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Inconsistent Responses of Rhizosphere Microbial Community Structure and Extracellular Enzyme Activity to Short-Term Nitrogen and Phosphorus Additions in Chinese Fir (*Cunninghamia lanceolata*) Plantations

Zhilong Hu [1,2] and Wenhua Xiang [1,2,*]

[1] Faculty of Life Science and Technology, Central South University of Forestry & Technology, Changsha 410004, China; huzhilong2020@163.com
[2] Huitong National Station for Scientific Observation and Research of Chinese Fir Plantation Ecosystem in Hunan Province, Huaihua 438107, China
* Correspondence: xiangwh2005@163.com; Tel.: +86-0731-85623350

Abstract: Rhizosphere is a hot zone formed by root–microbial interaction, and microbial activities in this zone differ from those in bulk soil. Nitrogen (N) and phosphorus (P) inputs are able to change forest soil nutrient availability, affecting microbial communities and extracellular enzyme secretion. However, the impact of N and P additions on the structure and functions of rhizosphere microbial community in Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) plantations are not yet clear. To reveal the impact of short-term N and P inputs on microbial community structure and functions in rhizosphere soil, soil physicochemical properties, phospholipid fatty acids, and seven hydrolytic enzyme activities were measured in Chinese fir rhizosphere soil after one year of nutrient addition. N addition reduced the rhizosphere's pH and increased ammonium N, but the rhizosphere's available N (AN) initially wentdown and then up along the P-addition gradient. The rhizosphere fungi:bacteria ratio showed a decline after N addition, while a concave peak change occurred as rhizosphere AN under P addition. Moreover, rhizosphere extracellular enzyme activities and microbial C limitation climbed markedly with N addition rates, while this also showed an obviously unimodal pattern along the P-addition gradient. P addition did not alleviate rhizosphere microbial P limitation. Our findings suggest inconsistent responses of rhizosphere microorganisms of Chinese fir soil to N and P additions. Rhizosphere N availability can regulate microbial community structure and extracellular enzymes by influencing microbial C limitation. The study provides more knowledge on microbial activities in rhizosphere soil of subtropical forests under global changes.

Keywords: nitrogen addition; phosphorus addition; Chinese fir; phospholipid fatty acids; extracellular enzymes; microbial nutrient limitations

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1. Introduction

Globally, reactive nitrogen (N) input in terrestrial ecosystems has risen two-fold over the past century since anthropogenic disturbances (e.g., fertilization and fossil fuel burning) and will continue to rise in hotspots in the future [1–3], which is bound to have a non-negligible impact on forest ecosystems [4]. N deposition can affect soil processes by acidifying soils, influencing soil nutrient status, increasing plant growth, and altering the composition and content of organic matter [5–9]. Meanwhile, phosphorus (P) is considered to be a key element in limiting plant growth and microbial activity, and frequently limits primary productivity in subtropical/tropical regions [10–12]. Low P availability occurs in subtropical/tropical forests due to limited access of P to soil and leaching of soil P caused by the high rainfall and strong soil weathering [13,14]. Generally, stoichiometric imbalances in forest ecosystems are caused by differences in element inputs (e.g., high N

and low P) [15], which may affect plant growth and ecosystem nutrient cycling [16,17]. Soil microorganisms play a critical role in mediating key ecosystem processes, such as organic matter degradation, nutrient flow, and plant primary productivity [18–21]. However, our understanding of microbiological mechanisms underlying the response of ecosystems to nitrogen and phosphorus deposition is poorly understood. The mechanistic knowledge of N and P inputs on soil microorganisms is important.

Rhizosphere is regarded as a hot zone formed by root–microbial interaction, and microbial community structure and functions in this zone are different from those in bulk soil [22–24]. Plant carbon (C) enters the input to the rhizosphere's environment via rhizodeposition and root exudates [25], which increases the C-containing substrate required for microbial proliferation and stimulates its secretory enzyme, resulting in a more active rhizospheric environment compared to bulk soil [26]. Microbial community structure and metabolism function in rhizosphere and bulk soil may have different patterns of response to N and P inputs because of plant–soil–microbial interactions. For example, plants may decrease underground C allocation under the presence of high soil N availability, leading to aggravated C restriction of microorganisms in the rhizosphere and reducing fungi and arbuscular mycorrhizal fungi (AMF) [25,27,28]. Other studies have reported that high N increases rhizosphere C flux when N addition intensifies secondary P restriction [29]. Furthermore, soil N enrichment changes the microhabitat of rhizosphere microorganisms through the input of leaf or root litter, thus influencing the rhizosphere microbial community structure [30]. Meanwhile, P-deficient soil is ubiquitous in subtropical forests [11,31], so the effect of P availability on microbial activities has been increasingly recognized under the increasing intensity of atmospheric P deposition [32]. AMF in rhizosphere carries on the meaningful function in P absorption by plants and is usually sensitive to P addition [33,34]. It is expected that an increase in P availability in subtropical rhizosphere soil may increase AMF and tree growth and alter microbial community structure towards a higher fungi:bacteria (F:B) ratio by alleviating microbial P limitation. Although previous experiments from subtropical and tropical forests observed improvement in the F:B ratio under the addition of exogenous P [32,34–36], most were focused on microbial communities in bulk soil. It is uncertain how rhizosphere microorganisms respond to P inputs. Consequently, how microbial communities respond to N and P inputs and their underlying mechanisms remains elusive.

Generally, the transformation in microbial communities is combined with shifts in soil extracellular enzyme activities (EEAs) [27,37,38]. Extracellular enzymes secreted by microorganisms into the surrounding environment rhizosphere are beneficial to the depolymerization of organic matter into small molecules to meet their own growth and metabolism [39]. These extracellular enzymes directly participate in soil organic matter mineralization and nutrient release [40]. Different enzyme activities are usually deemed indicators of microbial acquisition for C, N, and P [41,42]. For example, glycosidases undertake responsibility for obtaining C by degrading cellulose and polysaccharides into small molecular organic C. The N-cycle enzymes secreted by soil microorganisms can also degrade chitin and protein to obtain N. In the process of microbial degradation of organic P, acid phosphatases release inorganic P by hydrolysis of phospholipid, which can be absorbed and utilized by microorganisms. Therefore, soil enzymes can reflect functions of microorganisms and imply their nutrient requirements [43,44]. Notably, numerous studies have implied that soil nutrients are markedly related to extracellular enzyme activities [42,45,46]. For example, N addition significantly enhanced C- and P-acquisition enzyme activities [47], while exogenous P input can dramatically decrease P-acquisition enzyme activities [48,49]. These results are in line with the resource allocation theory for enzyme production where soil microbes can produce extracellular enzymes to attain the most needed resources, and reduce enzyme secretion when the availability of simple resources is high [50]. Therefore, EEAs are affected by soil nutrient status [42]. Many researchers of soil EEAs have also analyzed the ecoenzymatic stoichiometry among different hydrolytic enzymes [42]. Ratios of soil EEAs participating in nutrient cycling represent the stoichiometric

balance between microbial biomass and environmental resources [24]. Thus, ecoenzymatic stoichiometry can imply nutrient acquisition strategies for microbes and be commonly used to evaluate relative resource limitations of microbial metabolism [51]. For example, the enzyme C:P and N:P ratios increase with P availability [42], while the enzyme C:N ratio is positively relevant to soil available N [46]. Carrara et al. [28] discovered that long-term N fertilization improved rhizosphere BG:NAG and BG:ACP ratios to face intensification of microbial C limitation.

Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) is a native, rapid-growing species, which has been cultivated widely for economic demand in southern China [11]. Yet, owing to continuous rotation, these forests suffer from low soil nutrient availability, which limits microbial activities and productivity in Chinese fir [52]. Meanwhile, over the past few decades, ambient N and P inputs in subtropical China have risen [53,54]. Many experiments have demonstrated the effect of nutrient additions on functions of bulk soil microorganisms in forests, while rhizosphere responses remain unclear [55,56]. Understanding how rhizosphere microorganisms face the short-term N and P inputs is relatively rare. This research aims to estimate how rhizosphere microbial community structure and hydrolytic enzymes respond to changes in soil nutrients induced by ambient N and P inputs. We will resolve this issue in ongoing N and P addition plots in Chinese fir plantations. Considering previous research, we assumed that (a) short-term N addition could shift rhizosphere microbial community structure towards a lower F:B ratio owing to the decline of underground photosynthetic C and increase of microbe C limitation, while P addition could increase F:B ratio because of alleviating microbial P limitation, and (b) rhizosphere enzymatic activities and ecoenzymatic stoichiometry could change with the gradient of N and P additions. Specifically, increases in rhizosphere N availability may aggravate microbial C or P limitation, which would shift to higher investment in C or P acquisition enzymes, while increases in rhizosphere P availability maybe alleviate microbial P limitation and allocate less resources to P acquisition enzymes.

