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N2-Fixing Tree Species Help to Alleviate C- and P-Limitation in Both Rhizosphere and Non-Rhizosphere Soils in the Eucalyptus Plantations of Subtropical China

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Abstract: The extracellular enzyme activity (EEA) and enzymatic stoichiometry (EES) of soil are useful indicators of shifts in soil nutrition and microbial resource requirements. Nevertheless, it is uncertain how the limitation of soil microbial nutrients is altered by a *Eucalyptus* plantation mixed with a N2-fixing tree species. Our study examined the microbial nutrient limitation in two plantations: a pure *Eucalyptus* plantation (PP) and a mixed plantation (*Eucalyptus* and *Erythrophleum fordii*, MP) in rhizosphere and non-rhizosphere soils, beginning with two indicators, soil EEA and EES. In this study, the soil EEA was considerably ($p < 0.05$) greater in the MP contrasted to the PP, and the enzyme C:N:P ratios of the PP (1.12:1:1.10) and MP (1.07:1:1.08) both diverged from the global average EEA (1:1:1), and the deviation degree of the PP was greater. The results of the vector analysis demonstrated that the vector angle (VA) and vector length (VL) were considerably ($p < 0.05$) smaller in the MP contrasted to the PP. In comparison to the PP, the MP had a considerably ($p < 0.05$) poorer carbon quality index (CQI). Additionally, both microbial and soil properties have a considerable impact on soil EEA and EES, according to variance partitioning analysis (VPA) and redundancy analysis (RDA). In summary, our results show that the restriction of microorganisms on C and P in rhizosphere soils is usually weaker than that in non-rhizosphere soils and that the addition of N2-fixing tree species to *Eucalyptus* plantations can lessen but not completely remove the restriction of soil microorganisms on C and P. Future management practices involving mixed plantations with N2-fixing trees species could help decrease microbial nutrient limitation and promote sustainable plantations.

Keywords: enzyme activity; enzyme stoichiometry; rhizosphere soil; nutrient limitation; *Eucalyptus* plantation

1. Introduction

Plant roots and soil microorganisms are the primary sources of most extracellular enzymes found in soil. These enzymes play a pivotal role in terrestrial ecosystems by facilitating the decomposition of soil organic matter (SOM), thereby contributing to biogeochemical cycling [1]. Anterior research has shown that the measurement of soil extracellular enzyme activity (EEA) serves as a dependable indicator for assessing changes in microbial requirements and soil nutrient utilizability [2]. Evaluating the characteristics of soil microbial nutrient limitation is commonly achieved through the application of extracellular enzymatic stoichiometry (EES) [3,4]. Furthermore, Sinsabaugh et al. found that EES was

approximately 1:1:1 for C:N:P on a global scale [5]. The stability of microbial growth metabolism, nutrient demand, and ambient nutrient effectiveness is indicated by a ratio of enzyme activity that is near to 1:1:1 [6], while deviation from this ratio indicates that soil microorganisms are nutrient limited [7]. Some studies have demonstrated that biological and abiological variables, incorporating vegetation kinds, soil microorganisms, and soil physicochemical properties, influence soil enzyme activity [5,8]. Hence, the use of EEA and EES techniques has significantly improved our knowledge of the metabolic traits and nutrient restriction of soil microbes.

Soil N is usually a limiting nutrient, its availability directly affects the productivity of forest ecosystems [9]. An appealing option to increase long-term soil N availability is mixed-species plantations, which combine N_2-fixing trees with other highly productive tree species (e.g., *Eucalyptus* species), alleviating the soil-plant N limitation and increasing overall biomass production [10]. According to one study, the addition of N_2-fixing trees to *Eucalyptus* plantations has been found to alter the component of microbial community and boost biomass [11]. Soil microorganisms play a crucial role in mobilizing forest soil nutrients, acting as the main driving factor for nutrient cycling. Additionally, these microorganisms have beneficial effects on both soil nitrogen content and availability [11]. Alterations in nitrogen content and availability may also impact enzyme activity, thereby influencing the accessibility of soil nutrients [12]. Marklein and Houlton concluded that nitrogen addition promotes the secretion and increases the activity of phosphatase, which facilitates the uptake and utilization of phosphorus by plants [13]. Lammirato et al. also found that when the soil nutrient supply is insufficient, soil microorganisms will degrade organic compounds mainly by inducing the production of β-1,4-glucosidase (BG), thereby alleviating soil microbial nutrient limitation [14].

The rhizosphere, which serves as the focal point of soil-plant interactions, facilitates nutrient flow between the soil and the plant and enhances microbial activity [15]. Enzymes facilitate nutrient cycling between the soil, microbes, and plants by breaking down organic substrates [16]. Furthermore, the rhizosphere microbial population also changes the microenvironment and affects soil nutrient transformation [17]. In studies of plantation ecosystems, the bulk of the soil's microbial populations have gotten much of the focus, but little has been published about how different rhizosphere and non-rhizosphere soils are in terms of microbial metabolism and nutrient requirements. To fully comprehend the functions of root systems in soil nutrient cycles in plantation ecosystems, it is important to identify the ecological enzyme stoichiometry and nutrient turnover patterns in rhizosphere soil.

Due to their rapid growth rate, short rotation cycle, and high productivity, *Eucalyptus* species have been planted across numerous tropical and subtropical nations [18]. The use of fertilizers and pesticides, soil consolidation, and water and nutrient loss in these pure plantation systems have raised worries regarding their economic and environmental consequences [19]. In recognition of symbiotic N_2 fixation, the introduction of N_2-fixing tree species (e.g., *Erythrophleum fordii*) in *Eucalyptus* plantations may offer a substitute to mineral fertilizers [20]. While there has been extensive research on how acacias influence soil nutrient cycling in mixed-species plantations, little is known about the properties of EEA and EES related to soil nutrient looping in the rhizosphere of *Eucalyptus* plantations. Our understanding of the processes involved in converting nutrients after the addition of a N_2-fixing tree species will be enhanced through our investigation of EES in both rhizosphere and non-rhizosphere soils. The main aims of our study were to assess how the addition of a N_2-fixing tree species impacts the characteristics of soil EEA and EES in both rhizosphere and non-rhizosphere soils, and to pinpoint the environmental factors affecting EEA and EES parameters.

2. Materials and Methods

2.1. Study Area Situation

The study was carried out at the Guangxi Youyiguang Forest Ecosystem National Research Station in China (21°57′–22°19′ N, 106°39′–106°59′ E) [1]. The rain–heat synchronization and the temperature in this area are suitable and rainfall is abundant [21]. The soil type is red soil [11]; the main geomorphological type is low mountains and hills, and elevations range from 430 to 680 m [22]. Finally, the planting patterns are mainly pure and mixed plantations.

