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Introducing N_2-Fixing Tree Species into *Eucalyptus* Plantation in Subtropical China Alleviated Carbon and Nitrogen Constraints within Soil Aggregates

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Abstract: Soil extracellular enzymatic activity (EEA) and extracellular enzymatic stoichiometry (EES) within aggregates indicate variations in soil-nutrient effectiveness and the nutrient requirements of microorganisms. However, the responses of soil EEA and EES after introducing N_2-fixing tree species into *Eucalyptus* plantations are poorly understood. Therefore, we examined soils from a 15-year-old pure *Eucalyptus* urophylla plantation (PP) and mixed *E. urophylla* and *Acacia mangium* plantation (MP) based on the theory of EEA and EES at the aggregate scale. Aggregates were separated into four fractions using a dry-sieving procedure: >2, 1–2, 0.25–1, and <0.25 mm. We measured the EEA of soil carbon (C)-, nitrogen (N)-, and phosphorus (P)-acquiring enzymes, and examined potential factors (soil physicochemical properties, microbial biomass, and litterfall [LF]) that may influence EEA and EES. Significantly higher ($p < 0.05$) EEA levels in all aggregates were found in MP than in PP. The average natural logarithmic ratio of C-, N-, and P-acquiring enzyme activities in our study was 1.44:1.21:1, which deviated from the global mean ratio of 1:1:1 and implied that soil microbes were limited by C and N. Moreover, the enzyme C:N ratio ($E_{C:N}$), C:P ratio ($E_{C:P}$), and vector length (VL) were markedly lower ($p < 0.05$) in bulk soil and most aggregates in MP compared to PP, suggesting that C limitation was more serious in PP than in MP. Furthermore, while the vector angle (VA) of bulk soil and four aggregate sizes were all <45° in both the PP and the MP, they were markedly higher ($p < 0.05$) in bulk soil and >2 mm aggregate in MP than in PP. This indicated that mixing N_2-fixing species with *Eucalyptus* alleviated but did not eliminate N limitation. Our study also found that nitrate nitrogen (NO_3^--N), total nitrogen (TN), and microbial biomass C:P ratio (MBC:MBP) were the main factors driving changes in EEA, while LF was a key factor controlling EES ($p < 0.05$). Overall, introducing N_2-fixing species into the *Eucalyptus* plantation alleviated but did not eliminate C and N limitation. The results provide specific recommendations for soil-nutrient management in *Eucalyptus* plantations in subtropical China.

Keywords: *Eucalyptus* plantation; N_2-fixing species; ecoenzymatic activity and stoichiometry; soil aggregates

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1. Introduction

Soil extracellular enzymes synthesized by microbes and plant roots promote the biogeochemical cycles of terrestrial ecosystems, and extracellular enzymatic activity (EEA) provides an important indicator of microbial demand for resources [1,2]. Soil extracellular enzyme stoichiometry (EES) indicates microbial resource limitation status [3–6]. The global logarithmic ratio of carbon (C)-, nitrogen (N)-, and phosphorus (P)-acquiring enzyme is about 1:1:1 [7], but microorganisms change the extracellular enzymes they release to cope with the resource-limited conditions of different ecosystems, resulting in deviations in

EES. For example, the previous research found that the soil EES deviated from the global value in different seasons and thinning treatments [8], implying that the EES in the region is dependent on the availability of soil resources rather than homeostasis. In addition, changes in EES are related to the patterns of microbial nutrient effectiveness after forest stand transformation [2,9]. Some scholars had suggested that abiotic factors (e.g., soil nutrient status and pH) [10,11] and biotic factors (e.g., plant properties and microbial characteristics) [3,12] have a combined effect on soil EEA and EES. Although their effects on EEA and EES are of concern, the relative impacts of land-use changes (e.g., afforestation) on EEA and EES have been little investigated [13].

Being the essential unit of soil structure, aggregates are associated with soil nutrients. This has important implications for the composition and function of microbial communities [14,15], which may affect soil-nutrient allocation and utilization [16]. It has been shown that EEA varies in different aggregate sizes [17]. The content and availability of organic matter and nutrients varies among various aggregates sizes, and therefore the distribution of EEA in soil aggregates differs [18]. In addition, variations in EEA within aggregates reveal the effects of habitat structure on microbial biodiversity and activity [15,19]. Land-use changes have no direct impact on the variation of soil EEA, but since such changes alter the composition of aggregate sizes, the aggregates play crucial roles in the changes of EEA during land-use changes [20,21].

Eucalyptus is widely planted in subtropical China because it offers a fast growth rate and short rotation periods. Guangxi is an important *Eucalyptus*-growing region in China, accounting more than 45% of the country's *Eucalyptus* plantation area (about 4,500,000 hm^2) [22]. However, the short rotation period with continuous cropping of *Eucalyptus*, combined with long-term monoculture planting patterns, have resulted in the gradual emergence of various ecological issues (e.g., decline of forest productivity and land degradation) [23,24]. Generally, N is the main limiting factor affecting forest productivity [25]. Therefore, the "N-generation effect" resulting from continuous planting of *Eucalyptus* has received much attention from researchers. Previous studies have shown that changes in forest structure have an impact on biodiversity and are of great significance to the stability and productivity of forest ecosystems [26,27]. In addition, the introduction of N_2-fixing tree species into *Eucalyptus* plantations improved the quantity and quality of litter, changed the structure and function of microbial communities, enhanced soil C stability and N availability, and improved the structure of soil aggregates [28,29]. However, the impacts of land-use changes on EEA and EES remain unclear. This study investigated the impacts on EEA and EES within aggregates of introducing N_2-fixing species into *Eucalyptus* plantations. We hypothesized that (i) soil microbes would be limited by both C and N in our study area, and that (ii) EEA and EES would be changed by introducing N_2-fixing tree species, and illuminated by variations of soil abiotic and biotic factors. Thus, our main aims were to (i) determine the effects on EEA and EES within aggregates of introducing N_2-fixing tree species, and (ii) clarify soil microbial resource limitations and determine the essential drivers of EEA and EES.

2. Materials and Methods

2.1. Experimental Site

The research site was located in the Guangxi Youyiguang Forest Ecosystem Research Station (22°10′ N, 106°50′ E) of Tropical Forestry Experimental Center, Chinese Academy of Forestry, in Pingxiang City, China. The region has a subtropical monsoon climate, with distinct dry and wet seasons. The average annual rainfall is 1400 mm, with 60%–75% falling between April and September. The average annual temperature is 21 °C [30]. The landform type is mainly low hills and mountains. According to the Chinese soil classification, the soil type is predominantly a red soil that formed from granite, which is the equivalent of oxisol in the USDA Soil Taxonomy [28].

The forest types used in our research included 15-year-old mixed *Eucalyptus urophylla* and *Acacia mangium* plantation (MP) and 15-year-old *E. urophylla* pure plantation (PP)

as a control (CK). The plantations were adjacent to each other. The PP and the MP (mixed ratio = 1:1) were planted in 2004 after clear-cutting of *Pinus massoniana* plantations in 1977. Before planting, 500 g per plant of base fertilizer was applied and followed by semi-annually fertilization for the first 2 years, with total application of 200 kg hm^{-2} N, 150 kg hm^{-2} P, and 100 kg hm^{-2} K both in PP and MP. Similar stand management was adopted for the two stand types during the experiment. The detailed experimental design and basic information were reported by Huang et al. [29]. In August 2019, five 400 m^2 (20 m × 20 m) plots were installed at random in both the PP and the MP stands, considering variations in spatial representation and topography. Vegetation surveys were carried out on the test plots to determine the stand density (SD), diameter at breast height (DBH), and tree height (TH). Fine root samples to a depth of 10 cm were collected from each plot by ways of soil coring with a soil corer made of a cylindrical stainless hole cutter. Live roots ≤ 2 mm in diameter were manually picked and washed clean of soil residues. Six litter traps (1 m × 1 m) with 1 mm mesh size were randomly emplaced in every sample plot. Fine root and litterfall (LF) were weighed after oven-drying at 65 °C. The annual LF output during our study period was determined. Table 1 provides details of the sample plots.