2. Materials and Methods

2.1. Study Region

The experimental site is in the Huitong National Field Station for Scientific Observation Experiment for the Chinese Fir Ecosystem (26°41′50″~26°47′08″ N, 109°35′26″~109°38′45″ E), in the southwest border of the Hunan Province, China. Here, plantation has become a dominating forest landscape, and a prime operating tree species is *Cunninghamia lanceolata*. In the 1960s, a large-scale Chinese fir plantation was established in this region to meet the huge demand for wood for economic growth [11]. This study was carried out in Chinese fir plantations at an altitude of 330–482 m. The research site is a typical humid subtropical monsoon climate, with an annual average temperature of 16.8 °C and precipitation of 1268 mm [57].

The mean rates of total N deposition and total P deposition are 21.6 kg N ha^{-1} a^{-1} and 0.69 kg P ha^{-1} a^{-1}, respectively, in China's forests [53]. Like most subtropical forests, Chinese fir plantations in the Huitong County in China are experiencing increasing rates of atmospheric N and P input [37,38,58]. Chinese fir plantations were planted in 2006 with a planting density of 1.5 m × 1.5 m. Basic characteristics of the plots are shown in Table S2. The soils of the site are predominantly Alliti-Udic Ferrosols that developed from slate and shale parent rock [11].

2.2. Soil Sampling Measurements

Field fertilization experiments in Chinese fir plantations were established in August 2020, using a randomized block design: four random blocks for Chinese fir plantations, each including nine treatments (Figure 1). These treatments are CK, N1 (25 kg N ha^{-1} a^{-1}), N2 (50 kg N ha^{-1} a^{-1}), N3 (100 kg N ha^{-1} a^{-1}), N4 (200 kg N ha^{-1} a^{-1}), P1 (25 kg P ha^{-1} a^{-1}), P2 (50 kg P ha^{-1} a^{-1}), P3 (75 kg P ha^{-1} a^{-1}), and P4 (100 kg P ha^{-1} a^{-1}). Thirty-six plots (10 m × 10 m each) were set up, with a buffer zone ≥ 10 m wide between each

plot. N element was added to the plots as NH_4Cl, because the main input form of N deposition in China is ammonia nitrogen [54]. P addition was selected as NaH_2PO_4 due to its good solubility and frequent use in agriculture. N and P fertilizers were evenly sprayed once every three months for the whole year from September 2020. At each fertilization, the corresponding dose of solute was weighed, then dissolved in 6 L of deionized water. After fully dissolved, fertilizer was loaded into a knapsack sprayer. Each plot was manually sprayed twice under the canopy. Unfertilized plots were sprayed with 6 L of deionized water.

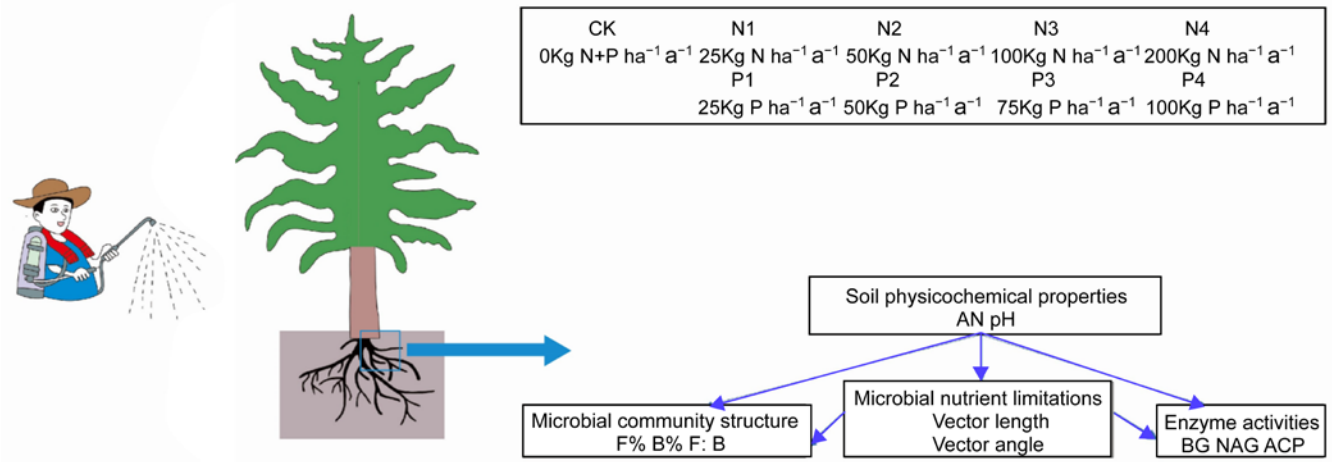

Figure 1. The conceptual experimental design in this study.

2.3. Soil Sampling

Soil sampling was conducted in August 2021. Four Chinese fir trees were sampled in each treatment plot. The selected tree had no other tree species within a radius of 2 m. Before sampling, the ground was carefully removed from plant litter, branches, and stones. Then, mineral soil samples were taken from under the four trees and combined into one composite sample. Due to the high root density of this layer, it is easy to collect enough rhizosphere soil samples, so sampling was carried out. All samples were kept in a refrigerated environment and delivered to the Huitong National Station for further processing within the 24 h. Back in the laboratory, rhizosphere soils were separated from mineral soil samples by the soil adhesion method, where the rhizosphere soil portion has operationally deemed soil that is still attached to roots after moderate shaking [59]. After root and rhizosphere separation, all rhizosphere soils were uniformly mixed with a 2 mm sieve, and moisture of each sample was quickly determined. Each rhizosphere soil sample was separated into three subsamples based on measurement requirements of indicators. The first subsample was air-dried for measuring rhizosphere pH, soil organic carbon (SOC), total N (TN), total P (TP), and available P (AP). The second subsample was held at −20 °C after freeze-drying for the determination of phospholipid fatty acids. The third subsample was refrigerated at 4 °C and used for analyzing extracellular enzyme activities, ammonium N (NH_4^+-N), and nitrate N (NO_3^--N).

2.4. Soil Properties

Soil water content (SWC) was determined by drying fresh soil samples to a constant weight in the oven (105 °C). Soil pH was determined in a 1:2.5 soil/water suspension with a pH meter. SOC content was determined using $K_2Cr_2O_7$–H_2SO_4 oxidation [60]. Soil TN was measured by applying a semi-micro Kjeldahl digestion with mixed catalysts. Soil TP was quantified using the Mo–Sb colorimetric method. The content of soil available N was the sum of NH_4^+-N and NO_3^--N. Soil available N was extracted with 2M KCl solution, and NH_4^+-N and NO_3^--N contents in extracts were analyzed by a flow injection analyzer (FIAstar 5000, Foss, Munkedal, Sweden). Soil AP was determined by ultraviolet and

visible spectrophotometry after extracting air-dried soil samples with 0.05 M HCl–0.025 M H_2SO_4 [61].

2.5. Soil Phospholipid Fatty Acids (PLFAs)

A second subsample was used for assessing the rhizosphere microbial community structure by PLFAs analysis [62]. The lipids from rhizosphere soil were collected with a monophasic extraction solution. The separation, quantification, and identification were carried out with the same equipment as Tian et al. [63]. The MIDI peak identification software (Microbial ID, Newark, DE, USA) was operated to calculate individual PLFAs in the sample according to internal standard 19:0, and it was measured in nmol per g of dry soil. All the fatty acids except those less than 0.5% were used for describing the rhizosphere microbial community structure. Given the inconsistency of markers applied in previous research, frequently used markers were employed to represent specific taxa (Table S1) [38,63]. Relative abundances of the microbial group were also calculated by dividing the PLFAs corresponding to the microbial group by the total PLFAs in each sample and expressed as a percentage.

