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13 Abstract:

14 *Aims* Broad-leaved tree species are important group building tree species of typical zonal vegetation in
15 subtropical China. Yet, the impacts of mixed broad-leaved plantations on microbial community and enzymatic
16 stoichiometry are not well understood.

17 *Methods* We evaluated soil microbial community structure and enzymatic stoichiometry using rhizosphere and
18 non-rhizosphere soil samples of *Machilus pauhoi* from three forest plantation (the pure *M. pauhoi* (CK), *Machilus*
19 *pauhoi* with *Cerasus campanulata* mixed forest (MC), *M. pauhoi* with *Manglietia glauca* mixed forest (MM)) in
20 subtropical China.

Results P concentration of rhizosphere soil in MC was higher than that in CK, and soil TC, TN, SOC, DOC of non-rhizosphere soil in MC were higher than those in MM. Different mixed plantations had significant effects on G^*/G , being its value lower in MC than in MM. The microbial P limitation in rhizosphere soil was higher than that in non-rhizosphere soil. The relative microbial C limitation did not change significantly amongst different

25 treatment. Soil TC, TN, SOC and DOC are critical factors affecting the microbial community structure.

26 *Conclusions* Mixing with *Cerasus campanulata* is more beneficial to reducing soil P deficiency and improving
27 soil microbial community structure of young *Machilus pauhoi* afforestation in a mid-subtropical region of China.

28

29 **Keywords** mixed forest, soil nutrient, microbial community structure, enzymatic stoichiometry, *Machilus*
30 *pauhoi*

31

32 **Introduction**

33 Plantations play a key role in sustainable forest management, while conventional plantation
34 management is generally characterized by the pure tree species plantations that face many
35 problems, including the loss of biodiversity, a decline in ecosystem stability, soil degradation, and
36 higher susceptibility to pests (Xu et al., 2021; Zhang et al., 2021a). Mixed plantations are proven to
37 promote biodiversity, provide more ecosystem functions, and enhance resilience to disturbances,
38 representing an essential forest management practice for the sustainable management and soil
39 health (Brunel et al., 2017; Santana et al., 2021; Li et al., 2022a). Forest species richness can
40 increase stand-level productivity (Huang et al., 2018). So, afforestation with mixed species has been
41 gradually became effective method to enhance ecosystem functions and develop sustainable
42 plantations in forestry production practice (Lucas-Borja et al., 2012; Pereira et al., 2019).

43 Microbes play a critical role in nutrients cycle and energy flow of terrestrial ecosystem through
44 their effects and links with their absorption, utilization and decomposition and nutrient recycling

45 and release to soil (Fanin et al., 2019). The phospholipid fatty acids (PLFAs) extracted from soil can
46 be used for the quantification and characterization of soil microbial community structure (Lucas-
47 Borja et al., 2012; Liu et al., 2022). The tree species diversity plays an important role in affecting
48 soil organic matter (Fekete et al., 2017), and thus shapes microbial community composition (Huang
49 et al., 2014). Mixed forest can produce more litters, change the composition of soil organic matter
50 and the circulation channel of organic matter between soil and plants, thus providing a large number
51 of carbon sources and nutrient elements, and were more conducive to metabolic activities of
52 microorganisms (Dai et al., 2021). And, microbial communities are also influenced by plant
53 community and root exudates. Due to the more developed root network of multiple tree species in
54 mixed forest, the soil physicochemical properties and soil fertility can be changed, and more root
55 exudates can provides carbon sources and water, the soil microbial community structure can be
56 improved indirectly (Zi et al., 2017; Dai et al., 2021). Mixed forest also affect the soil moisture and
57 pH (Li et al., 2022b), which directly and indirectly influences soil microbial community structure
58 via effects on soil properties, organic matter availability, diffusion and transport capacity (Zhu et al.,
59 2019). Fungi and bacteria have adopted different strategies to cope with water status, bacteria are
60 more affected by water conditions than fungi because bacteria require water films for motility and
61 substrate diffusion (Evans et al., 2012; Zhu et al., 2019). And most studies suggested that pH
62 controls the changes in microbial communities (Ji et al., 2020; Li et al., 2022). Huang et al., (2014)
63 proposed that the soil microbial community was primarily influenced by the below ground C and N
64 inputs, and the number of PLFAs were higher in an 8-year-old mixed *Eucalyptus urophylla* and