Table 1. Main properties of the two experimental stands (mean ± standard error, n = 5).

Stand Type	Elevation (m)	Slope (°)	Age (Years)	SD (Trees·hm^{-2})	DBH (cm)	TH (m)
PP	232	24	15	580 ± 23	20.43 ± 1.32	24.82 ± 0.93
MP	241	22	15	-	-	-
Eucalyptus urophylla	-	-	-	300 ± 14	21.22 ± 1.56	24.65 ± 2.31
Acacia mangium	-	-	-	310 ± 31	15.81 ± 1.08	20.82 ± 1.69

Note: PP, pure plantation of *Eucalyptus urophylla*; MP, mixed plantation of *E. urophylla* and *Acacia mangium*. SD, stand density; DBH, diameter at breast height; TH, tree height.

2.2. Soil Sampling

Soil samples were acquired in August 2019 from six systematic sampling points selected in each plot, each 5 m from the center of the plot. Specifically, every sampling point was set randomly at 0°, 60°, 120°, 180°, 240°, and 300° around the plot center. After removing dead leaves and other impurities (about 0.5–1.5 cm thick) from the mineral soil surface, six undisturbed soil samples were obtained at soil depths of 0–10 cm, mixed into a composite sample for each plot, stored in a sterile container, and delivered to the laboratory. Every sample was carefully broken into natural soil particles and impurities (e.g., gravels and plant roots) were eliminated. The soil sample was divided into two parts; one part was sieved through a 2 mm mesh and used to determine bulk soil properties, and the other part was used for isolation of soil aggregate [31].

2.3. Soil Aggregate Separation

The soil samples were dried at 4 °C until the gravimetric water concentration of ~100 g H_2O kg^{-1} was reached and gently broken apart along their natural structures. Soil aggregates were divided using the dry-sieving method described by Schutter and Dick [32]. Briefly, soil samples (500 g) were passed sequentially through sieves of 2, 1, and 0.25 mm diameter, followed by 15 min of vertical oscillation at the rate of 1 oscillation s^{-1}. Four different sizes of aggregate were obtained: >2 mm (large macro-aggregates), 1–2 mm (medium macro-aggregates), 0.25–1 mm (small macro-aggregate), and <0.25 mm (micro-aggregates). A portion of soil aggregates were stored at −20 °C for the analysis of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and biological properties, and the others were air-dried to determine physical and chemical properties.

2.4. Physicochemical Analyses of Litterfall and Soil

We determined the C and N contents of litterfall using the C/N elemental analyzer (Vario EL III, Elementar, Langenselbold, Germany). Soil pH was determined using a pH meter with a soil-to-water ratio of l:2.5 (m/v). Soil organic carbon (SOC) was measured

using the $K_2Cr_2O_7$-H_2SO_4 oxidation approach [33]. Total nitrogen (TN) was measured following the Kjeldahl digestion approach, with digestion at 380 °C for 60 min [34]. Total phosphorus (TP) was digested with H_2SO_4-$HClO_7$ and measured by the molybdenum blue colorimetric method [35]. Soil ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were extracted with a 0.01 M $CaCl_2$ solution and determined by a continuous flow analyzer (SEAL AA3, SEAL Analytical, Norderstedt, Germany).

2.5. Soil Microbial Biomass Analysis

Soil microbial biomass C (MBC) and N (MBN) were measured using the chloroform fumigation extraction approach [36]. In brief, soil samples were fumigated for 24 h, extracted using 0.5 M K_2SO_4, and shaken for 30 min. The samples were then filtered and the filtrate was analyzed in a Jena Multi N/C 3100 CN Analyzer (Analytik Jena, Jena, Germany). In addition, soil microbial biomass P (MBP) was determined using the chloroform fumigation extraction approach [37]. In brief, soil samples were fumigated for 24 h, extracted using 0.5 M $NaHCO_3$, and shaken for 30 min. The samples were then filtered and the filtrate was analyzed by the molybdenum blue colorimetric method.

2.6. Analysis of Soil Extracellular Enzyme Activity

The activities of five soil extracellular enzymes, including C-acquiring enzymes (β-1,4-glucosidase [BG] and β-D-cellobiosidase [CB]), N-acquiring enzymes (β-1,4-N-acetylglucosaminidase [NAG] and leucine aminopeptidase [LAP]), and P-acquiring enzymes (acid phosphatase [ACP]), were measured using 96-well microplate fluorescence technology [38]. Enzyme activity was calculated by reading the fluorescence after the enzyme reacted with the substrate. To prepare a soil suspension, we weighed 1.25 g (fresh weight) of soil, added 125 mL of sodium acetate buffer solution (50 mM, pH = 4.5), and stirred for 1 min. We set up eight replicate wells for each soil sample. After 3 h incubation at 25 °C under dark conditions, we added 5 μL NaOH (0.5 M) to stop the reaction, and the fluorescence was determined as described by Looby and Treseder [39]. We used nmol g^{-1} soil h^{-1} to express the EEA. Table S1 provides details of the various soil extracellular enzymes and associated substrates.

2.7. Data Calculation and Analysis

Soil aggregate stability is commonly measured by the mean weight diameter (MWD), which is calculated using the following formula [40]:

$$\mathrm{MWD} = \sum_{i=1}^{n} (X_i \times M_i) \quad (1)$$

where X_i is the average diameter of the ith size fractions (mm) and M_i is the percentage of the ith size fraction in total soil (%).

The relative enzyme activity index (REAI) and the relative enzyme activity comprehensive index (REACI) were used as indicators of the protective effects of soil aggregates on EEA. The REAI takes into account the EEA of both the bulk soil and the aggregates and can objectively reflect the protective effects of soil aggregates on EEA. When the REAI value is >1, soil aggregates have a protective effect on the EEA, and the higher the value, the stronger the protection. In addition, REACI values indicate the overall protective effect of aggregates on the activities of C-, N-, and P-acquiring enzymes [41].

$$\mathrm{REAI} = EA_i / EA_s \quad (2)$$

$$\mathrm{REACI} = (\mathrm{REAI_C} + \mathrm{REAI_N} + \mathrm{REAI_P})/3 \quad (3)$$

where EA_i denotes the enzyme activity in the ith-size aggregate; EA_s denotes the whole-soil enzyme activity; and $\mathrm{REAI_C}$, $\mathrm{REAI_N}$, and $\mathrm{REAI_P}$ represent the REAI related to soil C-, N-, and P-cycling, respectively.

Soil EES reflects the relative resource limitation of microbes. The soil enzyme C:N, C:P, and N:P ratios ($E_{C:N}$, $E_{C:P}$, and $E_{N:P,}$ respectively) were determined as follows [42]:

$$E_{C:N} = \ln(BG + CB) / \ln(NAG + LAP) \quad (4)$$

$$E_{C:P} = \ln(BG + CB) / \ln(ACP) \quad (5)$$

$$E_{N:P} = \ln(NAG + LAP) / \ln(ACP) \quad (6)$$

Vector length (VL) and vector angle (VA) were measured using the equations of Moorhead et al. [43]:

$$VL = SQRT\left(x^2 + y^2\right) \quad (7)$$

$$VA = \text{DEGREES}(ATAN2(x, y)) \quad (8)$$

where x = ln (BG + CB)/ln (ACP) and y = ln (BG + CB)/ln (NAG + LAP). The VL implies the degree of soil microbial C limitation. A VA of less than 45° was considered N-limited, while a VA above 45° was considered P-limited, and the greater the deviation, the stronger the limitation.