2.2. Sample Collection and Processing

In this study, the forest types included a pure *Eucalyptus grandis* × *E. urophylla* plantation (PP) and a mixed *E. grandis* × *E. urophylla* and *E. fordii* plantation (MP), with PP serving as a control (CK). Before the current stand was planted, the region had been covered by a *P. massoniana* plantation, which was planted in 1983 on a hill that had previously been deforested and was cleared in 2011. The PP plantation was established in 2012 with *E. grandis* × *E. urophylla* (3-month-old clones) at a separation of 2 m × 2 m (2500 trees·ha^{-1}) while the MP plantation consists of mixed *E. grandis* × *E. urophylla* and *E. fordii* (1-year-old container seedlings) at the same spacing using a mixed row planting model with a ratio of 2 (*E. grandis* × *E. urophylla*):1 (*E. fordii*). A native N_2-fixing tree species that is well adapted to the soil and environment is *E. fordii*. During the first three years following planting, both plantation types were given a comparable stand management regimen, which included weed control and surface soil plowing, and were then allowed to mature naturally. Five blocks were selected from stands with similar site conditions. A sampling plot measuring 20 m × 20 m (400 m^2) was established in each of the two distinct plantation types (PP and MP), one next to the other, inside each block. Table 1 provides more detailed information on the PP and MP.

Table 1. Basic information on sample plots in the PP and MP.

Plantation Type	Altitude (m)	Slope (°)	Age (yr)	DBH (m)	TH (m)	CD	LF (kg·hm^{-2})
PP	246–263	15–17	8	15.66 ± 0.07	16.62 ± 0.14	0.61 ± 0.01	3669.79 ± 98.00
MP	250–266	14–18	8			0.71 ± 0.01	4891.66 ± 132.17
E. urophyla				16.78 ± 0.10	17.74 ± 0.10		
E. fordii				8.62 ± 0.17	8.30 ± 0.18		

DBH: diameter at breast height; TH: tree height; CD: canopy density; LF: litterfall mass.

A total of 12 locations, located at distances about 5 m away from the midpoint of the quadrat in the 0°, 30°, 60°, 90°, 120°, 150°, 180°, 210°, 240°, 270°, 300°, and 330° directions, were sampled in August 2020. To collect soil samples, surface debris was first removed, followed by extraction using a cylindrical corer with a diameter measuring 10 cm at depths ranging from 0 to 10 cm. Each plot was blended to receive one composite sample. The rhizosphere soil was operationally defined as the soil that remained attached to the roots after loosening and shaking the core. Rhizosphere soil samples were obtained by shaking off loosely bound soil from roots, after which soil closely adherent to the roots within ~4 mm was brushed off and retained and the rest of the soil was collected as non-rhizosphere soil [23,24]. Each sample of soil was collected, immediately put in an incubator with an ice box, and brought back to the lab. After 2 mm soil screening, the soil samples were used for the two-part experiment. One portion was kept in a freezer at minus 20 degrees Celsius to determine soil microbial properties, while the other portion was preserved after natural air-drying to estimate the soil physicochemical characteristics.

2.3. Soil and Microbial Properties Analysis

Detection of soil water content (SWC) using the drying method. The soil specimens and water were stirred for one minute at a ratio of 1 to 2.5 and the pH value was measured

with a pH meter. The $K_2Cr_2O_7$-H_2SO_4 oxidation method was chosen to calculate soil organic carbon (SOC) content. Soil total nitrogen (TN) was detected using the Kjeldahl method for nitrogen determination [25]. Nitrate nitrogen (NO_3^--N) and ammonia nitrogen (NH_4^+-N) were extracted and filtered by 0.01 mol/L $CaCl_2$, and the filtrate was determined by a continuous-flow analyzer (SEAL Auto Analyzer 3, SEAL Analytical, Norderstedt, Germany). Molybdenum blue colorimetry can be used to determine soil available phosphorus (AP) and total phosphorus (TP) [26], but AP is somewhat different in that it uses HCl-H_2SO_4 to extract filtrate [27]. In general, a chloroform-fumigation extraction method is commonly used to measure soil microbial biomass [28]. Firstly, the soil samples were divided into two groups, one group was fumigated with chloroform for 24 h, while the other group was left for the same 24 h without fumigation to form a control. Then, 0.5 mol/L K_2SO_4 was added and filtered by shaking to obtain the extracts of microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN). In contrast, microbial biomass phosphorus (MBP) was extracted by 0.5 mol/L $NaHCO_3$. Finally, the difference between the two controls was used to calculate the microbial biomass.

2.4. Soil Extracellular Enzyme Characteristics Analysis

The soil EEA of C-acquiring enzymes (BG and CB), N-acquiring enzymes (LAP and NAG), and one of the P-acquiring enzymes (ACP) was determined using the 96-well microplate fluorescence method [29]. The method outlined by Tabatabai was used to assess the soil EEA of the other P-acquiring enzyme (PDE) [30]. Oxidases, including phenol oxidase (PO) and peroxides (PER), were determined according to the method described by Sinsabaugh et al. [31]. Soil slurry was prepared by shaking the soil sample and 0.05 mol/L sodium acetate with a ratio of 1 to 100 for 30 min. PO was determined from the reaction of 200 μL Levodopa (L-DOPA) solution and 800 μL soil slurry, while PER was measured by adding 50 μL more 0.3% H_2O_2 to PO. Specific extracellular enzyme functions and substrate information are shown in Table S1. EES was the relative proportion of enzymes participating in the C, N, and P cycles [5,32], calculated using the following formulas:

$$\text{Enzyme C : N} = \frac{\ln(BG + CB)}{\ln(LAP + NAG)} \tag{1}$$

$$\text{Enzyme C : P} = \frac{\ln(BG + CB)}{\ln(ACP + PDE)} \tag{2}$$

$$\text{Enzyme N : P} = \frac{\ln(LAP + NAG)}{\ln(ACP + PDE)} \tag{3}$$

Vector analysis of soil EEA reflects the state of soil microorganisms limited by C, N, and P [33]. Vector length (VL), vector angle (VA), and carbon quality index (CQI) are calculated as follows [34]:

$$\text{Vector Length} = \sqrt{\left[\frac{\ln(BG + CB)}{\ln(ACP + PDE)}\right]^2 + \left[\frac{\ln(BG + CB)}{\ln(LAP + NAG)}\right]^2} \tag{4}$$

$$\text{Vector Angle}(°) = \text{Degrees}\left\{\text{ATAN2}\left[\left(\frac{\ln(BG + CB)}{\ln(ACP + PDE)}\right), \left(\frac{\ln(BG + CB)}{\ln(LAP + NAG)}\right)\right]\right\} \tag{5}$$

$$\text{CQI} = \frac{\ln(PO)}{\ln(PO) + \ln(BG)} \tag{6}$$

The more restricted the microorganisms are by C, the longer is the VL. VA 45° is the dividing line, VA smaller than 45° demonstrates that the microorganisms are restricted by N, while VA larger than 45° demonstrates that the microorganisms are restricted by P; greater deviation from the dividing line indicates greater restriction [35]. CQI indicates

the percentage of recalcitrant C, and the larger the CQI is, the greater the recalcitrant C content [36].