65 *Acacia mangium* plantation than that in pure *E. urophylla* plantation. However, the PLFAs of soil
66 microbial community, bacteria, fungi, actinomycetes, arbuscular mycorrhizal fungi, Gram-positive
67 bacteria and Gram-negative bacteria in pure *Pinus massoniana* plantation were significantly higher
68 than that in the mixed plantations (Wen et al., 2019). Taken together, afforestation with mixed
69 species may induce inconsistent results of soil microbes biomass. And most studies have focused on
70 plot soils, but few have focused on soils of target species. However, whether and how the microbial
71 community structure and function changes in target species in the mixed forest of subtropic forest
72 ecosystems is a question still not fully understood.

73 Soil-derived enzymes were considered to be closely related to microbial biomass and nutrient
74 elements transformation process for plant growth in topsoil (Tian et al., 2010; Zhang et al.,
75 2019). Soil enzymatic stoichiometry was identified as an index to record changes in soil nutrient
76 availability and microbial demands for the biogeochemical processes (Sinsabaugh and Follstad
77 Shah, 2012; Zhou et al., 2020). Enzymatic stoichiometry is also used to characterize soil nutrient
78 limitation from microbial metabolism throughout the investment in the specific enzymes to
79 mineralize carbon, nitrogen and phosphorus from soil organic matter (Sinsabaugh et al., 2009;
80 Sinsabaugh and Follstad Shah, 2012; Cui et al., 2021). The vector analysis provides a quantitate
81 measure of the relative resource use limitation, the status of microbial resource limitation can help
82 to elucidate the factors controlling soil fertility and can increase the plant-microbial competition for
83 nutrients, affecting plant growth (Qi et al., 2022). Mixed-species plantations have been found to
84 increase, decrease or have no effect on extra-cellular enzyme activities. The Leucine

85 aminopeptidase (LAP) activities were significantly increased in the mixed *Eucalyptus* and
86 *Castanopsis hystrix* plantations, and the activities of β -1,4-glucosidase (BG) and acid phosphatase
87 (AP) were significantly reduced on the above layers, but had no significant effects on the activity of
88 β -1,4-N-acetylglucosaminidase (NAG) (Shao et al., 2022). The BG and NAG were significantly
89 higher in the mixed second-generation *E. urophylla* and *D. odorifera* plantation than that in pure *E.*
90 *urophylla* plantation, and there was no different in AP between the two plantations (Chen et al.,
91 2021). However, after the conversion of pure *P. massoniana* plantation to mixed plantations, the
92 BG and NAG activities decreased, and AP activities increased (Wen et al., 2019). The LAP
93 activities and the relative activity of C- versus N-acquiring enzymes [BG/(NAG + LAP)] at 10-20
94 cm soil layer of *P. massoniana* plantation was significantly higher than those of mixed plantations.
95 The vector length of enzyme stoichiometry have an order of mixed plantation > *P. massoniana*
96 plantation, and there was no significant difference in vector angle between the two forest types (Shi
97 et al., 2020). These contradictory results may be partly attributed to the distinct responses of soil
98 enzymes to aboveground tree species (Zhang et al., 2021). Furthermore, how microbial resource
99 limitation due to soil nutrients variable changes after mixed forests treatments, to some extent, has
100 also been scarcely studied in the subtropical forest.

101 The rhizosphere is one of the most complex and sensitive compartments of terrestrial
102 ecosystems, affected by plant roots and soil microorganisms (Kumar et al., 2022). As a hotspot of
103 microbial activity and enzymatic catalytic function, the rhizosphere plays an important role in
104 microbial metabolisms and enzyme reactions, because of frequent soil-microbial-plant interactions