2.6. Enzyme Assays

The potential activities of seven hydrolytic enzymes in rhizosphere soil related to the C (BG, β-1,4-glucosidase; CBH, cellobiohydrolase; BX, β-1,4-xylosidase; AG, α-1,4-glucosidase), N (NAG, β-1,4-N-acetyl-glucosaminnidase; LAP, leucine aminopeptidase), and P (ACP, acid phosphatase) cycles were measured using a 96-well micro-plate [64,65]. Approximately 1 g of fresh rhizosphere soil samples were suspended in a sodium acetate buffer. Suspensions were stirred sufficiently using a magnetic stirrer. Subsamples of soil suspension (200 µL) were distributed into the well of the micro-plate, with 8 duplicate wells for each sample. Enzyme substrate (200 mM) was then added to each well of soil suspension. After culturing all microplates in a dark environment at 20 °C for 4 h, 10 µL NaOH solution (1 M) was distributed into each well, and quantity of fluorescence was performed with the microplate reader (Synergy H4, BioTek, Washington, DC, USA) at 365 nm excitation and 450 nm emission for hydrolytic enzymes. The units for rhizosphere extracellular enzyme activity were nmol h^{-1} g^{-1} dry soil. The ratios of soil extracellular enzymes C:N, C:P, and N:P are indicated as BG:(NAG + LAP), BG:ACP, and (NAG + LAP):ACP, respectively.

2.7. Microbial Nutrient Limitation

In this study, the ecoenzymatic stoichiometry and vector analysis were used for assessing the relative microbial nutrient limitation in rhizosphere soil [66]. Vector length is often used to represent soil microbial C limitation, and relatively longer vector length demonstrates larger microbial C limitation. Vector angles > 45° show that microorganisms are limited by P relative to N, while vector angles < 45° indicate microbial N limitation. Microbial P limit increases with the increase of the vector angle, while microbial N limit decreases with the increase of the vector angle. The calculation formula for vector length and vector angle is as follows [51]:

$$\text{Vector length} = \sqrt{\frac{[\text{BG}+\text{CBH}+\text{BX}+\text{AG}]^2}{[\text{BG}+\text{CBH}+\text{BX}+\text{AG}+\text{NAG}+\text{LAP}]^2}+\frac{[\text{BG}+\text{CBH}+\text{BX}+\text{AG}]^2}{[\text{BG}+\text{CBH}+\text{BX}+\text{AG}+\text{ACP}]^2}}$$

$$\text{Vector angle}\ (^\circ) = \text{atan2}\left(\frac{\text{BG}+\text{CBH}+\text{BX}+\text{AG}}{\text{BG}+\text{CBH}+\text{BX}+\text{AG}+\text{ACP}'}\frac{\text{BG}+\text{CBH}+\text{BX}+\text{AG}}{\text{BG}+\text{CBH}+\text{BX}+\text{AG}+\text{NAG}+\text{LAP}}\right)\times 180/\pi$$

2.8. Data Analysis

This experiment aimed to check responses of rhizosphere microorganisms to N and P additions in Chinese fir plantations. Unless otherwise noted, the statistical significance in the chart in this paper was determined as $p < 0.05$. All analyses were performed applying R v.4.1.2 [67]. One-way ANOVA with Tukey's honestly significant difference (HSD) was applied to examine the effects of N or P addition on soil properties, hydrolytic enzymes and relative abundance of PLFAs in the rhizosphere. Pearson correlation coefficient was serviced for appraising relationships between relative abundances of microbial groups, extracellular enzyme activities, and soil physicochemical properties. Stepwise regression analysis was used to screen out soil physical and chemical indexes that could explain the variation in hydrolytic enzyme activities and in the enzyme stoichiometric ratio. In detail, the explaining variables were selected backward applying the "stepAIC" function in R till the *p*-value of modeling was notable. Additionally, the principal component analysis (PCA) was applied in R (Version 4.1.2 software) for assessing rhizosphere microbial community structure and seven extracellular enzyme activities in Chinese fir plantations. The entire microbial community structure and enzyme profiles were represented by the first and second principal components (PC1 and PC2) (Figure S4).

According to known and latent relationships, a structural equation model (SEM) was constructed for identifying relationships among microbial community structure, extracellular enzyme activities, and soil properties in Chinese fir rhizosphere soil. We applied the Chi-square ($\chi 2$) test, comparative fit index (CFI), and the root mean square error of approximation (RMSEA) to check the model's goodness of fit and retained significant pathways [49,68].

3. Results

3.1. Responses of Rhizosphere Soil Properties to Nutrient Additions

Rhizosphere pH decreased dramatically with N addition rates in Chinese fir plantations, while P addition effects on rhizosphere pH showed a unimodal change pattern (Figure 2a, $p < 0.01$). Along the P addition rates, rhizosphere pH initially increased and then gradually declined, and was highest at the medium level of P addition (P2).

Rhizosphere SOC and SWC were not dramatically affected by N and P additions (Table S3, $p > 0.05$), while the effects on rhizosphere TN and TP were responsive to nutrient additions significantly. N and P additions consistently increased rhizosphere TN and TP (Figure 2c,d, $p < 0.05$). P addition had greater impacts on their stoichiometric ratio in the rhizosphere. Rhizosphere C:N and C:P ratios declined and then rose with P addition rates, which was lowest under the medium addition (P2) (Figure 2g,h, $p < 0.05$). Nutrient additions significantly changed nutrient availability in the rhizosphere. Rhizosphere AN presented an increasing trend with N addition rates (Figure 2e, $p = 0.082$), while it initially declined and then climbed with P addition rates, with its maximum value at a high-P addition rate (P4) (Figure 2e, $p < 0.05$). Rhizosphere NH_4^+-N also followed similar patterns under nutrient additions (Table S1, $p < 0.1$). Rhizosphere AP increased markedly under P addition (Figure 2f, $p < 0.05$).

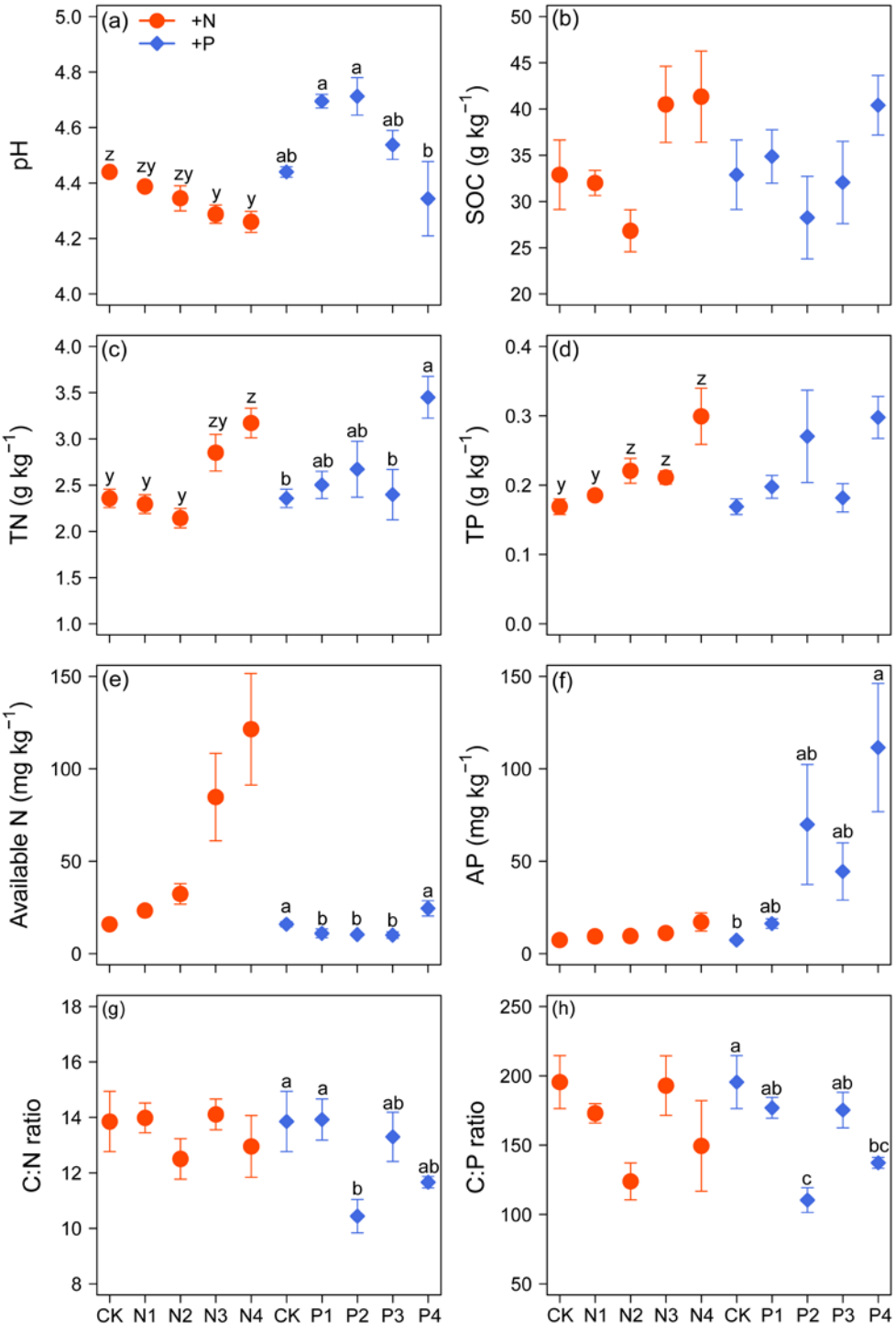

Figure 2. Effects of N and P additions on rhizosphere soil properties. (**a**) Soil pH; (**b**) soil organic carbon; (**c**) soil total nitrogen; (**d**) soil total phosphorus; (**e**) soil available nitrogen; (**f**) soil available phosphorus; (**g**) ratio of SOC to TN; (**h**) ratio of SOC to TP. Values are means ± SE (n = 4). Different letters indicate significant differences among N- or P-addition treatments in the same below ($p < 0.05$).