2.5. Statistical Analyses

The variances of soil physicochemical characteristics, soil microbial biomass, soil EEA and EES of the PP and MP in rhizosphere and non-rhizosphere soil were contrasted by independent-sample *t*-tests. The relationships among soil physicochemical characteristics, microbial biomass, soil extracellular enzymes, VL, VA, and CQI were analyzed by Pearson correlation analysis. SPSS 21.0 (IBM, Chicago, IL, USA) was used for data analysis, and then Origin (2023) was used to present the results. The cut-off for all significance levels was set at 0.05. The individual and synthetic effects of soil physicochemical properties and microbial properties on EEA and EES were detected by variance partition analysis (VPA), and the results were presented as Venn diagrams. In order to establish whether there was a significant variation in EEA and EES between the rhizosphere and non-rhizosphere and between the PP and MP, principal component analysis (PCA) was utilized. Redundancy analysis (RDA) was used to determine environmental parameters that affect soil EEA and EES. VPA, PCA, and RDA were calculated using the multivariate statistical program Canoco4.5 for Windows. Before analysis, all data were converted logarithmically [1].

3. Results

3.1. Soil Physicochemical Properties and Microbial Biomass

In the rhizosphere soil, the MP had considerably greater levels of NO_3^--N, NH_4^+-N, TN, SOC, TP, AP, MBC, MBN, and MBP than did the PP by 187.28, 14.27, 11.11, 8.07, 5.21, 29.38, 25.72, 111.89, and 78.33%, respectively (Table 2). In the non-rhizosphere soil, NO_3^--N, NH_4^+-N, TN, SOC, AP, MBC, MBN, and MBP were remarkably (all $p < 0.05$) greater in the MP contrasted to the PP by 159.39, 16.46, 14.05, 12.44, 17.70, 23.61, 105.07, and 60.15%, respectively (Table 2).

Table 2. Soil characterization in rhizosphere (R) and non-rhizosphere (NR) soil in the PP and MP.

Soil Type	R		NR	
Plantation Type	**PP**	**MP**	**PP**	**MP**
SWC (%)	21.57 ± 0.59 a	23.15 ± 0.60 a	22.49 ± 0.90 a	23.33 ± 0.93 a
pH	4.90 ± 0.14 a	4.88 ± 0.14 a	4.89 ± 0.11 a	4.85 ± 0.12 a
SOC (g·kg^{-1})	19.72 ± 0.32 b	21.31 ± 0.29 a	12.75 ± 0.42 b	14.34 ± 0.44 a
TN (g·kg^{-1})	3.12 ± 0.06 b	3.47 ± 0.08 a	2.44 ± 0.07 b	2.78 ± 0.09 a
NO_3^--N (mg·kg^{-1})	2.36 ± 0.17 b	6.78 ± 0.25 a	0.18 ± 0.02 b	0.48 ± 0.03 a
NH_4^+-N (mg·kg^{-1})	206.06 ± 6.56 b	235.47 ± 5.64 a	175.94 ± 5.47 b	204.89 ± 6.70 a
TP (g·kg^{-1})	0.56 ± 0.01 b	0.58 ± 0.01 a	0.54 ± 0.01 a	0.56 ± 0.01 a
AP (mg·kg^{-1})	5.92 ± 0.16 b	7.66 ± 0.18 a	4.46 ± 0.14 b	5.25 ± 0.11 a
Soil C:N	6.33 ± 0.12 a	6.16 ± 0.18 a	5.25 ± 0.22 a	5.20 ± 0.31 a
Soil C:P	35.50 ± 0.93 a	36.45 ± 0.78 a	23.61 ± 1.01 a	25.81 ± 0.81 a
Soil N:P	5.62 ± 0.17 a	5.92 ± 0.10 a	4.52 ± 0.20 a	5.01 ± 0.18 a
MBC (mg·kg^{-1})	226.05 ± 7.55 b	284.19 ± 8.47 a	215.22 ± 7.58 b	266.03 ± 7.53 a
MBN (mg·kg^{-1})	10.63 ± 0.72 b	22.52 ± 0.55 a	9.48 ± 0.74 b	19.44 ± 0.73 a
MBP (mg·kg^{-1})	6.43 ± 0.56 b	11.46 ± 0.92 a	4.56 ± 0.65 b	7.30 ± 0.70 a
MBC:MBN	21.74 ± 1.79 a	12.67 ± 0.60 b	23.18 ± 1.68 a	13.76 ± 0.58 b
MBC:MBP	36.40 ± 3.85 a	25.34 ± 1.84 b	50.77 ± 6.39 a	38.26 ± 4.91 a
MBN:MBP	1.72 ± 0.20 a	2.02 ± 0.17 a	2.24 ± 0.34 a	2.80 ± 0.37 a

SWC, Soil water content; pH, pH value; SOC, soil organic carbon; TN, total nitrogen; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; TP, total phosphorus; AP, available phosphorus; Soil C:N, carbon to nitrogen ratio of soil; Soil C:P, carbon to phosphorus ratio of soil; Soil N:P, nitrogen to phosphorus ratio of soil; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus; MBC:MBN, microbial biomass carbon to microbial biomass nitrogen ratio; MBC:MBP, microbial biomass carbon to microbial biomass phosphorus ratio; MBN:MBP, microbial biomass nitrogen to microbial biomass phosphorus ratio. Different lowercase letters within the same row indicate significant differences within the PP and MP, $p < 0.05$.

3.2. Enzyme Activities

We discovered that mixing with N_2-fixing tree species dramatically impacted the soil EEA linked to C, N, and P acquisition. In particular, in the rhizosphere soils of the MP compared to the PP, BG and CB were greatly ($p < 0.001$) increased by 166.17 and 51.65%, respectively. Moreover, BG and CB were considerably greater in the non-rhizosphere soils of MP than in PP, increasing by 127.93% ($p < 0.01$) and 27.07% ($p < 0.05$), respectively (Figure 1a). In addition, LAP and NAG were both significantly ($p < 0.001$) higher by 85.44 and 215.80% in rhizosphere soils and by 54.17% ($p < 0.01$) and 102.26% ($p < 0.001$) in non-rhizosphere soils, respectively, of the MP compared to those of the PP (Figure 1b). Furthermore, ACP and PDE were both greatly increased by 90.52% ($p < 0.001$) and 110.70% ($p < 0.01$) in rhizosphere soils and by 65.97% ($p < 0.001$) and 52.29% ($p < 0.05$) in non-rhizosphere soils, respectively, of the MP compared to those of the PP (Figure 1c). Additionally, PO and PER were both greatly increased ($p < 0.001$) by 64.24 and 44.16% in rhizosphere soils and by 89.56 and 40.42% in non-rhizosphere soils, respectively, of MP compared to those of the PP (Figure 1d).
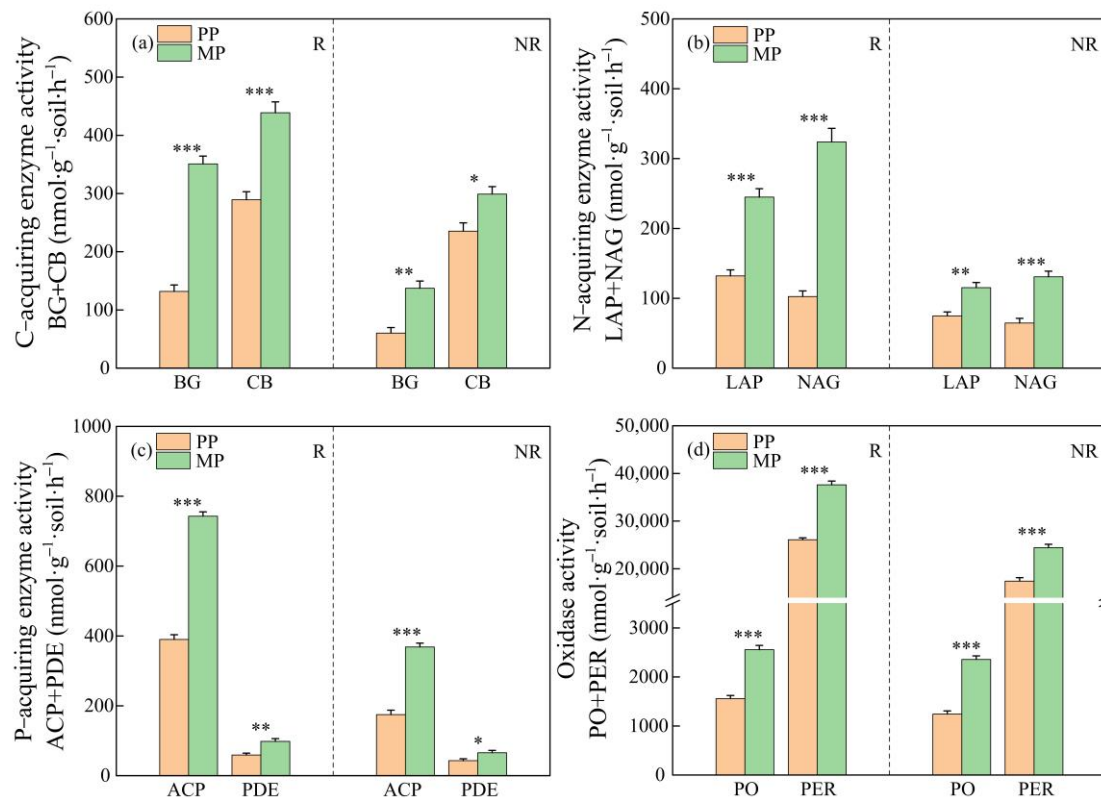