105 and the transfer of energy among them (Kuzakov et al., 2007; Zhang et al., 2019; Cui et al., 2021).
106 Differently, non-rhizosphere soils generally have relatively oligotrophic features, showing low rates
107 of microbial and enzymatic activities (Ai et al., 2012; Zhang et al., 2019). The microbial
108 community structures of rhizosphere soils were different considerably from those in non-
109 rhizosphere soils, due to the various propensity to absorb and use nutrients of the root systems (Ai
110 et al., 2012; Zhang et al., 2019), which may significantly affect microbial metabolic patterns and
111 enzymatic reaction. These differences maybe showed distinct variations in the metabolisms of
112 microbial communities between rhizosphere and non-rhizosphere soils. Previous studies showed
113 rhizosphere soil has high soil microbial biomass than non-rhizosphere soil in the central Himalaya
114 (Kumar et al., 2022). The microbial relative C and P limitations also varied significantly between
115 rhizosphere and non-rhizosphere soils at the high altitudes in alpine ecosystems (Cui et al., 2021).
116 However, Cui et al. (2019) suggested that the similar microbial communities between rhizosphere
117 and non-rhizosphere soils were observed in the cold and high nutrient contents environments. The
118 responses of microbial communities to rhizosphere effect in subtropical forest ecosystems may
119 differ considerably from these in other ecosystems. Identifying the variation characteristics of
120 microbial communities and enzymatic activities in the rhizospheric microenvironment and non-
121 rhizosphere soils is consequently vital for our understanding the roles of microorganisms and
122 enzyme in the cycles of soil nutrient in the mixed forest ecosystem.

123 Evergreen broadleaved forests are the typical and widespread zonal vegetation type in
124 subtropical China. *Machilus pauhoi* (Lauraceae) is a subtropical evergreen broadleaved native tree

125 species of China and is a national second-class protected precious plant with high economic value
126 for its wood, bark and seed (Guo et al., 2017). Previous studies have mainly concentrated on
127 seedling cultivation techniques, and plant eco-physiology characteristics (Zhong et al., 2009), with
128 less focus on the mixed plantations. *Manglietia glauca* (Blume) is an endemic evergreen single tree
129 species, and is an excellent ornamental and economic species with straight stem form, beautiful
130 flowers and multi-purpose wood (Zhang et al., 2011). *Cerasus campanulata*, a deciduous tree of the
131 rosaceae family, is an important popular ornamental tree species (Yi et al., 2020), which is usually
132 used to establish mixed broadleaved forests. These three tree species are important group building
133 species of zonal vegetation evergreen broadleaved forests in this region. We conducted a mixed tree
134 species experiment, which was designed with three different species community compositions
135 (monoculture *M. pauhoi* plantations, and two mixed plantations; *M. pauhoi* with *C.campanulata*,
136 and *M. pauhoi* with *M. glauca*). We aim to understand the impact of mixed plantation between *M.*
137 *pauhoi* and other broad-leaved tree species on rhizosphere and non-rhizosphere soil nutrient and
138 microbial variables, the concrete objectives of this study are (1) to analyse whether the mixing of
139 *M. pauhoi* with different broadleaved species in the early stage change the rhizosphere and non-
140 rhizosphere soil nutrient, microbial community composition and enzyme activity characteristics
141 with respect to the monoculture stands of *M. pauhoi*, and (2) to explore the key driving factors that
142 control the microbial variables. We hypothesized: (1) rhizosphere soil nutrients and microbial
143 community increased in the mixed forest with deciduous tree species, since the litter quantity
144 increase and the litter quality improves compared with pure stand; (2) Carbon is key factor affecting

microbial community due to more C input. This study provides practical suggestions for selecting suitable mixed species combinations for future multi-tree afforestation to improve the soil concentrations of the limiting nutrient in the early stage of ecological restoration.

Materials and methods

Study site and experimental design

The study was conducted within the Anxi Fengtian State-owned Forest Farm (25°19' - 25°50' N, 117°59' - 118°00' E) in the southeast of Fujian province, China. Mean annual temperature was in the range of 19-21°C. Mean annual precipitation was 1600 mm, with a summer season from long to hot and a winter season of short without cold. The soil was predominantly typical lateritic soil.

The studied forests were afforested in February 2016. *M. pauhoi* was used as the main broad-leaved afforestation tree species, and *C. campanulata* and *M. glanca* were selected as associated tree species to construct mixed plantations. The mixed ratio of the plantation forest was set to 1:1, and the plant-row spacing was 2 m × 2 m. The three reforestation treatments were as follows: pure *M. pauhoi* (CK), *M. pauhoi* × *C. campanulata* (MC), *M. pauhoi* × *M. glanca* (MM) plantation, and each treatment sets up three repeated sites with a size of 50m*50m. All plantations were performed by using the same general management measures (forest tending once in first year of afforestation and two times in the second and follow years, nitrogenous-phosphatic-potassium compounded fertilization once from the second and follow year of afforestation).