3.2. Responses of Rhizosphere Microbial Community Structure to Nutrient Additions

Generally, short-term nutrient additions shifted rhizosphere microbial community structure in Chinese fir plantations. N addition had a greater negative impact on microbial community structure with significant decreases in relative abundances of fungi and AMF, inducing declines in the F:B ratio (Figure 3b,c,e, $p < 0.05$). Meanwhile, N addition decreased

the Gram-positive bacteria:Gram-negative bacteria (G+:G−) ratio and G+, while it also increased the relative abundance of actinobacteria and G− (Table S1, $p < 0.1$). The proportion of bacteria did not respond to N addition (Table S3, $p > 0.05$). Along the P addition rates, relative abundance of fungi and F:B ratio tended to first decrease and increase at the medium P addition (P2), while the proportion of bacteria followed a single trend (Figure 3a,b,e, $p < 0.05$). Compared with no fertilization, P1 and P4 increased significantly the proportion of actinobacteria, while P3 lowered significantly the proportion of G+ (Table S3, $p < 0.05$). Total PLFAs did not show significant responses to nutrient additions.

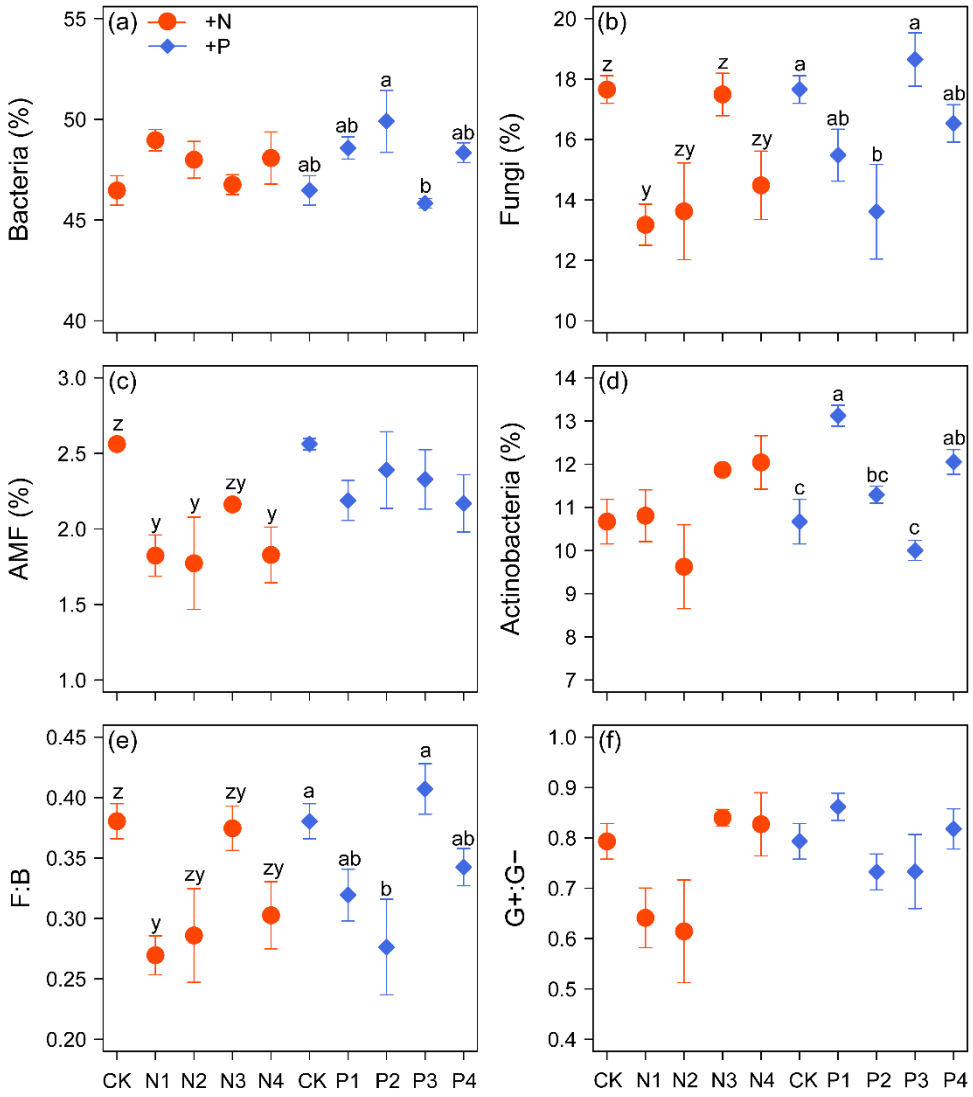

Figure 3. Effects of N and P additions on rhizosphere microbial community structure (presented by relative abundance of individual PLFAs). (**a**) The relative abundance of bacteria; (**b**) the relative abundance of fungi; (**c**) the relative abundance of arbuscular mycorrhizal fungi; (**d**) the relative abundance of actinobacteria; (**e**) the fungi: bacteria ratio; (**f**) the Gram-positive bacteria: Gram-negative bacteria ratio.Error bars indicate ± SE ($n = 4$). Different letters indicate significant differences among N- or P-addition treatments in the same below ($p < 0.05$).

3.3. Responses of Rhizosphere Extracellular Enzymes, Ecoenzyme Stoichiometry, and Microbial Nutrient Limitations to Nutrient Additions

Rhizosphere EEAs responded variously to N and P inputs. Short-term N addition generally increased rhizosphere enzyme activities. Seven extracellular enzyme activities in the rhizosphere showed a great increase with N addition gradient, being highest at high N

addition (N3) (Figure 4, $p < 0.001$). However, they showed similar variation as AN and F:B ratio under P addition, and tended to first decline and then rise, reaching their maximum value at P4 and minimum at P2 (Figure 4, $p < 0.01$).