Figure 1. (**a–d**) Soil extracellular enzymatic activity (EEA) in rhizosphere (R) and non-rhizosphere (NR) soil in the PP and MP. *, significant at $p < 0.05$; **, significant at $p < 0.01$; ***, significant at $p < 0.001$.

3.3. Enzymatic Stoichiometry and Vector Characteristics

In the rhizosphere soil, the ratio of enzyme C:N of the PP and MP was greater than 1, while that of the MP was considerably ($p < 0.01$) lower than that of the PP. The ratio of enzyme C:P of the PP and MP was less than 1, but there was no significant difference between them ($p > 0.05$). The N:P ratio of the enzyme was less than 1 in both the PP and the MP, and the ratio in the MP was considerably ($p < 0.01$) larger than that in the PP (Figure 2). In the non-rhizosphere soil, the enzyme C:N ratios both exceeded 1 in the PP and MP, while that of the MP was remarkably ($p < 0.05$) lower than that of the PP. Both the PP and MP had an enzyme C:P ratio larger than 1, but the enzyme C:P ratio of the MP was remarkably ($p < 0.05$) lower than that of the PP. Although the enzyme N:P ratios in the PP and MP

were both less than 1, there was no difference between them that was significant ($p > 0.05$) (Figure 2).
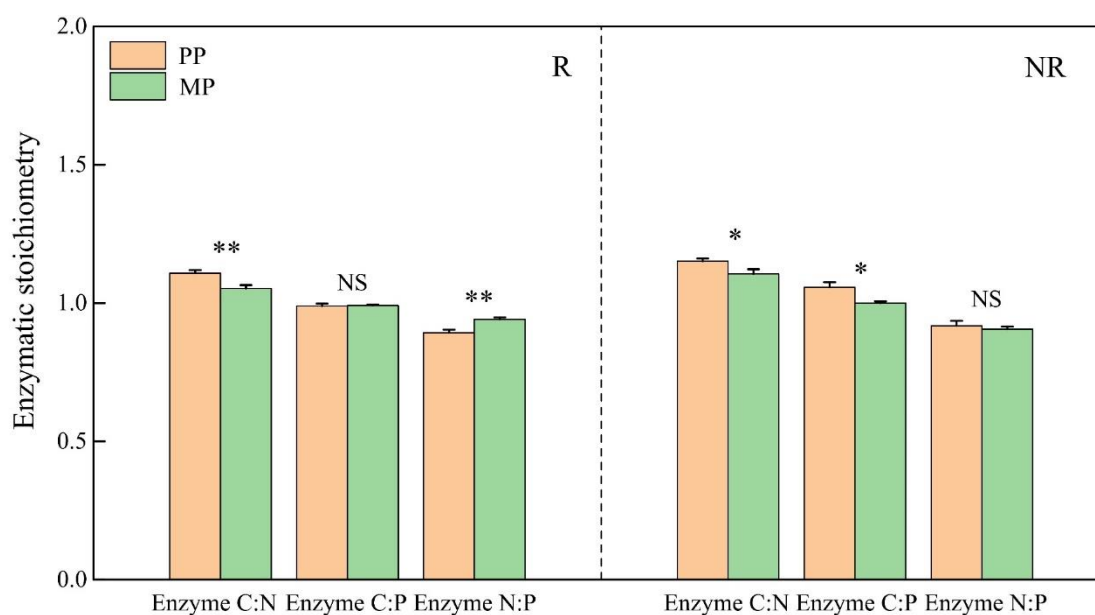

Figure 2. Soil extracellular enzymatic stoichiometry (EES) in rhizosphere (R) and non-rhizosphere (NR) soil in the PP and MP. Enzyme C:N, ln(BG + CB):ln(LAP + NAG); enzyme C:P, ln(BG + CB): ln(ACP + PDE); enzyme N:P, ln(LAP + NAG):ln(ACP + PDE). NS, not significant; *, significant at $p < 0.05$; **, significant at $p < 0.01$.

According to vector analysis, the VL (microbial C limitation) range of the PP and MP is between 1.44 and 1.56 (VL > 1), and the microbial C limitation was remarkable ($p < 0.05$) lower in MP than that in PP, in both the rhizosphere and non-rhizosphere soils (Figure 3). VA was greater than 45° in both the PP and MP, and decreased considerably ($p < 0.01$) in the MP for rhizosphere soils, but there was no difference between the PP and MP that was significant ($p > 0.05$) in non-rhizosphere soil (Figure 3). The mean CQI of the MP in rhizosphere soil was remarkably ($p < 0.05$) lower than that of the PP, and there was no difference between the PP and MP that was significant ($p > 0.05$) in non-rhizosphere soil (Figure 3).

3.4. Relationship between Soil Enzymes and Soil Properties

When comparing the soil EEA in the PP and MP, there were strong positive correlations ($p < 0.0001$), but not between VL and VA ($p > 0.05$) (Figure 4).

