [47]. There is a positive correlation between fungal abundance and vector length. Thus, we conclude that the reduction in SOC and N content in *P. armandii* soils following Sanqi cultivation affects the EES and further decreases fungal abundance. In addition, fungi can assist plants in acquiring soil nitrogen, while nitrogen serves as the primary factor influencing the assembly of fungal communities in both pine and Sanqi soils [47]. Consequently, the decline in soil nitrogen levels directly contributes to the reduction in fungal abundance.

Seasonal variations are pivotal in shaping soil enzyme dynamics, as acknowledged in biogeochemical models [15,35]. The soil EEA in both *P. armandii* systems was significantly higher in spring/summer than in autumn/winter, which is inconsistent with other research results indicating that EEA peaks during summer in *Quercus*, wetland, and *Betula platyphylla* soil [36,66,67]. Seasonal variations can influence abiotic factors, such as temperature and moisture, leading to differences in soil enzyme acquisition activities [32–35,37]. In particular, the soil water content demonstrated a significant negative correlation with EEA, likely because of the distinct alternation of drought and rainy seasons in subtropical forests, resulting in alternating changes in soil moisture. Concurrently, alterations in the quality and quantity of plant inputs to the soil affect the nutrient utilisation efficiency of microorganisms, consequently modulating EEA and EES [2,13]. Therefore, the absence of a well-defined growing season in subtropical forests, which results in increased plant inputs, has emerged as a primary factor influencing soil enzyme activity.

P. armandii soils in the SPA system experienced a significant reduction in C limitation (indicated by decreases in C:N and C:P ratios and vector length) and an increase in N limitation (evidenced by decreases in the C:N ratio, increases in the N:P ratio, and decreases in vector angle) during autumn, which was inconsistent with previous studies [34,37,67]. This discrepancy can be attributed to seasonal shifts in the biological factors affecting EEA and EES [2,11,13,34,36]. Correlation analyses revealed a positive association between vector length and fungal abundance, suggesting that reduced fungal abundance in *P. armandii* soils during autumn, possibly due to Sanqi planting, plays a crucial role in alleviating carbon restriction. Moreover, soil water content was negatively correlated with vector length, whereas temperature was positively correlated with vector angle. Soil water can

modulate soil extracellular enzyme activities by changing substrate diffusion rates (mainly C) and inhibitor levels [68], as well as the relative utilisation rates of different nutrient elements [69]. Furthermore, the soil temperature exerts substantial control over enzymatic activity, thereby affecting both EEA and EES [2,35,36,70]. Therefore, the observed alleviation of carbon limitations in autumn may be attributed to heavy rainfall and adequate water replenishment during Sanqi planting. In contrast, higher temperatures exacerbated N-limitation during this season. In addition, a strong interrelationship exists among the EEA related to C, N, and P in the soil; that is, restrictions on one element can affect the acquisition of another [13,71,72]. Therefore, the reduction in C limitation in autumn may exacerbate N limitation.

Compared to seasonal variations and plant introduction, the impact of the soil compartments on C:N, N:P, vector angle, and vector length was relatively minor (Figure 5). Rhizosphere soil exhibits higher soil enzyme activity [12,42], which is consistent with our findings. Moreover, compared to the rhizosphere soil, the bulk soil of *P. armandii* in agroforestry systems showed significant decreases in C:N, C:P, and vector length compared to the MPA system, indicating that the planting of Sanqi effectively reduced C limitation in bulk *P. armandii* soil. Our observations are consistent with those of a previous study, which noted a smaller C limit in *Pinus sylvestris* rhizosphere soil than in bulk soil [12]. In addition, the interplay of nutrient competition among different plants [46], the mutual transfer of soil microorganisms [47], and the diffusion of root exudates [48] could complicate the plant–soil–plant relationship in agroforestry systems [18], significantly affecting both rhizosphere and non-rhizosphere soils.

5. Conclusions

Our hypotheses regarding the effect of Sanqi planting on EEA in *P. armandii* soil in an agroforestry system were confirmed. The planting of Sanqi decreased the EEAs in *P. armandii* soil in the agroforestry system. However, the C:N:P ratios (1:1.30:1.24 and 1:1.17:1.17) did not significantly deviate from the global mean EES (1:1:1). After Sanqi cultivation, both the bulk and rhizosphere soils of *P. armandii* exhibited a significant decrease in vector length, indicating a reduction in microbial metabolic C limitations. Moreover, the vector angle $<45^\circ$ in *P. armandii* soils under both systems revealed that microbial metabolism was primarily restricted by N rather than P. Furthermore, the strongest factors affecting the vector length and vector angle were plant introduction ($\eta^2 = 0.248$, $p < 0.001$) and season ($\eta^2 = 0.343$, $p < 0.001$), respectively. Structural equation modelling (SEM) indicated that F:B (fungi/bacteria) ratios and soil chemical properties as direct factors positively influenced vector length. Therefore, we recommend increasing the application of organic fertiliser in the management of Sanqi cultivation to alleviate the microbial N limitations, thereby promoting the sustainable development of the SPA system. Concurrently, the introduction of plants into an agroforestry system significantly influences microbial nutrition limitations; thus, it is essential to ensure the judicious selection and integration of plants to maintain the optimal design of the agroforestry system. As a next step, we will employ multi-omics techniques to analyse the microbiome, thereby confirming the sustainability of the agroforestry system.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/f16010158/s1>. Table S1. The primer sequences and PCR conditions for bacteria and fungi; Table S2. Soil physicochemical properties and bacterial/fungal abundance in the MPA and SPA systems; Table S3. Seasonal changes in soil physicochemical properties and bacterial/fungal abundance in the MPA and SPA system; Table S4. EES, the vector length, and angle in the MPA and SPA systems; Table S5. Pearson correlation between soil extracellular enzyme activity and enzyme stoichiometry, soil properties, and fungal and bacterial abundance; Table S6. The permutation test of the redundancy analysis (RDA); Table S7. Analysis of the interaction between soil extracellular enzymes and stoichiometry with seasons and treatments; Table S8. Summary of standardized direct, indirect, and total effects of predictor variables on enzymatic vector length and angle in the SEM; Figure S1. One-year changes in soil physicochemical properties and bacterial/fungal abundance in the MPA and SPA systems; Figure S2. Annual changes in soil extracellular enzyme activity and stoichiometry in the MPA and SPA systems.

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