Statistical analysis and regressions were conducted using SPSS 25.0 (IBM, Chicago, IL, USA) software. We used independent-sample *t*-tests to examine the degree of deviation in soil physicochemical characteristics, EEA, and EES between the PP and the MP. Differences were considered significant at $p < 0.05$. Principal component analysis (PCA) was used to identify whether there were significant variations in EEA and EES between the PP and the MP. The main factors affecting EEA and EES were determined by a redundancy analysis (RDA), using the Monte Carlo permutation test to determine the significance of each environmental factor in the ranking ($p < 0.05$). The PCA and RDA were performed using CANOCO 5.0 software. Graphs were plotted using Origin Pro 9.0 (OriginLab, Northampton, MA, USA) software.

3. Results

3.1. Plant and Bulk Soil Characteristics

Litterfall was 71.89% higher in MP than in PP ($p < 0.01$). However, the C/N ratio of LF was 25.23% lower in MP than in PP ($p < 0.01$). There was no significant difference in root biomass between PP and MP ($p > 0.05$). The soils were acidic, with pH values of 4.67–5.22, and the values were markedly higher in MP compared to PP. In addition, SOC, TN, NO_3^--N, MWD, $C:P_{soil}$, and $N:P_{soil}$ were also markedly higher in MP compared to PP ($p < 0.05$; Table 2).

Table 2. Plant and bulk soil properties in PP and MP (mean ± standard error, n = 5).

Sample	Item	Stand Type	
		PP	MP
Litter	Amount ($kg \cdot hm^{-2} \cdot years^{-1}$)	4907.19 ± 127.25 b	8434.85 ± 199.90 a
	$C:N_{litter}$	50.47 ± 1.34 a	37.74 ± 1.09 b
Root	Biomass ($kg \cdot hm^{-2}$)	1124.08 ± 60.82 a	995.67 ± 42.78 a
Soil	SOC ($g \cdot kg^{-1}$)	15.54 ± 0.36 b	19.01 ± 0.79 a
	TN ($g \cdot kg^{-1}$)	1.23 ± 0.06 b	1.72 ± 0.09 a
	$C:N_{soil}$	12.74 ± 0.70 a	11.16 ± 0.63 a
	TP ($g \cdot kg^{-1}$)	0.23 ± 0.01 a	0.22 ± 0.01 a
	$C:P_{soil}$	67.21 ± 3.65 b	89.04 ± 4.75 a
	$N:P_{soil}$	5.36 ± 0.47 b	8.00 ± 0.29 a
	NH_4^+-N ($mg \cdot kg^{-1)}$	28.80 ± 1.43 a	24.82 ± 0.94 a
	NO_3^--N ($mg \cdot kg^{-1}$)	5.51 ± 0.13 b	12.26 ± 0.65 a
	pH	4.67 ± 0.06 b	5.22 ± 0.12 a
	MWD (mm)	1.66 ± 0.01 b	1.98 ± 0.04 a

Note: $C:N_{litter}$, litter carbon to nitrogen ratio; SOC, soil organic carbon; TN, total nitrogen; $C:N_{soil}$, soil organic carbon to total nitrogen ratio; TP, total phosphorus; $C:P_{soil}$, soil organic carbon to total phosphorus ratio; $N:P_{soil}$, soil total nitrogen to total phosphorus ratio; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; MWD, mean weight diameter. Different lowercase letters show marked variations between the PP and the MP ($p < 0.05$).

3.2. Soil Enzyme Activity within Bulk Soil and Aggregates

The mixed N_2-fixing species and *Eucalyptus* plantation significantly increased soil EEA ($p < 0.05$; Figure 1). Specifically, C-acquiring enzyme (BG + CB) activity increased markedly ($p < 0.05$) in the MP by 45.88%, 48.37%, 42.09%, 40.34%, and 34.18% for bulk soil and >2, 1–2, 0.25–1, and <0.25 mm aggregates, respectively (Figure 1a). Furthermore, N-acquiring enzyme (NAG + LAP) activity increased markedly ($p < 0.01$) in the MP by 83.62%, 87.61%, 89.72%, 87.09%, and 68.37% for bulk soil and >2, 1–2, 0.25–1, and <0.25 mm aggregates, respectively (Figure 1b). Finally, P-acquiring enzyme (ACP) activity increased markedly ($p < 0.01$) in the MP by 107.55%, 91.02%, 52.23%, 102.15%, and 56.55% for the bulk soil and >2, 1–2, 0.25–1, and <0.25 mm aggregates, respectively (Figure 1c).

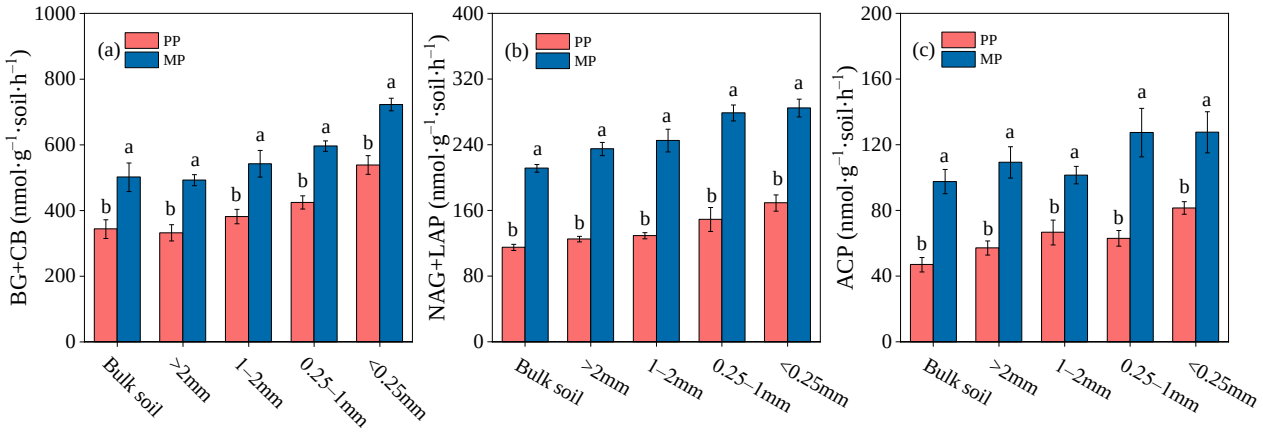

Figure 1. Soil extracellular enzymatic activity (EEA) within bulk soil and aggregates in PP and MP (**a**–**c**). Different lowercase letters denote marked variations between the PP and the MP ($p < 0.05$). Error bars denote standard errors (n = 5).

We found that most aggregates were protective of soil EEA. The $REAI_C$ and $REAI_N$ ranged from 0.84 to 1.37 and 0.91 to 1.23, respectively, and increased with decreasing aggregate particle size. The $REAI_P$ ranged from 0.89 to 1.29, with almost no distinction among the aggregate fractions ($p > 0.05$). In addition, REACI values were more than 1 in the 0.25–1 and <0.25 mm aggregates and tended to raise as the aggregate size decreased (Table 3).

Table 3. Soil relative enzyme activity index (REAI) and relative activity comprehensive index (REACI) within soil aggregates in PP and MP (mean ± standard error, n = 5).