On the correlation heatmap, there were clear correlations between the soil EES, vector analysis indicators, the majority of the soil nutrient ratios, and the microbial biomass ratios. As opposed to the enzymatic N:P ratio, which had no associations that was significant with soil nutrient ratios ($p > 0.05$), the enzymatic C:N and C:P ratios were considerably ($p < 0.01$) negatively linked with the soil nutrient ratios (Figure 5). Enzymatic C:N and MBC:MBN ratios demonstrated strong positive relationships ($p < 0.01$), and enzymatic C:N and C:P ratios demonstrated prominent positive correlations ($p < 0.05$) with the MBC:MBP ratio. A substantial ($p < 0.05$) negative association between the enzymatic N:P and MBC:MBN ratios was found, despite the fact that there was no prominent ($p > 0.05$) connection found between soil EES and the MBN:MBP ratio. VL and the soil C:N and C:P ratios both had strong inverse correlations ($p < 0.001$) with each other, and there was a substantial ($p < 0.01$) inverse correlation between the soil N:P ratio and VL. Additionally, there was a strong ($p < 0.001$) inverse correlation between the soil C:N and C:P ratios and CQI, and there was a substantial ($p < 0.05$) negative correlation between the soil N:P ratio and CQI. Additionally, the MBC:MBN ratio showed a significant ($p < 0.05$) positive relationship with both VL and

VA; the MBC:MBP ratio showed a strong ($p < 0.01$) positive relationship with VL; and the MBN:MBP ratio showed a significant ($p < 0.05$) positive relationship with CQI (Figure 5).
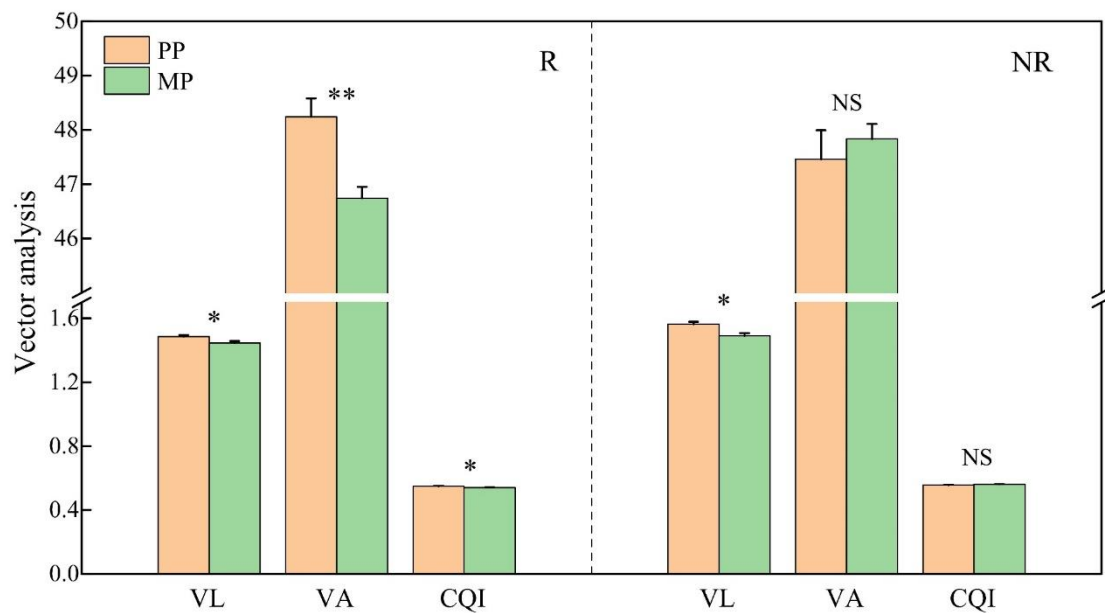

Figure 3. Vector analysis in rhizosphere (R) and non-rhizosphere (NR) soil in the PP and MP. VL, vector length; VA, vector angle (°); CQI, carbon quality index. NS, not significant; *, significant at $p < 0.05$; **, significant at $p < 0.01$.
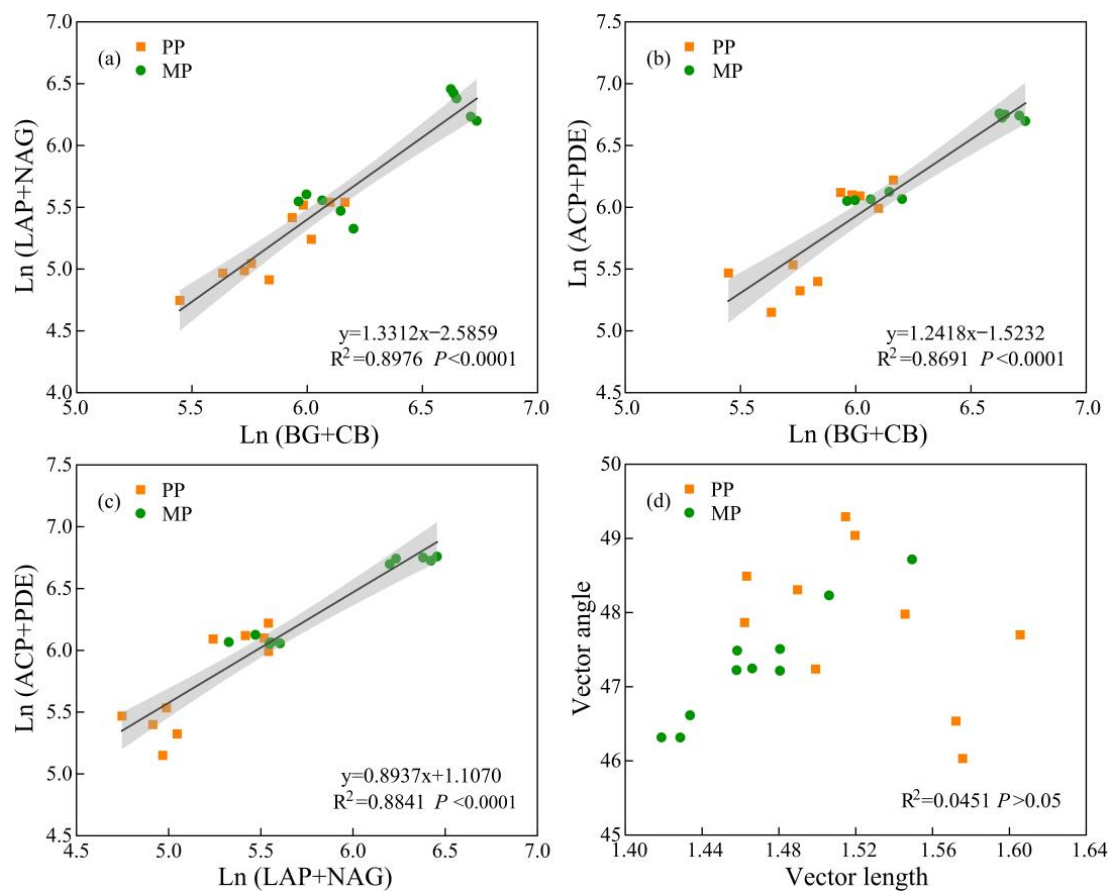