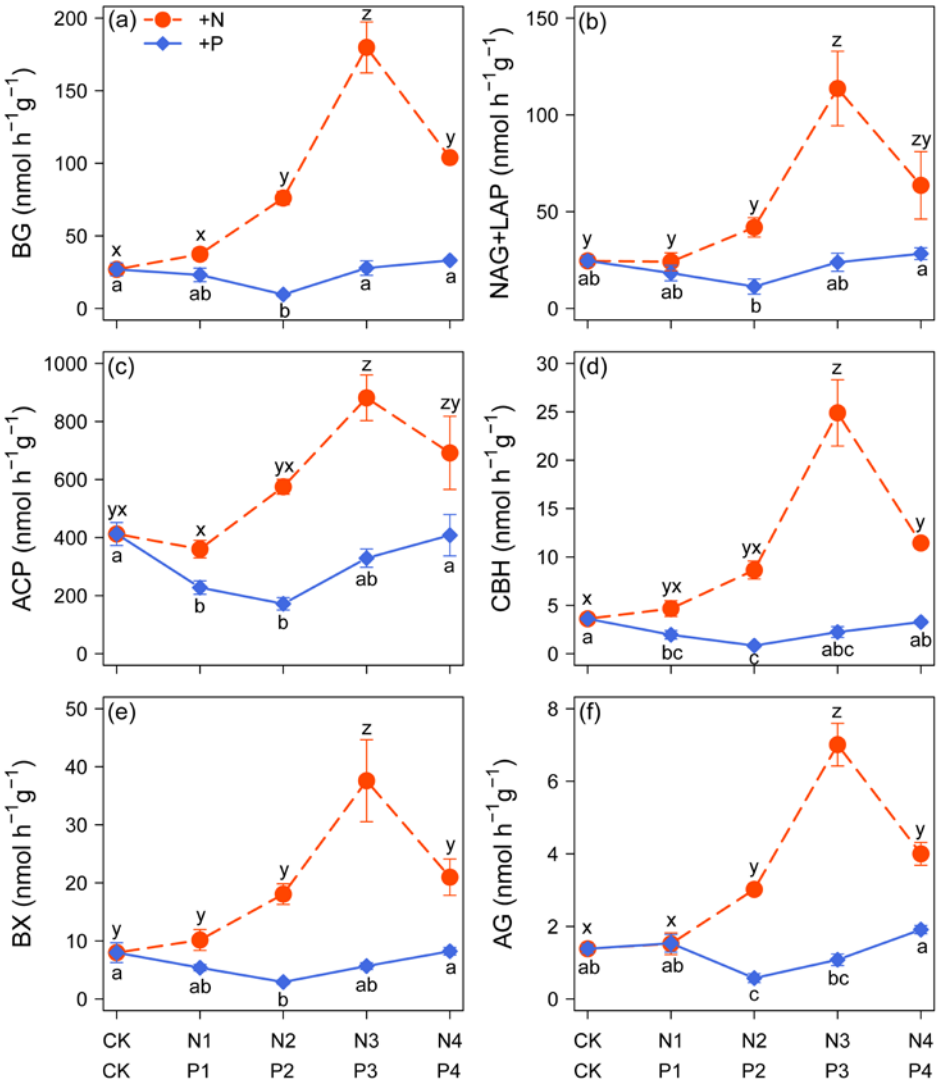

Figure 4. Effects of N and P additions on rhizosphere enzyme activities. (**a**) The activity of β-1,4-glucosidase; (**b**) the activity of β-1,4-N-acetyl-glucosaminnidase and leucine ami-nopeptidase; (**c**) the activity of acid phosphatase; (**d**) the activity of cellobiohydrolase; (**e**) the activity of β-1,4-xylosidase; (**f**) the activity of α-1,4-glucosidase. Error bars indicate ± SE ($n = 4$). Different letters indicate significant differences among N- or P-addition treatments in the same below ($p < 0.05$).

The characteristics of enzymatic stoichiometry differed among nutrient addition treatments. Rhizosphere BG:ACP and (NAG + LAP):ACP showed increasing trends with elevated N addition levels (Figure 5b,c, $p < 0.01$). Under P addition, the rhizosphere BG:(NAG + LAP) ratio showed a similar variation to rhizosphere pH and relative abundance of bacteria, initially increasing and then declining (Figure 5a, $p < 0.01$), while the rhizosphere BG:ACP ratio and the rhizosphere (NAG + LAP):ACP ratio remained constant. Vector length and angle expressed both microbial C and P vs. N constriction, respectively. All vector angles were greater than 45° in the rhizosphere soil of Chinese fir plantations, illustrating that rhizosphere microbes in the experimental area were restricted by P rather than N. Furthermore, rhizosphere vector length increased significantly and vector angle reduced markedly with N addition rates (Figure 5d,e, $p < 0.05$). P addition did not obvi-

ously influence the rhizosphere vector angle, while the variation pattern of vector length was similar to the rhizosphere BG:(NAG + LAP) ratio (Figure 5d, $p = 0.072$).

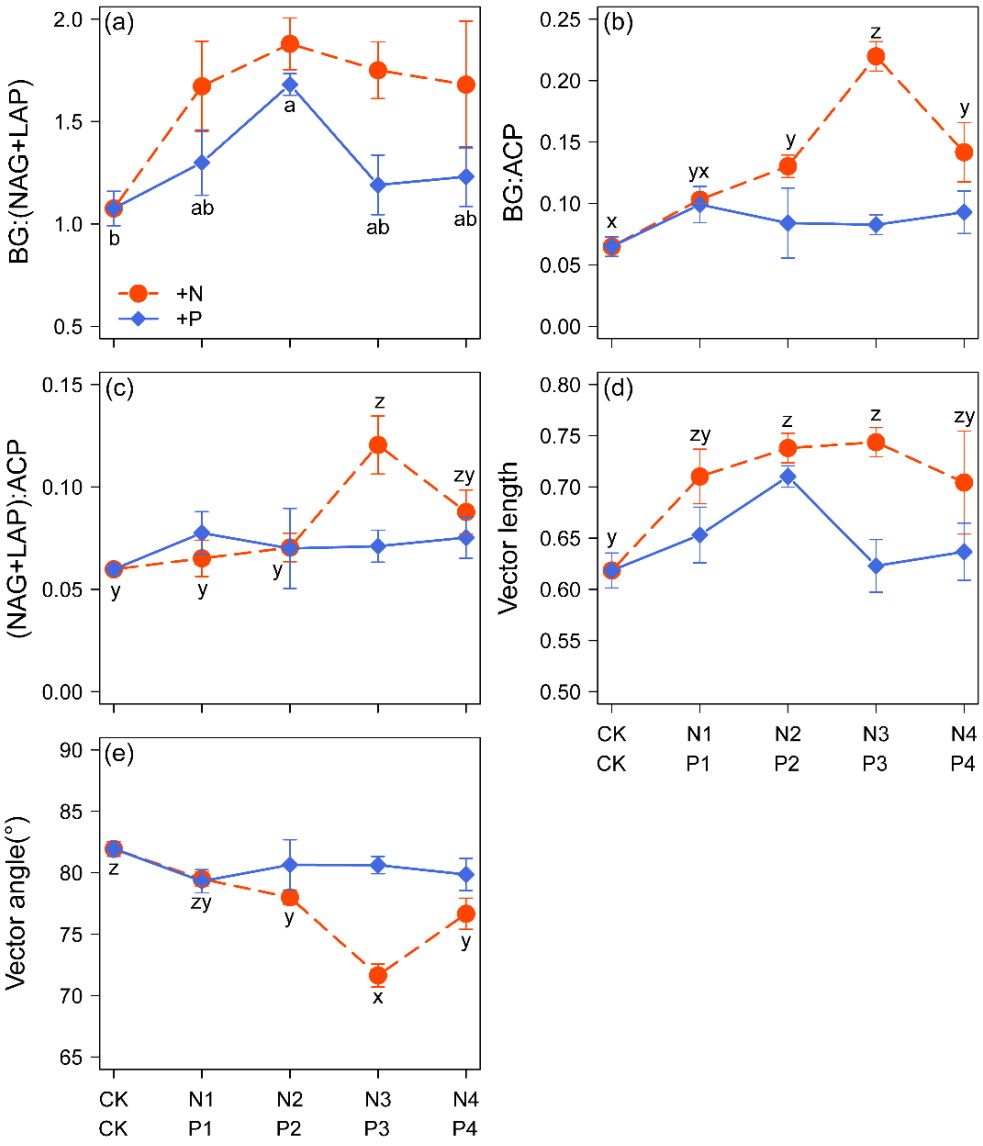

Figure 5. Effects of N and P additions on stoichiometric ratio of rhizosphere enzymes and microbial nutrient limitation. (**a**) The ratio of BG to the sum of NAG and LAP; (**b**) the ratio of BG to ACP; (**c**) the ratio of the sum of NAG and LAP to ACP; (**d**) vector length; (**e**) vector angle. Error bars indicate ± SE ($n = 4$). Different letters indicate significant differences among N- or P-addition treatments in the same below ($p < 0.05$).

3.4. Relationships among Rhizosphere Soil Properties, Microbial Community Structure, and Extracellular Enzyme Activities

Rhizosphere microbial community structure was linked to soil properties in Chinese fir plantations. The results exhibited that relative abundances of bacteria and G− were negatively relevant to the rhizosphere N:P ratio (Figure S2, $p < 0.05$). Relative abundances of fungi and AMF were positively relevant to the rhizosphere N:P and C:P ratio and negatively to NO_3^--N (Figure S2, $p < 0.05$). Total PLFAs were negatively relevant to rhizosphere SWC (Figure S2, $p < 0.05$).

Correlation analyses showed that most of the EEAs were positively relevant to rhizosphere N availability and SOC, and negatively to pH. For example, rhizosphere BG activities were positively correlated with AN and NH_4^+-N, and negatively with pH (Figure 6,

$p < 0.05$). Regression analysis results ulteriorly clarified that changes for most of the EEAs were strongly associated with rhizosphere available N and SOC (Table 1, $p < 0.05$). Furthermore, correlation analyses showed that rhizosphere vector length, BG:(NAG + LAP), and BG:ACP were positively linked to rhizosphere AN, NH_4^+-N, and NO_3^--N, and negatively to C:N and C:P ratio (Figure 6, $p < 0.05$). Regression analysis results ulteriorly clarified that changes for rhizosphere vector length, BG:(NAG + LAP), and BG:ACP were strongly associated with rhizosphere NH_4^+-N, NO_3^--N, and C:N ratio (Table 1, $p < 0.05$).