Stand Type	Aggregate Size	$REAI_C$	$REAI_N$	$REAI_P$	REACI
PP	>2 mm	0.84 ± 0.05 c	0.91 ± 0.01 b	0.89 ± 0.04 b	0.88 ± 0.02 c
	1–2 mm	0.97 ± 0.04 bc	0.94 ± 0.04 b	1.03 ± 0.09 b	0.98 ± 0.03 bc
	0.25–1 mm	1.08 ± 0.07 b	1.07 ± 0.07 ab	0.99 ± 0.05 b	1.05 ± 0.05 b
	<0.25 mm	1.37 ± 0.06 a	1.23 ± 0.06 a	1.29 ± 0.10 a	1.29 ± 0.05 a
MP	>2 mm	0.90 ± 0.02 c	0.94 ± 0.02 b	0.96 ± 0.05 a	0.93 ± 0.02 b
	1–2 mm	1.00 ± 0.07 bc	0.97 ± 0.03 b	0.91 ± 0.08 a	0.96 ± 0.06 b
	0.25–1 mm	1.10 ± 0.04 b	1.11 ± 0.04 a	1.12 ± 0.08 a	1.11 ± 0.05 a
	<0.25 mm	1.33 ± 0.04 a	1.13 ± 0.02 a	1.13 ± 0.09 a	1.20 ± 0.03 a

Note: $REAI_C$, relative enzyme activity index of soil C-acquiring enzyme; $REAI_N$, relative enzyme activity index of soil N-acquiring enzyme; $REAI_P$, relative enzyme activity index of P-acquiring enzyme; REACI, relative enzyme activity comprehensive index of soil enzymes. Lowercase letters in each column denote marked variations ($p < 0.05$) among soil aggregate fractions within the PP or the MP.

3.3. Soil Enzyme Stoichiometry within Bulk Soil and Aggregates

$E_{C:N}$, $E_{C:P}$, and $E_{N:P}$ values were all greater than one (Figure 2). Specifically, the $E_{C:N}$ values of bulk soil and all aggregates were markedly ($p < 0.05$) lower in MP than in PP

(Figure 2a). Furthermore, the $E_{C:P}$ values of bulk soil and most aggregates (except the 1–2 mm aggregate) were markedly ($p < 0.05$) lower in MP than in PP (Figure 2b). Finally, the $E_{N:P}$ values of bulk soil and >2 mm aggregate were markedly ($p < 0.05$) lower in MP than in PP, while there were no obvious ($p > 0.05$) differences among the 1–2, 0.25–1, and <0.25 mm aggregates (Figure 2c).

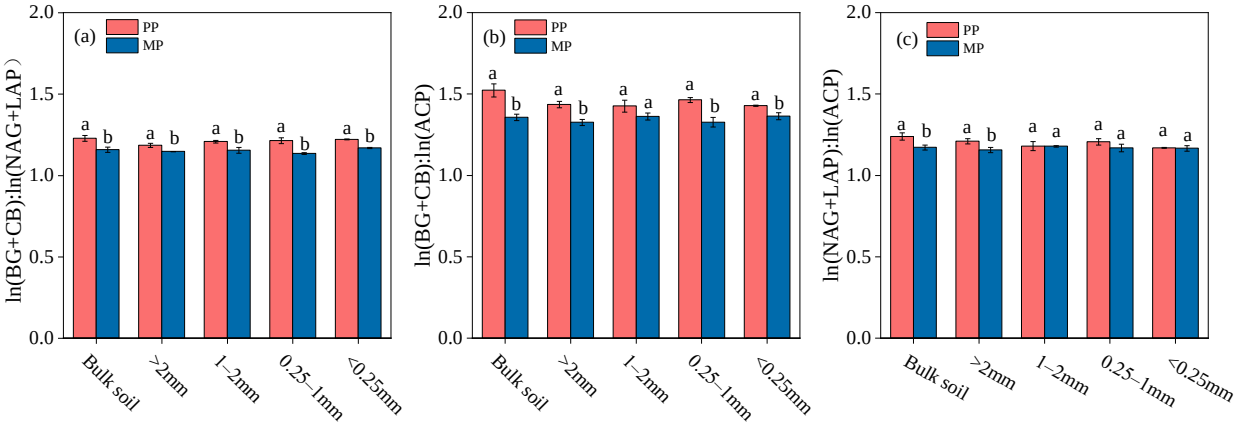

Figure 2. Soil extracellular enzymatic stoichiometry (EES) within bulk soil and aggregates in PP and MP (**a**–**c**). Different lowercase letters denote marked variations between the PP and the MP ($p < 0.05$). Error bars denote standard errors (n = 5).

VL and VA differed between the PP and the MP (Figure 3). The VL of bulk soil and most aggregates were markedly ($p < 0.01$) lower in MP than in PP (except 1–2 mm aggregate) (Figure 3a). The VA of bulk soil and all aggregate sizes were <45° in both the PP and the MP. Furthermore, the VA of bulk soil and >2 mm aggregate size were markedly ($p < 0.05$) higher in MP compared to PP, while there were no marked differences ($p > 0.05$) among the 1–2, 0.25–1, and <0.25 mm aggregate sizes (Figure 3b).

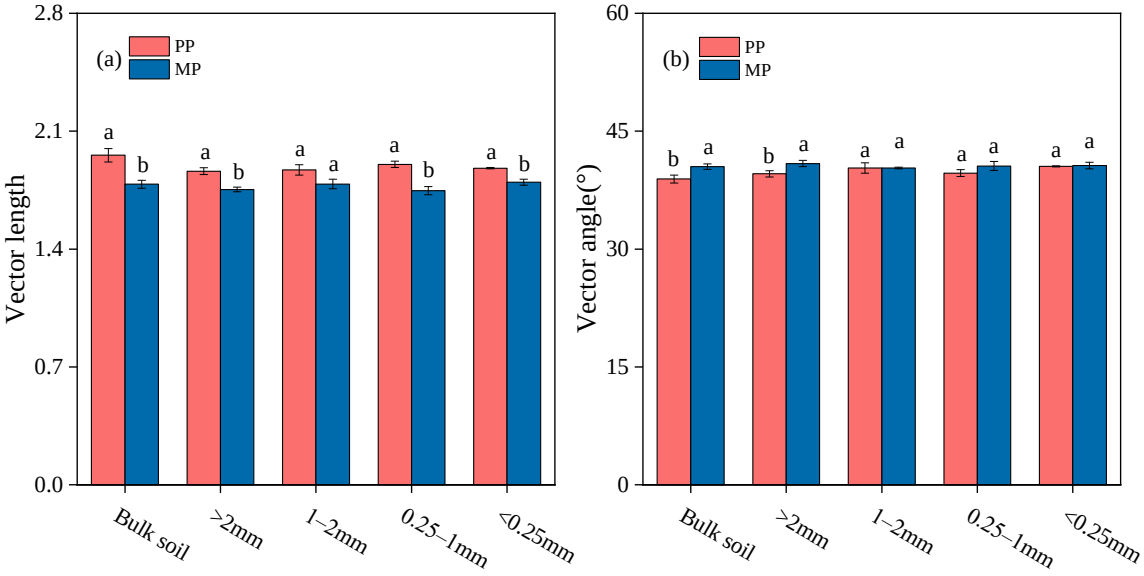

Figure 3. Vector length (VL) (**a**) and vector angle (VA) (**b**) in bulk soil and aggregates in PP and MP. Different lowercase letters denote marked variations between the PP and the MP ($p < 0.05$). Error bars denote standard errors (n = 5).