Figure 4. The relationship among soil extracellular enzyme activity (EEA) in PP and MP (**a–c**) and the relationship between vector length (VL) and vector angle (VA) in PP and MP (**d**).
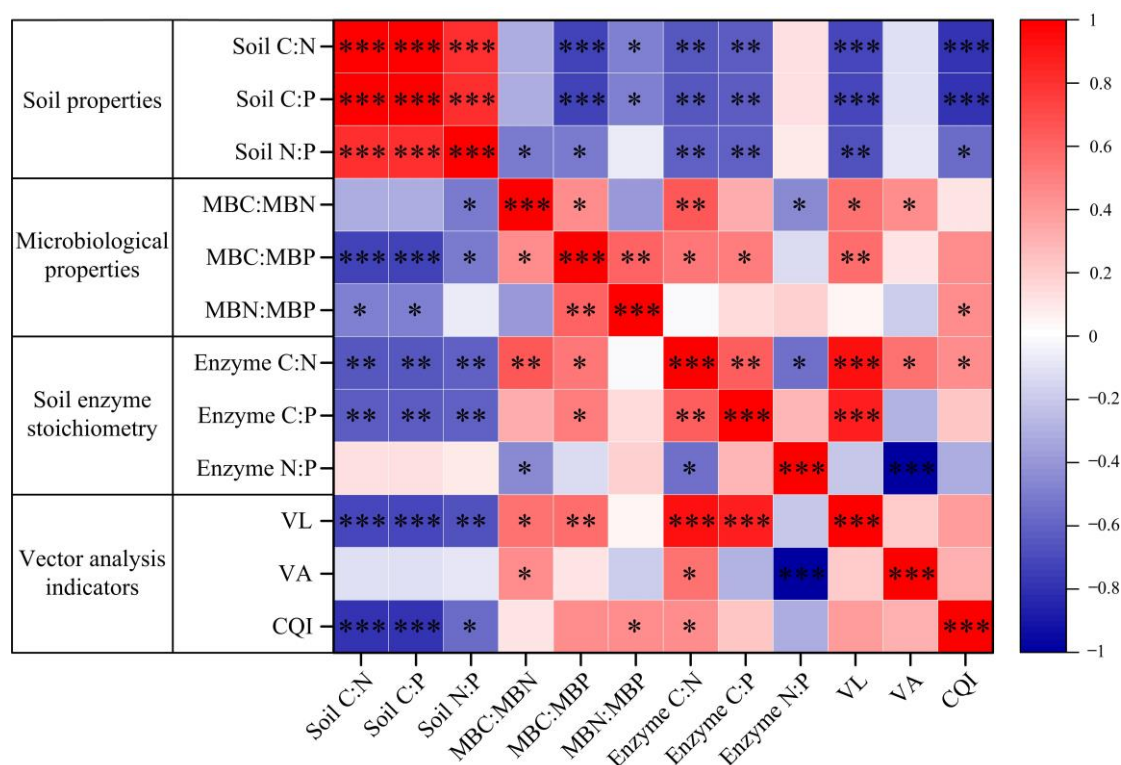
Figure 5. Correlations among soil EES and vector analysis indicators and soil nutrients and microbial biomass ratios within the PP and MP. *, significant at $p < 0.05$; **, significant at $p < 0.01$; ***, significant at $p < 0.001$. The abbreviations are shown in Table 1 and Figures 1–3.

3.5. Factors Affecting EEA and EES

An integration of soil physicochemical and microbiological variables, as determined by the variance partitioning analysis (VPA) for the soil EEA, was found to be responsible for 94.0% of the variation. The individual effects of soil physicochemical and microbial properties could only explain for 28.0% and 2.7% of the variance, respectively, while the combined effect of soil physicochemical and microbiological characteristics could account for 63.2% of the EEA variance (Figure 6a). Furthermore, RDA also showed connections between soil EEA and its physicochemical and microbiological characteristics. The variance related to soil EEA was described by the first and second major axes, respectively, by 93.47% and 1.54%. The soil EEA variance, which was primarily driven by AP, MBN, and TN, was strongly linked with seven of the soil physicochemical and microbiological variables studied ($p < 0.01$, Figure 6b).

Similarly, VPA revealed that the physicochemical and microbiological properties of the soil contributed remarkably (66.1%) to the difference in soil EES (Figure 7a). Only 18.7% and 6.5% of the variation in soil EES was described by the corresponding individual categories, while 41.0% of the variation in EES was explained by the shared effects of the two categories (Figure 7a). The correlations between soil EES and soil physicochemical and microbiological parameters were demonstrated by the RDA ordination biplot. The variance related to soil EES was described by the first and second major axes, respectively, by 57.67% and 28.69%. Nine soil physicochemical and microbiological parameters were substantially linked with soil EES change, with TN ($p < 0.01$), TP ($p < 0.05$), and MBN ($p < 0.05$) being the main drivers (Figure 7b).
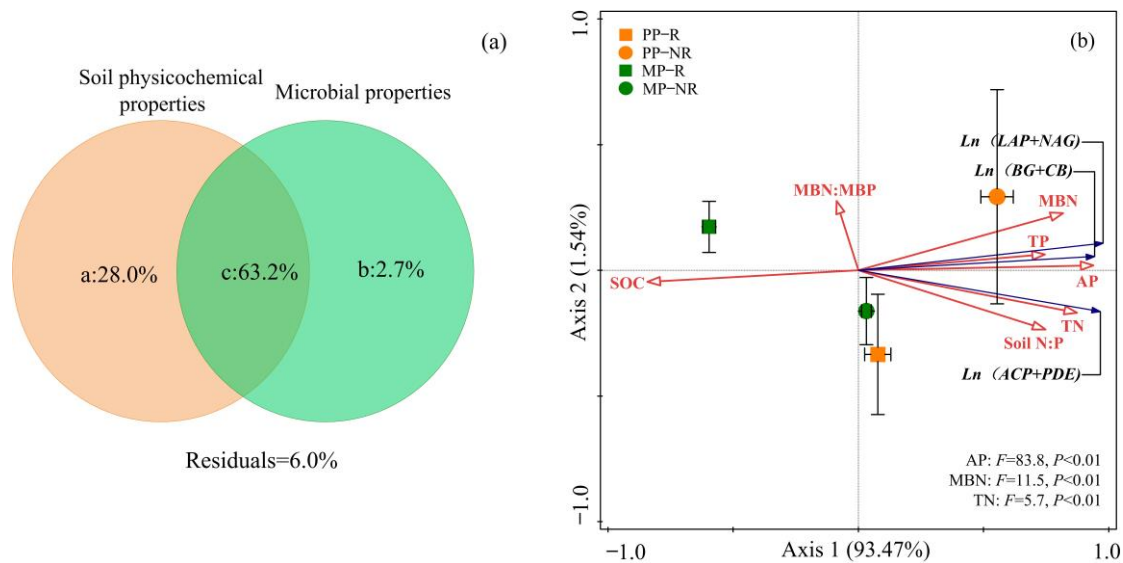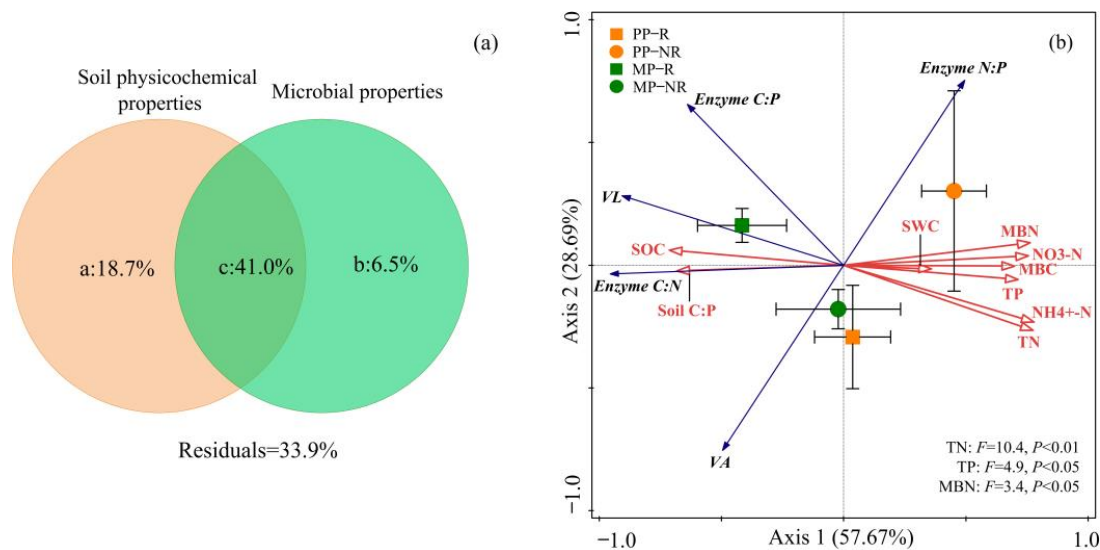
Figure 6. VPA of soil physicochemical and microbial properties on soil EEA (**a**). RDA reveals the variability of these two types as environmental factors on soil EEA (**b**). The abbreviations are shown in Table 1.