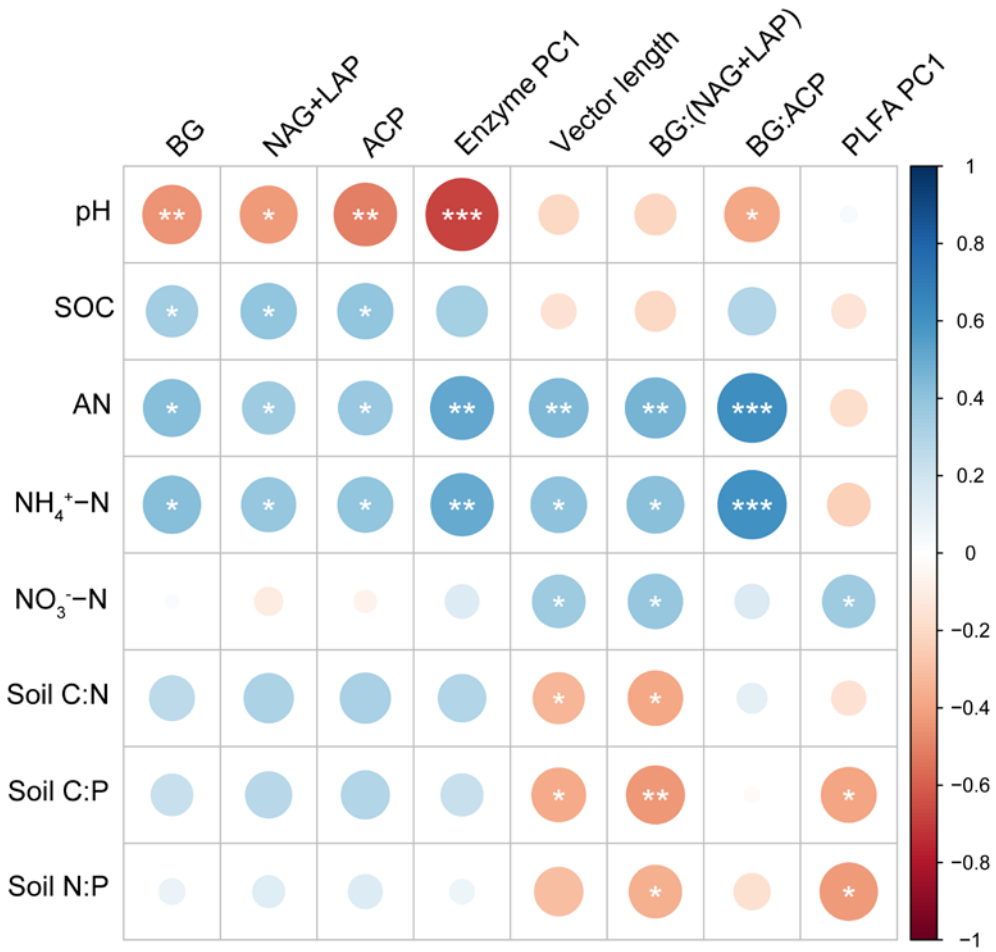

Figure 6. The correlation between soil physicochemical properties and microbial community structure, hydrolytic enzymes, stoichiometric ratio of enzymes, and vector length in the rhizosphere. PLFA PC1: the first principal component of the entire microbial community structure according to relative abundance of individual PLFAs. Enzyme PC1: the first principal component of enzyme profiles according to seven rhizosphere enzymes. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Rhizosphere soil properties responded significantly to nutrient additions, so we quantified the various pathways of rhizosphere soil properties on microbial community structure and hydrolytic enzymes in Chinese fir plantations by constructing a structural equation model. The SEM revealed that rhizosphere pH directly affected the EEAs under nutrient additions (Figure 7, $p < 0.01$). In addition, variation of rhizosphere AN induced by nutrient additions affected microbial C limitation, thus leading to changes in extracellular enzyme secretion (Figure 7, $p < 0.05$). Moreover, rhizosphere AN drove altered microbial community structure by influencing microbial C restriction (Figure 7, $p < 0.05$).

Table 1. Stepwise regression analysis of hydrolytic enzyme activities, ecoenzymatic stoichiometry, and soil properties in Chinese fir rhizosphere soil. † $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Response Variable	Standardization Regression Equations	R^2	p
BG	$BG = 0.45NH_4^+ - N^{**} + 0.64SOC^{*} - 0.43TN - 1.31^{*}10^{-16}$	0.27	0.005
NAG + LAP	$NAG + LAP = 0.42NH_4^+ - N^{*} + 0.49SOC^{**} - 0.33TP^{\dagger} - 1.76^{*}10^{-16}$	0.29	0.003
ACP	$ACP = -0.58pH^{***} + 0.25SOC^{\dagger} - 0.38NO_3^- - N^{*} - 4.78^{*}10^{-16}$	0.38	0.000
CBH	$CBH = 0.41NH_4^+ - N^{*} + 0.61SOC^{*} - 0.42TN^{*} - 2.169^{*}10^{-16}$	0.21	0.015
BX	$BX = 0.45NH_4^+ - N^{**} + 0.29C/N^{\dagger} - 9.86^{*}10^{-16}$	0.22	0.007
AG	$AG = 0.51NH_4^+ - N^{**} + 0.45SOC^{**} - 0.31TP^{\dagger} - 2.02^{*}10^{-16}$	0.34	0.001
Vector length	$Vector\ length = -0.67AP^{***} + 0.47TP^{**} + 0.32NO_3^- - N^{*} - 0.33C/N^{*} + 9.28^{*}10^{-16}$	0.42	0.000
BG:(NAG + LAP)	$BG:(NAG + LAP) = 0.37NH_4^+ - N^{**} - 0.44NO_3^- - N^{**} - 0.35C/N^{*} - 0.37SWC^{*} - 9.72^{*}10^{-17}$	0.42	0.000
BG:ACP	$BG:ACP = 0.56NH_4^+ - N^{***} + 0.2TP + 0.21C/N - 1.07^{*}10^{-16}$	0.37	0.000

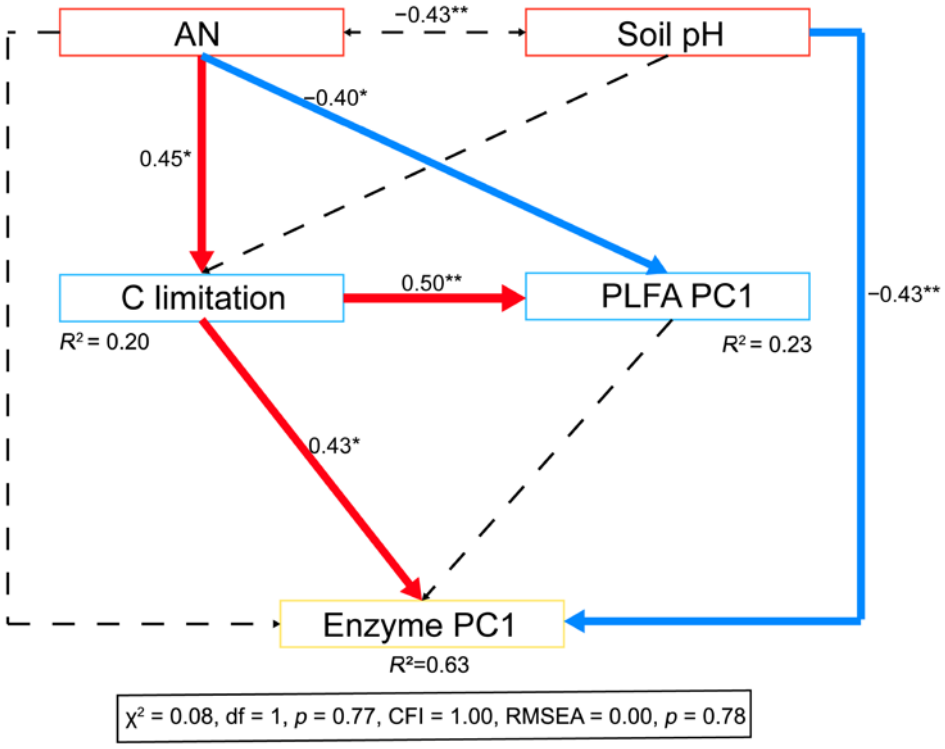

Figure 7. The structure equation model (SEM) investigates multi-variate effects on extracellular enzyme activities in Chinese fir rhizosphere soil. C limitation: the rhizosphere vector length. Red and blue arrows express the significantly positive and negative directions of causality, respectively. Black solid arrows indicate insignificant causal relationships. Values adjacent to solid arrows indicate standardized path coefficients. Black double arrows indicate correlation. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