3.4. Factors Influencing Soil Enzyme Activities and their Stoichiometry

The linear regression analysis indicated that enzyme N vs. C activities, enzyme C vs. P activities, and enzyme N vs. P activities were significantly and positively correlated

in both the PP and the MP ($p < 0.001$; Figure 4a–c). In contrast, VA was markedly and negatively related to VL ($p < 0.001$; Figure 4d). Furthermore, $E_{C:N}$ was markedly and positively related to soil C:N and MBC:MBN ratios ($p < 0.01$; Figure 5a,d). Similarly, $E_{C:P}$ was markedly and positively related to MBC:MBP ratios ($p < 0.01$; Figure 5e), while the $E_{C:P}$ and soil C:P ratios and $E_{N:P}$ and soil N:P ratios were markedly negative correlations ($p < 0.05$; Figure 5b,c). However, there was no marked relationship between the $E_{N:P}$ and MBN:MBP ratios ($p > 0.05$; Figure 5f).

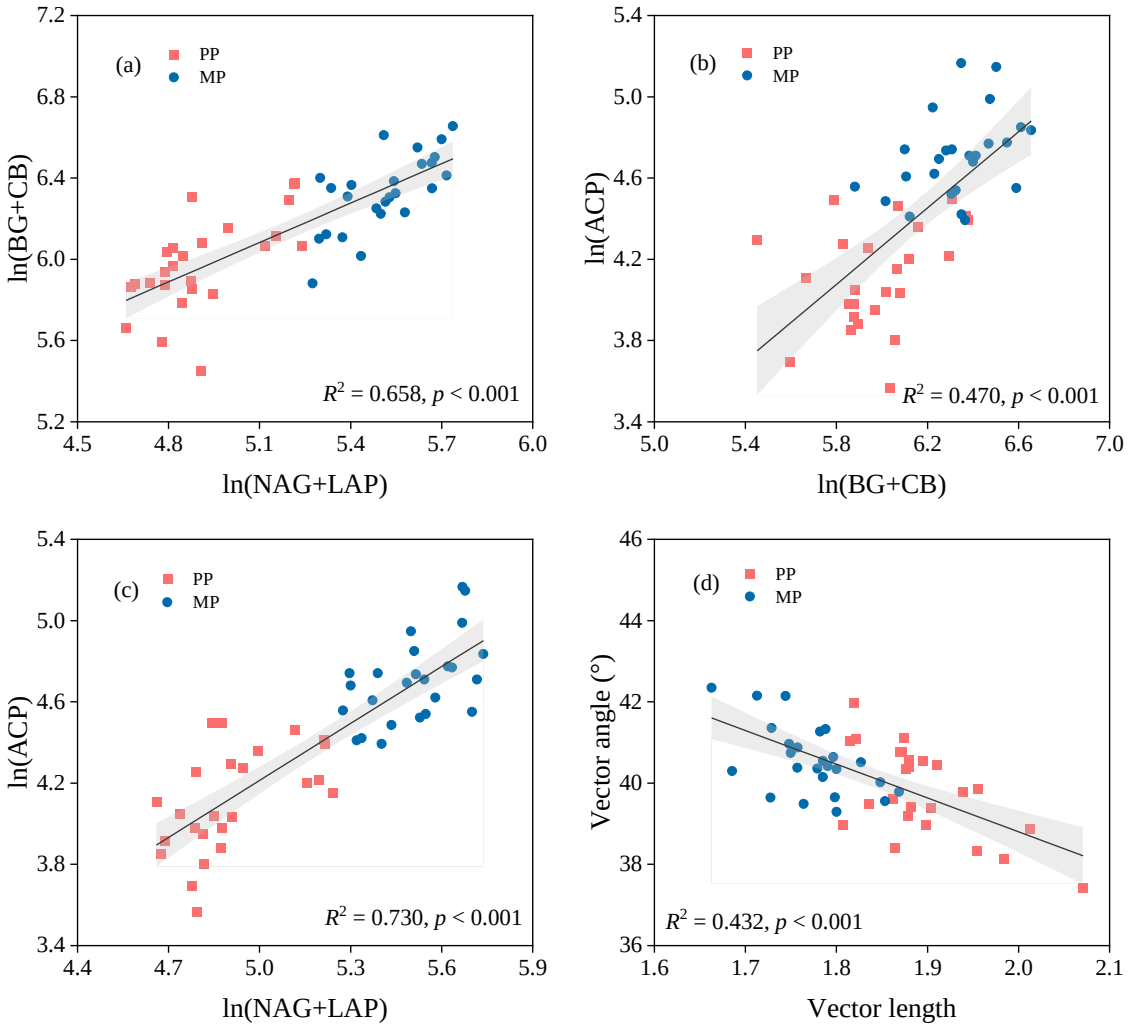

Figure 4. Relationships among soil extracellular enzymatic activity (EEA) (**a**–**c**), and between vector length (VL) and vector angle (VA) (**d**).

The RDA revealed that soil physicochemical characteristics, LF, and microbial characteristics together explained the total variation in EEA and EES. Specifically, the first and second axis explained 77.45% and 1.46% of the total effect in EEA, respectively (Figure 6a). More than 75% of the effect in EEA was explained by 14 factors (sum of Lambda-B in Table 4), with NO_3^--N ($F = 95.2$; $p = 0.002$), TN ($F = 9.2$; $p = 0.002$), and MBC:MBP ($F = 4.4$; $p = 0.018$) significantly ($p < 0.05$) correlated with EEA (Figure 6a; Table 4). Furthermore, the first and second axis explained 44.88% and 4.22% of the total effect in EES, respectively (Figure 6b). The 14 variations jointly explained almost 50% of the effect in EES (sum of Lambda-B in Table 5). Finally, forward selection of the 14 variations in the RDA demonstrated that EES was affected mainly by LF ($F = 29.6$; $p = 0.002$) (Figure 6b; Table 5).