Figure 7. VPA of soil physicochemical and microbial properties on soil EES (**a**). RDA reveals the variability of these two types as environmental factors on soil EES (**b**). The abbreviations are shown in Table 1.

4. Discussion

4.1. Effects of N_2-Fixing Tree Species on Soil EEA in Rhizosphere and Non-Rhizosphere Soils

There is a strong link between soil EEA and microorganisms, which can reflect changes in microbial nutrient uptake capacity and clarify how vegetation type influences soil structure and function [37]. Most studies have only looked at bulk soil, which may overlook the role of plant roots and their secretions in the soil, as well as the direct and indirect effects on soil enzymes [38,39]. In our research, the extracellular enzyme activities of the MP significantly outperformed those of the PP, especially in the rhizosphere soils (Figure 1). This phenomenon has two causes. The first is that the increase in litter of N_2-fixing tree species not only provides richer organic matter for microbial growth [40], but also maintains a certain level of soil moisture, which is conducive to increasing microbial activity and promoting the production of extracellular enzymes [4,41]. Second, the increased

microbial biomass, which was sustained by root exudates, encouraged the soil EEA in the rhizosphere [42]. Furthermore, the MBC, MBN, and MBP levels in the rhizosphere we studied were significantly better than those in non-rhizosphere soil (Table 2). Numerous studies have discovered positive connections between enzyme activities and microbial biomass [43,44], and this is also true for many other plant species. Additionally, the NO_3^--N, NH_4^+-N, TN, SOC, TP, and AP in the MP were all significantly increased (Table 2), which indicated that N_2-fixing tree species improve N availability [17], increasing the soil nutrient content and thereby improving soil quality [45]. Furthermore, the level of available N content affects the activity of enzymes involved in the C cycle [46]. Increased N supply can also encourage soil microorganisms to take up N and considerably raise the activity of N-acquiring enzymes [47]. Simultaneously, the improved N availability will stimulate phosphatase secretion, making more P available for microbial growth, and promoting an increase in P-acquiring enzyme activities [48]. Based on extensive research, microorganisms require external N inputs in order to degrade resistant substances like lignin [33]. This suggests that N_2-fixing tree species can speed up the degradation of lignin by encouraging soil oxidase activity [49]. The fact that soil EEAs positively influence one another is further supported by the fact that we discovered strong positive correlations between the three acquiring enzyme activities (Figure 4), which is in line with the majority of other research [50].

We carried out spatial ordination analysis (VPA and RDA) in order to more precisely pinpoint the variables that influence EEA. The findings demonstrated that the individual effects of categorical soil physicochemical and microbial properties with regard to explaining the variation in soil EEA and mineralization are much weaker than the shared effects of the factors investigated (Figure 6a). This shows the intricate mechanisms behind the effects of N_2-fixing tree species on soil EEA in *Eucalyptus* plantations and suggests interaction effects of soil physicochemical and microbiological features in soil EEA. Changes in soil AP, TN, and MBN that came along with the addition of a N_2-fixing tree species had the strongest associations with the EEA among the abiotic and biotic components (Figure 6b). Yang et al. found that variations in soil EEA are mostly caused by changes in soil nutrients and microorganisms, which is accordance with our view [51]. This suggests that the positive associations between the variables likely reflect the impacts of the addition of a N_2-fixing tree species into *Eucalyptus* plantations.

4.2. Soil EES Shows Microbial Nutrient Limitation in Eucalyptus Plantations Mixed with N$_2$-Fixing Tree Species

Our understanding of plant–microbe interactions under improved N availability is constrained by the absence of data on rhizosphere stoichiometry in response to the introduction of N_2-fixing tree species [52]. Here, we draw specific evidence from our research that a N_2-fixing tree species differentially affects EES and microbial stoichiometric ratios between rhizosphere and non-rhizosphere soils (Table 2, Figure 2). In addition, C:N:P acquisition ratios can link nutrient effectiveness to microbial biomass [53], and vector analyses can quantify nutrient limitation by soil microbes. Sinsabaugh et al. have shown that the C:N:P ratio in soil enzymes is roughly 1:1:1 globally, though it may fluctuate somewhat from region to region depending on how well soil microbial organisms are able to acquire nutrients [54]. Enzyme C:N:P ratios of the PP (1.12:1:1.10) and the MP (1.07:1:1.08) in this study both deviated from 1:1:1, showing that soil microorganisms in this study region are not only restricted by C but also by P, and they were more restricted by C than by P. Microorganisms are susceptible to microbial nutrient limitation when they require more nutrients for development than are available or when the rate of growth is faster than the rate of nutrient acquisition [55]. Additionally, the C:N:P ratio was closer to the global average in the MP than that in the PP, implying that the MP experienced less soil microbial nutrient limitation. VL > 1 and VA > 45° can further evidence that soil microorganisms are not only restricted by C but also by P. Meanwhile, the reduced VL and VA in the MP than in the PP also indicated that soil microbial nutrient limitation was

alleviated in the MP. CQI is an indicator of the percentage soil recalcitrant C content [36]. CQI in the MP was less than in the PP, indicating a reduction in the recalcitrant C content in the MP from roots and root exudates, and an increase in the microbially available C content, thereby alleviating soil microbial C limitation [56].