4.1. Effects of N and P Additions on Rhizosphere Soil Properties

After short-term nutrient additions, we found obvious changes in rhizosphere pH in Chinese fir plantations. The soil acidification by exogenous N input was not surprising, as it was often examined in forest soil, which was attributed to the absorption and consumption of NH_4^+ by soil microorganisms and roots, resulting in releasing more H^+ into the rhizosphere [63,69]. Contrary to N addition, short-term P addition effects on rhizosphere pH showed a roughly unimodal pattern of first increasing and then declining, which is out of line with other studies indicating that P addition significantly increased soil pH in subtropical and tropical forests [48,58]. This might be due to the fact that nitrification in

rhizosphere was stimulated, resulting in the generation of hydrogen, potentially increasing soil acidification [69]. Although the rates of nitrogen cycling were not measured in our study, the results show that high P addition (P4) increased significantly AN and NO_3^--N, implying increased N nitrification and soil acidification.

Rhizosphere SOC did not change significantly under nutrient additions, and is inconsistent with many studies in forests which showed that nutrient additions enhances SOC [37,38]. Two mechanisms may explain minor responses of rhizosphere SOC to nutrient additions in Chinese fir plantations. Firstly, nutrient additions would strengthen plant photosynthetic C synthesis and stimulate more litterfalls into soil. Meanwhile, increases in soil nutrient availability could also enhance plant absorption of N and P and it may cause less photosynthetic C secreted into rhizosphere as rhizodeposition. Therefore, more input of litter C may be offset by the reduction of plant C to the rhizosphere [37,69]. Secondly, nutrient additions lasted only one year in this study, but there is the abundant fact that long-term nutrient additions can influence soil nutrients [27,38]. So it may be necessary to undergo a longer fertilization period to detect significant changes in rhizosphere SOC.

Unlike SOC, rhizosphere TN or TP were sensitive to nutrient additions. The nutrient additions directly increased rhizosphere TN and TP, especially under N addition. These were also detected in similar N and P addition research in other forests [38,69]. Ratios of soil C:N, C:P, and N:P are mainly decided by the nutrient contents condition. In particular, rhizosphere nutrient stoichiometry did show a meaningful change under P addition. The shifts in rhizosphere nutrient stoichiometry may have significant effects on microbial activities which deserves further investigation.

Rhizosphere available nutrients improved under N or P addition. However, rhizosphere AN significantly decreased under low and medium P addition rates, but it rose under high P addition. The pattern is in line with other studies [69,70]. For instance, a short-term P addition treatment in an Alpine Meadow also showed that AN initially declined and then increased with P addition rates [69]. Low P input may promote tree growth and the absorption of mineral N from rhizosphere soil, lowering soil's available N [70]. A study showed that the addition of P affected soil N cycling and it dramatically facilitated expression of the *nifH* gene [71].

4.2. Effects of N and P Additions on Rhizosphere Microbial Community Structure

Although total PLFAs did not change significantly, relative abundances of the microbial taxa were significantly responsive to nutrient additions. Relative abundances of different microbial taxa were described by a PLFAs approach. The results showed that N addition decreased significantly relative abundances of fungi and AMF, and these reductions resulted in a lower F:B ratio, indicating significant shifts in rhizosphere microbial community structure to N enrichment. This was consistent with the expectation that N addition could decrease the rhizosphere F:B ratio and results from other experiments [49,72]. There may be two reasons for this response of rhizosphere microbial structure to exogenous N input. Firstly, soil fungi are characterized by low N metabolism, so rich soil N content would be conducive to bacterial activities [73]. A global meta-analysis implied that decreases in the F:B ratio after N addition may be because oligotrophic fungi need less N than copiotrophic bacteria for the same biomass synthesis [73]. Secondly, changes in rhizosphere microbial structure might be influenced by microbial nutrient restrictions [49]. Nitrogen and phosphorus cycles have a complex coupling relationship. The introduction of additional nitrogen into the system can interact with microbially directed N cycling and C mineralization. Chen et al. [74] believed that N addition delayed the mineralization of recalcitrant organic C by reducing the quality of soil organic matter and the oxidase activity, thus aggravating microbial carbon restriction. The decreases in relative abundances of fungi and AMF were likely linked to increases in microbial C limitation [63]. The results stood by this finding that rhizosphere BG:ACP and vector length increased significantly with N addition rates, indicating greater microbial C limitation induced by N addition. In the present study, the SEM further showed that rhizosphere N availability shifted rhizosphere

microbial community structure by strengthening C limitation. So, N deposition may shift in microbial community structure to a less fungal community and lower F:B ratio through increasing vector length thought commonly to be related to the C limiting.

Microbial processes are generally limited by P in subtropical forests [11]. Although P addition increased rhizosphere AP, ratios of C:P, N:P enzyme and vector angle had no response, indicating that P addition did not alleviate microbial P limitation at the sampling times. Meanwhile, P addition effects on rhizosphere microbial community structure were obviously unimodal in Chinese fir plantations. It was unexpected since it was contrary to our hypothesis that the rhizosphere F:B ratio would increase due to alleviated microbial P limitation under an elevated P supply. The changes in percentages of fungi and bacteria caused the F:B ratio to decrease first and then increase with the P addition level. This rhizosphere microbial variation pattern under P addition primarily stemmed from rhizosphere soil nutrient availability. Rhizosphere available N significantly declined under the low P addition (P1, P2), indicating that low P addition may enhance the uptake of mineral N by plants. Therefore, P addition may cause microbial N restriction [70]. Our results elaborated that the rhizosphere EEAs decreased markedly under low P addition. Meanwhile, enzyme ratio of C:N and vector length first climbed significantly along the P-addition gradient, indicating intensified microbial C restriction. Decreases in rhizosphere C:N and C:P ratio in low P addition also implied the decrease in rhizosphere C availability and intensification of microbial C limitation. Therefore, the intensification of microbial C restriction contributed to the shift of rhizosphere microbial community towards bacteria dominance under low P addition. On the other hand, elevated P availability may lead to a decrease in the F:B ratio. Mycelium growth makes it easier for fungi to access rhizosphere nutrients because it has the ability to transfer rich nutrients into nutrient-constrained soil patches. Adding P to the soil perhaps weakened this benefit. Therefore, higher nutrient availability is beneficial for energy channels based on bacteria [36]. However, the rhizosphere F:B ratio and relative abundance of fungi then increased in medium and high P addition. This may be due to greater soil C availability with a high P input. High P addition could increase root biomass and litterfall into the rhizosphere, thus leading to the improvement of C availability [35], which could alleviate microbial C limitation. Greater available N and TN may indicate an increase in rhizosphere C availability induced by high P addition. Increased rhizosphere C:N and C:P also supported this assumption. Finally, alleviated microbial C limitation changed microbial community structure [36]. Although the SEM showed that rhizosphere AN affected microbial community structure by microbial C limitation, through analysis, rhizosphere N availability in this experiment may also reflect C availability. Thus, P addition may induce the unimodal change pattern of the F:B ratio in Chinese fir rhizosphere soil by regulating nutrient availability and microbial C limitation.