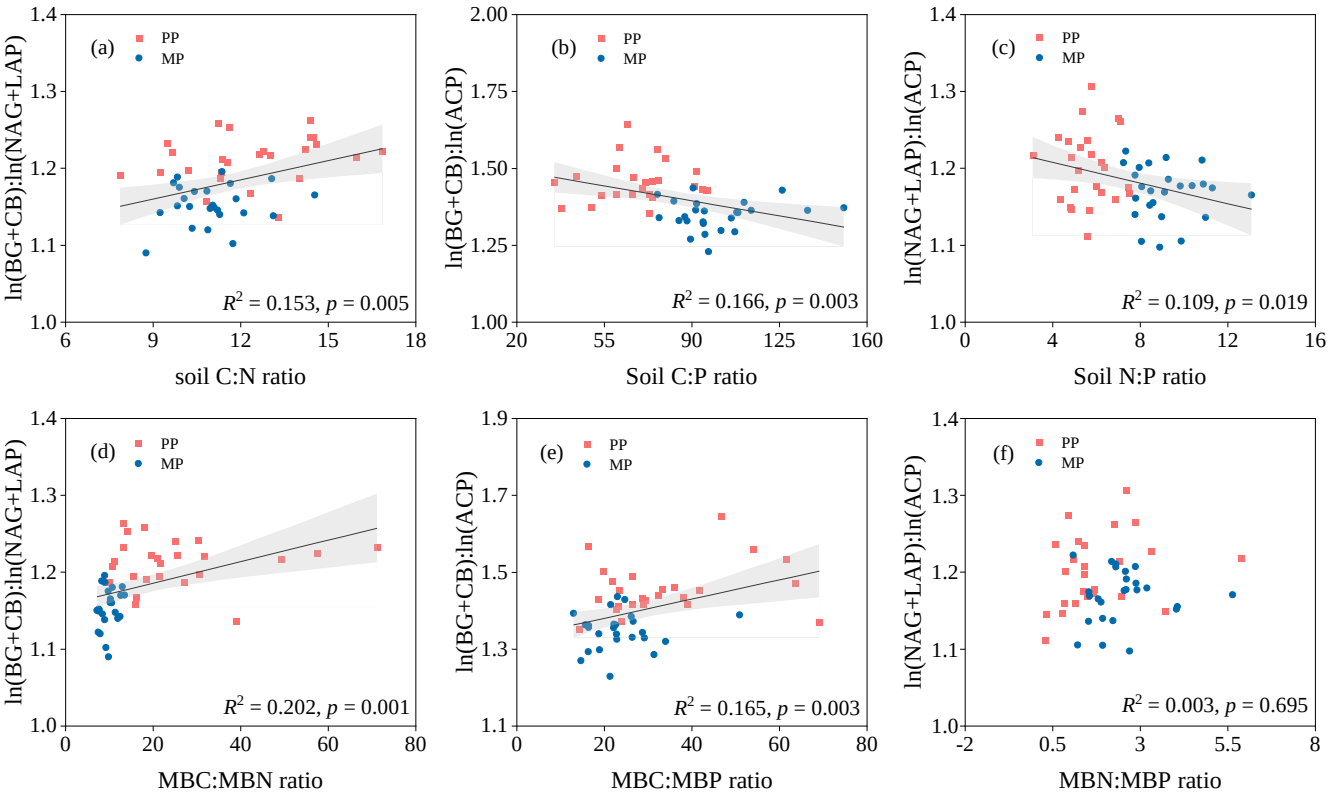

Figure 5. Relationship between soil extracellular enzymatic stoichiometry (EES) and soil nutrient stoichiometry ratios (**a**–**c**), and microbial biomass stoichiometry ratios (**d**–**f**).

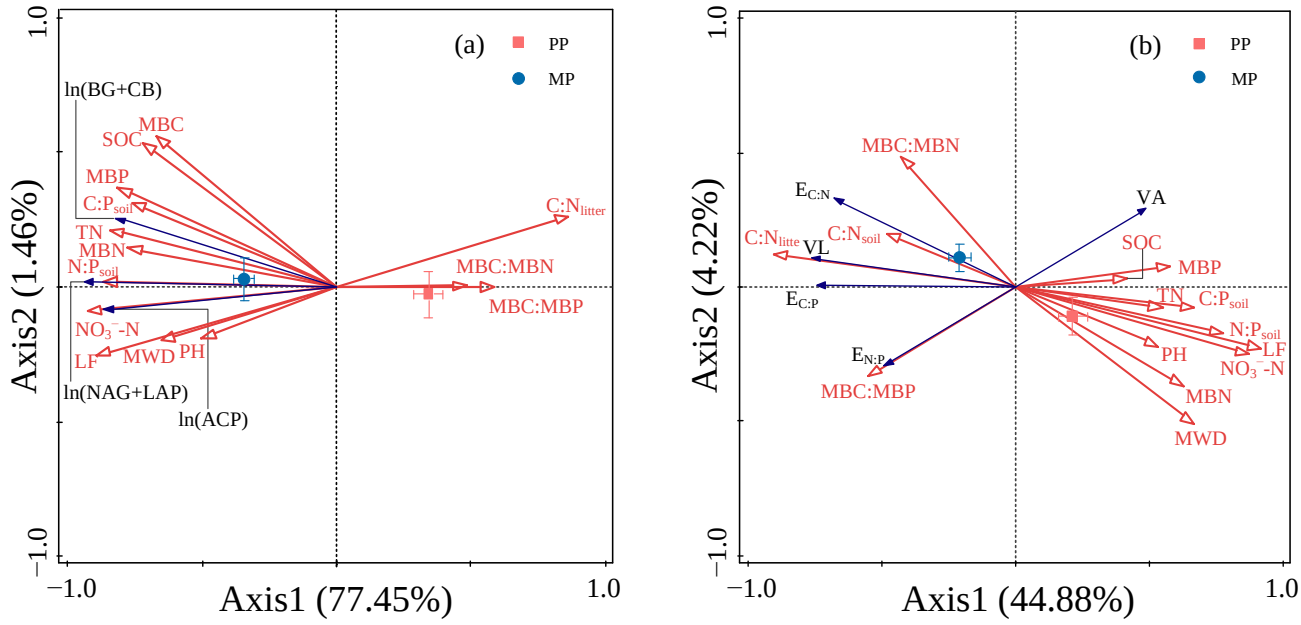

Figure 6. Results of a redundancy analysis (RDA) that was used to identify the relationship between extracellular enzymatic activity (EEA) and environmental variables (**a**), and between extracellular enzymatic stoichiometry (EES) and environmental variables (**b**). LF, litterfall; C:N_{litter}, litter carbon to nitrogen ratio; SOC, soil organic carbon; TN, total nitrogen; C:N_{soil}, soil organic carbon to total nitrogen ratio; C:P_{soil}, soil organic carbon to total phosphorus ratio; N:P_{soil}, soil total nitrogen to

total phosphorus ratio; NO_3^--N, nitrate nitrogen; MWD, mean weight diameter; 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. $E_{C:N}$, ln(BG + CB):ln(NAG + LAP); $E_{C:P}$, ln(BG + CB):ln(ACP); $E_{N:P}$, ln(NAG + LAP):ln(ACP); VL, vector length; VA, vector angle.

Table 4. Simple and conditional effects of environmental factors on soil extracellular enzymatic activity (EEA) identified in the redundancy analysis (RDA).

Variables	Lambda-A	Lambda-B	*p*	*F*
NO_3^--N	66.5	66.5	**0.002**	95.2
LF	61.9	1.2	0.110	2.2
$N:P_{soil}$	58.4	0.1	0.792	0.2
$C:N_{litter}$	58.0	0.2	0.592	0.4
TN	55.1	5.5	**0.002**	9.2
MBP	51.4	<0.1	0.958	<0.1
MBN	46.6	0.2	0.662	0.4
$C:P_{soil}$	44.9	1.2	0.134	2.1
SOC	40.6	0.2	0.710	0.3
MBC	35.3	1.1	0.114	2.1
MWD	33.3	0.5	0.380	0.9
MBC:MBP	26.6	2.5	**0.018**	4.4
pH	19.6	0.2	0.734	0.3
MBC:MBN	18.4	0.2	0.750	0.3

Note: Bold values denote marked influences at $p < 0.05$. Lambda-A denotes a simple effect, which shows the variance explained when the variable was used as the only factor. Lambda-B denotes a conditional effect, which shows the additional variance explained when each variable was included in the model. *p* denotes the level of significance corresponding to Lambda-B when conducting the Monte Carlo test at the 0.05 significance level. *F* denote the Monte Carlo test statistics corresponding to Lambda-B at the 0.05 significance level.

Table 5. Simple and conditional effects of environmental factors on soil extracellular enzymatic stoichiometry (EES) in the redundancy analysis (RDA).