Similarly, enzyme C:N:P ratios of rhizosphere (1.08:1:1.09) and non-rhizosphere (1.13:1:1.10) soils in this study both diverged from 1:1:1, and the rhizosphere C:N:P ratios were closer to the global average, suggesting that soil microorganisms in rhizosphere soils are less sensitive to C and P limitation. The main reason for this may be the rhizosphere effect. Additionally, SOM breakdown also provides the majority of the P that microorganisms need [57]. Additionally, root secretions provide a rich substrate for rhizosphere microorganisms and promote rhizosphere soil P acquisition [58]. Our research supposed that the addition of a N_2-fixing tree species might alter the vegetation types and microbial community structure, influencing soil microbial resource acquisition strategies, and thereby alleviating their nutrient limitation, which resembles the previous view [59]. Additionally, studies have shown that nutrient limitation due to microorganisms is readily transferred between C, N, and P [60,61]. Our research revealed strong relationships between the different soil EES (Figure 5), indicating that introducing N_2-fixing tree species can have a big impact on the soil metabolic activity and nutrient requirements.

The stoichiometric ratios of microbial biomass are useful indications for assessing the condition of soil nutrient availability because they show how well microorganisms can absorb and break down soil nutrients [62]. The soil EES and microbial biomass stoichiometry ratios in this investigation showed a substantial association (Figure 5). This may be mostly attributable to the addition of N_2-fixing tree species increasing the amount of organic matter (through litter decomposition and root exudates), which is very advantageous to microbial biomass. The N usage efficiency is encouraged by reduced microbial competitive ability [62]. Therefore, having more nutrients available in the MP may lessen the rivalry between plants and microbes. Soil microorganisms have access to more C, N, and P and lower MBC:MBN and MBC:MBP ratios in rhizosphere or non-rhizosphere soils [63]. Noteworthy relationships were also found between VL and CQI and soil nutrient ratios, as well as between VL, VA and CQI, and microbial stoichiometry ratios (Figure 5). The findings demonstrated that the stand conversion modifies soil and microbial properties, modifies the strategies for acquiring soil microbial resources, and may even harmonize soil microbial nutrient restriction [51,64].

Similar to soil EEA, soil and microbiological characteristics best explained the discrepancy in soil EES (Figure 7a). Specifically, the soil TN, TP, and MBN were the most important factors affecting EES (Figure 7b). Accordingly, soil chemical characteristics may significantly affect soil EES by changing the concentrations of substrates that are available [65], indicating that edaphic variables are substantially responsible for controlling soil EES (Figure 8), which is in line with what Qiu et al. found [64]. This is due to the fact that the existence of a direct relationship between soil nutrients and microorganisms has a contributing effect on soil enzyme production, and consequently on soil EES [54]. The processes of soil nutrients and microbes in regulating soil EES are complex [41], so we chose to explain them clearly and intuitively in the form of conceptual diagrams. Both microbial and soil factors change as a result of the introduction of *E. fordii* into *Eucalyptus* stands; however, the changes in soil EES are best explained by the changes in the soil factors. By increasing nutrient content and availability, soil characteristics carry weight on soil EEA and consequently soil EES [66]. In terms of microbial properties, microbial biomass reflects microbial activity and physiological metabolism [67], with a greater microbial biomass contributing to the production of soil enzymes [65]. Generally, the addition of N_2-fixing species into *Eucalyptus* plantations, to create mixed forests, can increase the N availability and soil nutrient content [68], enhance microbial activity, encourage the synthesis of soil enzymes [69], and alleviate nutrient limitation [51,64]. Overall, the rhizosphere is a critical microzone that creates a relationship between the plant and microbial communities that is essential for controlling ecoenzymatic stoichiometry and nutrient cycling [70]. The study

of enzyme stoichiometric features under different plant and soil types is important for elucidating root and soil effects on ecological enzymes.
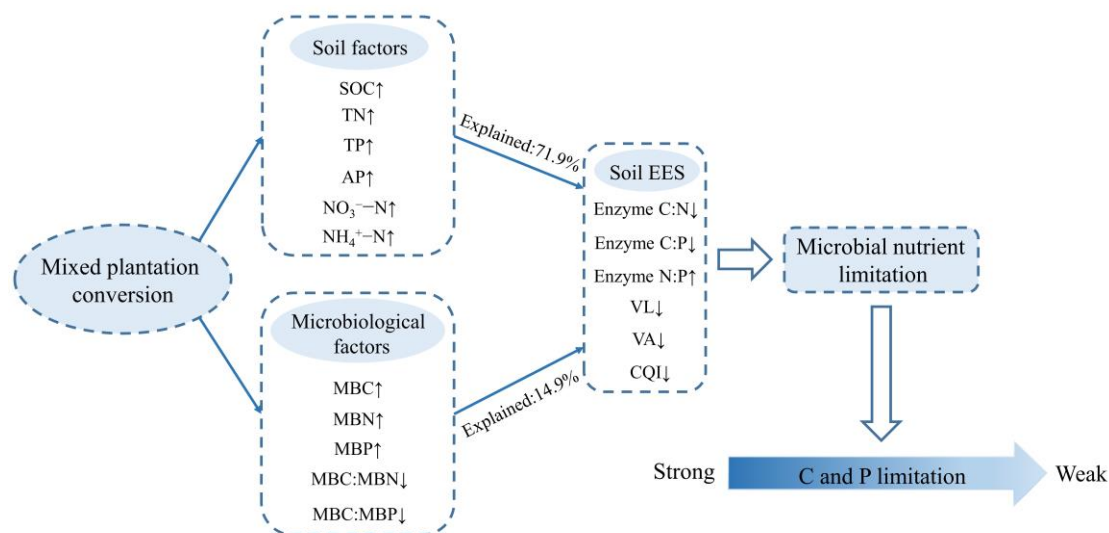

Figure 8. Conceptual map of soil factors and microbiologial factors on microbial nutrient limitation following the introduction of a N_2-fixing tree species into *Eucalyptus*, as indicated by soil extracellular enzyme stoichiometry (EES). SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus; MBC:MBN, microbial biomass carbon to microbial biomass nitrogen ratio; MBC:MBP, microbial biomass carbon to microbial biomass phosphorus ratio; Enzyme C:N, ln(BG + CB):ln(LAP + NAG); Enzyme C:P, ln(BG + CB):ln(ACP + PDE); Enzyme N:P, ln(LAP + NAG):ln(ACP + PDE); VL, vector length; VA, vector angle (°); CQI, carbon quality index. Arrow up indicates an increase; arrow down indicates a decrease.

5. Conclusions

In conclusion, the enigmatic stoichiometry method offers a practical method for analyzing interactions in the ecology of microbial resources. In *Eucalyptus* plantation ecosystems, C and P can co-limit soil microorganisms, but the limitation was reduced by the introduction of a N_2-fixing tree species, especially in the rhizosphere soils. This work sheds important light on how soil EEA and EES differ between rhizosphere and non-rhizosphere soils in *Eucalyptus* plantations. In addition, we discovered that microbial and soil characteristics both affect soil EEA (AP, MBN and TN) and EES (TN, TP and MBN). Our research should aid in the creation of management plans, such as those for the control of soil nutrients in subtropical Chinese *Eucalyptus* plantation ecosystems. While N_2-fixing tree species could help *Eucalyptus* plantations with their C and P shortages, judicious fertilization (e.g., applying phosphate fertilizer appropriately) and management (e.g., harvest residue retention) practices are also required for optimal growth.

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