4.3. Effects of N and P Additions on Rhizosphere Enzyme Activities and Microbial Nutrient Limitation

Enzymes in soil are mostly secreted by soil microorganisms, allowing them to acquire necessary nutrients from organic matter [42]. Soil microorganisms obtain resources most in demand by optimizing the investment of extracellular enzymes according to their own needs and nutrient environment [75]. Accordingly, following the theory of resource allocation for enzyme production, we observed that activities of rhizosphere C- and P-acquisition enzymes enhanced significantly with N addition rates. However, N addition also increased the N-acquisition enzyme activity, which did not follow the resource allocation theory. This difference was confirmed in a recent meta-analysis in which exogenous N input boosted NAG activity [76]. Additionally, N addition in this study increased significant rhizosphere TP and (NAG + LAP):ACP, indicating there was less investment of microorganisms in the P enzyme and declines in microbial P limitation. Decreases in vector angle under N addition also showed mitigation of microbial P restriction. This result suggested that soil microbial P restriction is common in subtropical forests. Furthermore, BG and BG:ACP increased significantly with N addition rates, implying that rhizosphere microorganisms secreted relatively

more C-acquiring enzymes to obtain C element for growth and metabolism. Along the N addition rates, vector length showed significant increasing trends, which meant that C became limiting and microbial C limitation was intensified by N addition. These are in line with our expectations that N addition could aggravate microbial C and make them shift to higher investment in C acquisition enzymes. These findings suggest that rhizosphere soil microorganisms in Chinese fir plantations may undergo the transition from P restriction to C restriction or P and C co-restriction. Taken together, our results implied that field N supply provoked a lot of secretion of extracellular enzymes and increased microbial C limitation. A large amount of N input directly led to greater rhizosphere N availability, and vector length, BG:(NAG + LAP) and BG:ACP were positively relative to rhizosphere N availability (NH_4^+-N and NO_3^--N). This suggested that microbial C constriction was regulated by N availability. So increased N availability subjected microorganisms to great levels of C limitation. Two mechanisms may explain such positive impact of N addition on the EEAs. Firstly, increased availability in soil N may affect microbial nutrient limitation and increase plant C supply to the rhizosphere as rhizodeposition and root exudates, which would encourage microorganisms to secrete more extracellular enzymes to degrade SOC. Our results showed that changes in EEAs and microbial C limitation were significantly correlated with rhizosphere N availability. Previous studies have reported that rhizosphere C flux increased when N addition intensified P restriction [29]. More plant C supply into the rhizosphere could mitigate microbial C limitation, thus increasing extracellular enzyme secretion [56]. However, our findings suggested that rhizosphere microbial C limitation aggravated with increasing rhizosphere AN. This may be because although N addition may increase plant belowground C allocation, a great increase in rhizosphere N availability decreased relative C availability in the rhizosphere. The SEM further showed that rhizosphere N availability affected rhizosphere EEAs through microbial C limitation. Therefore, the relationship between hydrolytic enzymes and microbial C limitation under N addition requires further research. Additionally, the results also revealed that EEAs were negatively correlated with rhizosphere pH, indicating that short-term N input increased enzyme activities by acidifying soil. Wang et al. [64] reported that enhanced enzyme activities were induced by decreased soil pH and increased bacterial stress under long-term N addition. Consequently, great increases in rhizosphere N availability under N addition induced higher extracellular enzyme activities by regulating microbial C limitation and pH.

P addition effects on rhizosphere EEAs and enzymatic stoichiometry were obviously unimodal in Chinese fir plantations. Most of the EEAs initially lowered and then grew along with the P addition rates. However, the BG:(NAG + LAP) ratio and vector length showed a unimodal pattern of first rising and then falling. Additionally, there was no response from the ratios of C:P, N:P enzyme, and vector angle to P addition, demonstrating that P addition did not alleviate microbial P limitation. It was contrary to our hypothesis that P addition could alleviate microbial P limitation and allocate less resources to P acquisition enzymes. Changes in rhizosphere EEAs under P addition may derive from soil nutrient availability. Appropriate application of P fertilizer can encourage trees to absorb N, thereby decreasing rhizosphere N content and intensifying the competition of plants and microorganisms for N, which will limit the production of enzymes by microorganisms [70]. This conjecture was supported by the present study that available N was lower and microbial C limitation was stronger in the low P addition than in the unfertilized addition. However, rhizosphere EEAs then increased in medium and high P addition. This might be caused by the increased availability of soil nutrients after high P input. High P addition may allow more root biomass and litterfall to enter the rhizosphere, thus leading to the improvement of nutrient availability [77]. We observed increases in available N and TN, and rhizosphere C:N and C:P also increased induced by high P addition, indicating greater rhizosphere C and N availability. The higher nutrient availability mitigated microbial C limitation to make microbes produce more extracellular enzymes [36]. Our SEM also further showed that variations in rhizosphere N availability induced under P addition changed EEAs by affecting microbial C limitation in Chinese fir plantations. Hence, under P addition,

changes of rhizosphere N availability can reflect extracellular enzymes through microbial C limitation.

5. Conclusions

The effects of short-term nitrogen (N) and phosphorus (P) inputs on soil properties, microbial community structure, and extracellular enzymes were examined in Chinese fir rhizosphere soil. Our study revealed diverse responses of rhizosphere microorganisms to field N and P additions. N addition diminished the rhizosphere F:B ratio and strengthened the microbial C limit as hypothesized, indicating shifts in microbial community structure. Surprisingly, along the P-addition gradient, the rhizosphere F:B ratio initially decreased and then increased, and the microbial P-limitation was not alleviated by P addition, implying that changes in microbial community structure are not due to P restriction. Rhizosphere microbial community structure was closely related to soil N availability and microbial C restriction. Moreover, N addition significantly enhanced BG:ACP, while BG:(NAG + LAP) strengthened first and then weakened as the rate of P addition was increased, meaning that nutrient additions were more likely to affect investment in C-acquiring enzymes. Consequently, rhizosphere N availability plays an essential role in mediating microbial community structure and extracellular enzymes after N and P fertilization. Overall, our study provides new evidence for better understanding the response mechanism of rhizosphere microbial activities to global changes, and provides a reference for the rational management of Chinese fir plantations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14081532/s1, Table S1. The fatty acids detected in this study. Table S2. General site and soil characteristics (0–10 cm soil depth) in Chinese fir plantation sites at the Huitong National Forest Ecosystem Research Station used in the present study. Table S3. Summary statistics (F statistic and probability level) of a one-way ANOVA on the effects of N addition and P addition on soil nutrients properties, soil enzymes and relative abundance PLFAs (mol %) in rhizosphere soil of Chinese fir plantations. Figure S1. Effects of N and P additions on rhizosphere soil properties and microbial community structure (indicated by relative abundance of individual PLFAs). Values are means ± SE (n = 4). Different lowercase letters indicate significant difference among the levels of N or P addition ($p < 0.05$). CK, 0 kg N ha^{-1} a^{-1} + 0 kg P ha^{-1} a^{-1}; N1, 25 kg N ha^{-1} a^{-1}; N2, 50 kg N ha^{-1} a^{-1}; N3, 100 kg N ha^{-1} a^{-1}; N4, 200 kg N ha^{-1} a^{-1}; P1, 25 kg P ha^{-1} a^{-1}; P2, 50 kg P ha^{-1} a^{-1}; P3, 75 kg P ha^{-1} a^{-1}; P4, 100 kg P ha^{-1} a^{-1}. Figure S2. The correlation between soil properties and microbial groups, enzyme activities, stoichiometric ratio of enzymes, vector angle in the rhizosphere. Other B%, relative abundance of other bacteria. B%, relative abundance of bacteria. F%, relative abundance of fungi. AMF%, relative abundance of arbuscular mycorrhizal fungi. Act%, relative abundance of actinobacteria. G+%, relative abundance of Gram-positive bacteria. G-%, relative abundance of Gram-negative bacteria.* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Figure S3. The correlation among microbial groups, enzyme activities and stoichiometric ratio of enzymes in the rhizosphere. F%, relative abundance of fungi. Enzyme PC1, the first principal component of enzyme profiles according to seven rhizosphere enzymes. PLFA PC1, the first principal component of the entire microbial community structure according to relative abundance of individual PLFAs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Figure S4. Principal component analysis (PCA) for rhizosphere microbial community structure (a), in which PC1 explains 52% and PC2 explains 23.8% of total variations. PCA for rhizosphere extracellular enzyme actives (b), in which PC1 explains 80.5% and PC2 explains 13% of total variations. PERMANOVA statistics refer to significant N and P treatment effects. CK, 0 kg N ha^{-1} a^{-1} + 0 kg P ha^{-1} a^{-1}; N1, 25 kg N ha^{-1} a^{-1}; N2, 50 kg N ha^{-1} a^{-1}; N3, 100 kg N ha^{-1} a^{-1}; N4, 200 kg N ha^{-1} a^{-1}; P1, 25 kg P ha^{-1} a^{-1}; P2, 50 kg P ha^{-1} a^{-1}; P3, 75 kg P ha^{-1} a^{-1}; P4, 100 kg P ha^{-1} a^{-1}.

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Abbreviations

AN	available N
EEAs	extracellular enzyme activities
BG	β-1,4-glucosidase
NAG	β-1,4-N -acetyl-glucosaminnidase
LAP	leucine aminopeptidase
ACP	acid phosphatase
CBH	cellobiohydrolase
BX	β-1,4 -xylosidase
AG	α-1,4 -glucosidase
PLFAs	phospholipid fatty acids
F:B	fungi: bacteria
G+:G−	gram-positive bacteria: gram-negative bacteria
AMF	arbuscular mycorrhizal fungi

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