Variables	Lambda-A	Lambda-B	*p*	*F*
LF	38.1	38.1	**0.002**	29.6
$C:N_{litter}$	36.7	0.4	0.726	0.3
NO_3^--N	34.5	1.1	0.396	0.8
$N:P_{soil}$	27.1	0.5	0.618	0.4
MWD	21.1	0.8	0.542	0.6
TN	20.1	0.5	0.672	0.4
MBN	18.2	0.5	0.708	0.3
MBP	15.1	0.4	0.778	0.3
MBC:MBP	14.0	2.7	0.136	2.2
$C:P_{soil}$	13.6	0.3	0.768	0.2
pH	13.0	0.6	0.628	0.5
$C:N_{soil}$	10.5	2.0	0.162	1.6
MBC:MBN	9.1	1.0	0.420	0.8
SOC	7.8	0.2	0.822	0.2

4. Discussion

4.1. Effect of Introducing N_2-Fixing Species on EEA within Soil Aggregates

Soil enzymes are the main catalysts for many biochemical reactions in soil. The level of their activities are characterized as soil microbial activity, and they can be regarded as an essential biological indicator of soil nutrient dynamics [44]. Enzymes can be physically protected in soil aggregates because they are generally adsorbed by, or bound to, soil colloids, thereby reducing the risk of degradation or denaturation [21,45]. Our results showed that EEA was markedly higher in MP than in PP (Figure 1), which was similar to the findings of Li et al. [46]. This may have been because introducing N_2-fixing species increased the LF, which may have enhanced the content of N and other nutrients. Earlier

researches had shown that both LF inputs and N addition can affect soil EEA [47–49]. In our study, stand conversion resulted in an obvious increase in SOC, which provided more substrate for C-acquiring enzymes, resulting in an obvious improvement of C-acquiring enzyme activity (BG + CB). It is also possible that N_2-fixing species increased N effectiveness, stimulating the production of C-acquiring enzymes and increasing their activities, accelerating the decomposition and conversion of SOC, and facilitating C sequestration [28,50]. The symbiosis of N_2-fixing bacteria with N_2-fixing plant roots improves the N_2-fixing capacity of bacteria, enhancing soil N content and availability [28], thereby increasing N-acquiring enzyme activity (NAG + LAP). This may because the improvement of N availability significantly increased N-acquiring enzyme activity [44]. Furthermore, N addition increased P-acquiring enzyme activity due to improved N availability [51], and higher N content may increase plant demand for P, stimulating soil microbes to secrete more ACP to obtain effective P for plants and microbes. This would explain the significantly higher ACP values in the MP in our study [52]. Our results were similar to those of Pereira et al. [53], who found that soil phosphatase activity increased in *Eucalyptus* mixed with *A. mangium*. In addition, a previous study implied that N_2-fixing species could facilitate the synthesis of phosphatase in P-deficient areas, thus acquiring more effective P for plants to use [54].

There was significantly positive correlation among soil EEA (Figure 4a–c), indicating that the changes of soil C, N, and P were strongly related [46], which was consistent with the findings of Zhang et al. [55], who found that soil microbial communities have similar patterns of assignment to nutrient acquisition. Furthermore, Waring et al. [2] reported the same regulation in a tropical dataset and found strong positive correlations between enzyme activities, emphasizing the important connections between soil nutrients and microorganisms. The RDA revealed that variations in soil and microbial characteristics may cause changes in EEA (Figure 6a). In our study, soil properties (e.g., NO_3^--N and TN) and microbial characteristics (e.g., MBC:MBP) were the main factors influencing EEA. Soil nutrient contents and available substrates may influence the composition and function of soil microbial communities which are directly related to EEA. [13,42,56]. This was consistent with existing findings showing that EEA increased with increasing levels of soil N [57,58], illustrating the significance of NO_3^--N and TN in driving changes in EEA [59]. Moreover, it has been reported that soil microorganisms play essential roles in enzymes, as they produce them directly, and the microbial biomass and microbial stoichiometry ratios directly influence soil enzyme activity [8,60].

The EEA of different soil aggregate particles are generally considered related to SOC concentrations and various physical and chemical protections [61,62]. We surmise that the larger specific surface areas of small aggregates allow more SOC to be adsorbed and that soil enzymes are susceptible to adsorption by organic-inorganic complexes in small aggregates, resulting in a greater impact on EEA. Previous studies have found that different aggregate particle sizes have different effects on EEA [45,63]. In our study, we found that EEA tended to increase with the decrease in aggregate particle size in both the PP and the MP (Figure 1). These results may be explained by the high content of organic substances in small aggregates, which is conducive to improving soil microbial activities and thereby contribute to increased enzyme activities. Differences in the soil types and biomes of various study areas lead to inconsistent distributions of EEA in aggregates, and the distribution of EES in aggregates is complex. The enzyme relative activity index considers the enzymatic activity of both bulk soil and aggregates and provides a more complete view of the influence of soil aggregates on EEA. Our study showed that REAI and REACI values were greater than 1 in most soil aggregates (Table 3), indicating that most aggregates play protective roles for enzyme activities [41], and the protection tends to increase as the particle size of the aggregates decreases. This may be driven by the chemical and physical protection mechanisms of soil aggregates on organic substances and microorganisms.

4.2. Introducing N_2-Fixing Species into Eucalyptus Plantations Alleviates C and N Limitation

Soil EES reflects microbial demand for energy and nutrients [42]. Microorganisms regulate EES by adjusting their production of specific extracellular enzymes or by changing the efficiency of nutrient utilization [64,65]. The soil enzyme C:N:P ratio close to 1:1:1 at the global scale, and the ratio may deviate in different regions, indicating the situation of microbial resource limitation. In our study area, the EES was 1.44:1.21:1, which clearly deviated from 1:1:1. The $E_{C:N}$ of 1.19 was lower than the global average (1.41), while the $E_{C:P}$ of 1.44 and $E_{N:P}$ of 1.21 were higher than the global averages (0.62) and (0.44), respectively. This indicated that the study area was more susceptible to C and N limitation than to P limitation. This may have been because the area has long been planted with *Eucalyptus*, which are fast-growing trees with a high nutrient demand, but the low quantity and quality of their LF and low rate of nutrient return have led to a low SOC content, and the area has therefore become vulnerable to nutrient limitation [28,29,66]. Furthermore, both $E_{C:P}$ and $E_{N:P}$ were higher than their respective global averages in the study site, implying that ACP activity in the region is relatively low and that microbes may be more likely to produce C- and N-acquiring enzymes to improve their nutrient availability. The scale of the local region, stand age, stand type, and soil parent material all impact soil nutrients and the EEA, causing variations in microbial resource limitation at the local regional scale [67].

$E_{C:N}$ and $E_{C:P}$ ratios reflect the relative acquisition of resources by microorganisms. Our study found that $E_{C:N}$ and $E_{C:P}$ ratios in bulk soil and most aggregates were markedly lower in MP than in PP (Figure 2a,b). In addition, the VL of bulk soil and most aggregates were markedly lower in MP than in PP (Figure 3a). These results implied that C limitation was more severe in PP than in MP. We also found $E_{N:P}$ in bulk soil and >2 mm aggregate were markedly lower in MP than in PP (Figure 2c). Moreover, VA were less than 45° in both the MP and the PP, and the VA of bulk soil and >2 mm aggregate were markedly higher in MP than in PP (Figure 3b), implying that both the PP and the MP were N-limited, but the limitations were alleviated by the introduction of N_2-fixing species in our study. We found that VL was significantly negatively correlated with VA (Figure 4d), implying that microbial C and N limitation was coupled. Soil microbes adjusting C acquisition through regulating enzyme activities is one of the most important strategies for alleviating microbial N limitation to maintain internal elemental balances [68]. Moreover, we also found that $E_{C:P}$ and $E_{N:P}$ ratios were lower in MP compared to PP, implying a high P requirement in the MP and relative P limitation of microbes. This may have been due to the higher P requirement of *A. mangium* [69] and the high N_2 fixation capacity of legumes that further enhanced soil P limitation [70,71].

EES is influenced by both biotic and abiotic factors [60,72]. Transformation of forest stands can change the vegetation status and stand environment, which can impact soil physicochemical properties, LF, and root secretions, subsequently affecting the structure and function of the soil microbial community and ultimately impacting EES [73]. Our results showed that $E_{C:N}$ and soil C:N ratios were positively correlated (Figure 5a). In contrast, $E_{C:P}$ and soil C:P ratios, and $E_{N:P}$ and soil N:P ratios, had a markedly negative correlation (Figure 5b,c). This result may be explained as the stoichiometric ratios of soil nutrients have strong impacts on the composition and function of microbial communities, ultimately effect soil EES. [60]. Our results also implied that $E_{C:N}$ and MBC:MBN ratios, and $E_{C:P}$ and MBC:MBP ratios, were significantly and positively correlated (Figure 5d,e). Soil microbes are dependent on soil nutrient availability, which is eventually expressed in the synthesis of ecoenzymes [74]. It was worth noting that there was a similar relationship among soil microbial biomass stoichiometry ratios, nutrient stoichiometry ratios, and EES. However, $E_{N:P}$ was not significantly correlated with MBN:MBP (Figure 5f), and it may have been that other biotic or abiotic factors exerted a stronger influence on soil EES, thereby masking the influence level of microbial biomass stoichiometry ratios on soil EES [75,76]. The correlation between soil EES and soil nutrient stoichiometry ratios is complex and there is no strict correspondence between them, with the response of EES to soil nutrient

stoichiometry ratios varying in different ecosystems. In addition, our results prove that LF is the main influencing factor shaping EES. Fifteen years after introducing N_2-fixing species into *Eucalyptus* plantation, the quantity and quality of LF had improved. LF is an important source of organic matter as an input-output system of nutrients, and the organic matter input from LF to the forest floor leads to an increase of soil nutrients, which affects the soil microbial community and changes the microbial resource availability [47,48]. Furthermore, prior research indicated that the symbiosis of legumes and soil bacteria enables N_2 fixation; introducing N_2-fixing species increased the N effectiveness, and microbial N limitation is diminished with an increasing level of N availability [77]. In our study, while NO_3^--N had relatively high simple effect, it had a minor conditional effect on soil EES, and was not confirmed as an important explanatory variable in the changes of EES. It is possible that other environmental factors masked the effects of NO_3^--N on EES. Finally, we created a conceptual map to summarize the impacts of mixing N_2-fixing species with *Eucalyptus* on soil EES and microbial resource limitation (Figure 7). Introducing N_2-fixing species into the plantation resulted in changes in biotic and abiotic factors, which together affected EES. Significantly, variations in EES were better explained by biotic factors, such as LF and microbial biomass, which were immediately involved in decomposition of organic matter and improved soil EEA, thereby influencing EES. $E_{C:N}$, $E_{C:P}$, $E_{N:P}$, and VL all decreased, while VA increased, implying that 15 years after introducing N_2-fixing species, microbial C and N limitation were gradually being alleviated. Prior researches have shown that increasing N effectiveness can alleviate N limitation [56,78]. Due to the ability of N_2-fixing species to increase N effectiveness, introducing N_2-fixing species to create a mixed forest is a viable management option in silviculture [79,80]. Generally, the primary productivity and microbial growth of young soils are susceptible to N limitation and rely on external N inputs [2], while anthropogenic N additions help to reduce N limitation [78,81]. At the same time, planting leguminous shrubs in the understory increases soil microbial biomass and microbial community diversity; plants can obtain N through plant inter-rhizosphere N-fixing bacteria that enhance the N-acquiring enzyme activity [82,83], thereby alleviating N limitation. Due to the important influences of N_2-fixing species on soil nutrient cycling, the relationships between N_2-fixing species, soil microbial community structure, and biogeochemical process should be further explored.

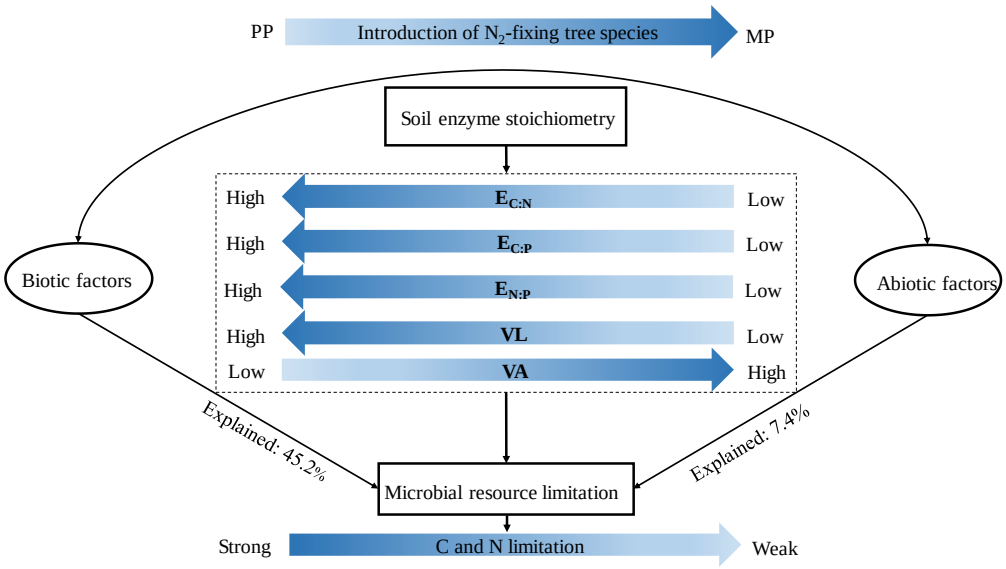

Figure 7. Conceptual map of the resource limitation of soil microbes following introducing N_2-fixing tree species as revealed by the soil extracellular enzymatic stoichiometry (EES). $E_{C:N}$, ln(BG + CB):ln(NAG + LAP); $E_{C:P}$, ln(BG + CB):ln(ACP); $E_{N:P}$, ln(NAG + LAP):ln(ACP); VL, vector length; VA, vector angle.

5. Conclusions

This study investigated EEA and EES and the biotic and abiotic factors affecting them, revealing the status of microbial resources limitation after introducing N_2-fixing species into *Eucalyptus* plantation for 15 years in subtropical China. The soil EEA was markedly increased in MP, in contrast to PP, and introducing N_2-fixing species to create a mixed forest would therefore have changed the EEA and affected the microbial resources limitation. Our results showed that soil aggregates can protect soil enzyme activities from denaturation and inactivation. Furthermore, analysis of the soil EES revealed that the study area was co-limited by C and N, and introducing N_2-fixing tree species alleviated the co-limitation status. In addition, we found that soil EEA and EES were more influenced by biotic than abiotic factors. Specifically, NO_3^--N, TN, and MBC:MBP were the main factors affecting EEA, while LF was the main factor affecting EES. In terms of nutrient management, forest managers should consider applying a moderate amount of N fertilizer to improve soil fertility. The findings of this study have significant implications for managing soil nutrients in *Eucalyptus* plantations in subtropical China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13122102/s1, Table S1: Details of the various soil extracellular enzymes and associated substrates.

Author Contributions: Conceptualization, J.Y. and Y.Y.; methodology, J.Y. and Y.Y.; software, X.S. and G.G.; validation, W.Z.; formal analysis, Y.Y.; investigation, X.S.; resources, X.H. and Y.Y.; data curation, J.Y.; writing—original draft preparation, J.Y.; writing—review and editing, J.Y. and X.S.; visualization, G.G.; supervision, W.Z. and Y.Y; project administration, X.H. and Y.Y.; funding acquisition, X.H. and Y.Y. All authors have read and agreed to the published version of the manuscript.

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