Table. 1 General status of experimental sites

Plantation	Age	Altitud	Slop	Slope	Slope	Average height(m)	Average DBH(cm)	Canopy
	(a)	e(m)	e	aspect	position	(mean± SE)	(mean±SE)	density
Pure forest(CK)	5	205	25°	Northeast	Middle slope	<i>M. pauhoi</i> =5.81± 0.65	<i>M. pauhoi</i> =5.51± 0.39	0.8
MC	5	205	25°	Northeast	Middle slope	<i>M. pauhoi</i> =6.50± 0.67	<i>M. pauhoi</i> =6.67± 0.77	0.8
						<i>C. campanulata</i> =6.23± 0.29	<i>C. campanulata</i> =6.27± 0.33	
MM	5	205	25°	Northeast	Middle slope	<i>M. pauhoi</i> =5.78± 0.37	<i>M. pauhoi</i> =4.95± 0.39	0.8
						<i>M. glanca</i> = 4.81± 0.42	<i>M. glanca</i> = 4.28± 0.45	

164 CK: pure *M. pauhoi* plantation, MC: *M. pauhoi* × *C. campanulata* mixed plantation, MM: *M. pauhoi* × *M. glanca*

165 mixed plantation. The same below.

166 Soil sampling

167 Soil samples were collected from the plots with a size of 20 m × 20 m at each site in October 2020.

168 The descriptions of sampling sites were shown in Table 1. Three healthy trees with the diameter at

169 breast height (DBH) and height close to the mean DBH and mean height in each plot were selected

170 to represent sample trees. To make sure that soil samples indicate more accurate and reveal the

171 mechanism of tree growth, we collected soil samples from two directions from sample trees,

172 respectively. The directions were 40-60 degrees perpendicular to slope aspect of the sample tree,

173 with the bottom of the sample tree, which was selected by the standard of medium growth in the

174 forest stand. And the sampling was performed at the 0-20cm soil layer perpendicular to one third of

175 the tree canopy. The rhizosphere and non-rhizosphere soils from different directions were taken for

176 the sample tree of *M. pauhoi*, and mixed *in situ* to form one pooled sample per sample tree, and the

177 samples of sampled trees per plot were mixed into a composite sample as one for the plot. The non-
178 rhizosphere soil not directly attached to roots was collected by gently shaking, and the soil that
179 tightly adhered to the root after gentle shaking was considered rhizosphere soil (Phillips et al., 2008;
180 Fu et al., 2020). After removing impurities such as stones, debris and other visible items, the soil
181 samples were sieved to 2 mm, and then divided into three parts for future analysis. One part was
182 immediately stored at -70°C for phospholipid fatty acid (PLFA) analysis. The second part was
183 stored at 4°C for assays of extracellular enzymatic activity and the remaining was air-dried for soil
184 physicochemical properties.

185 Measurement of soil physicochemical properties

186 Soil moisture content was determined by oven-drying method. Soil pH was measured for a 1:2.5
187 (w/v) soil:water mixture with a glass electrode meter. Soil total C (TC), organic C (SOC) and soil
188 total N (TN) concentrations were determined using an elemental analyzer (Elementar Vario EL III;
189 Elementar, Langenselbode, Germany). The soil total P (TP) concentrations were determined by using
190 the molybdenum-antimony anti-colorimetric method with a Continuous Flow Analytic System
191 (Skalar San++, Breda, the Netherlands). Soil dissolved organic C (DOC) was determined by total
192 organic carbon analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, Japan). Soil ammonium N (NH_4^{+} -
193 N) and nitrate N (NO_3^{-} -N) concentrations were determined by Continuous Flow Analyzer (Skalar
194 San++, Breda, the Netherlands) (Carter et al., 1993).

195 Measurements of soil phospholipid fatty acids (PLFAs)

196 Soil Phospholipid Fatty Acids (PLFAs) were determined to evaluate microbial community structure

197 in soils (Singh et al., 2021; Steinbeiss et al., 2009). We applied a chloroform-methanol extraction
198 method with some modification to incorporate a chloroform:methanol: phosphate buffer(1:2:0.8) to
199 extract microbial lipids from fresh soil samples (8.0 g). The same separation and quantification
200 procedures were adopted by the methods reported by Cao et al. (2011) and slightly modified (after a
201 pilot run) to maximize extraction of fatty acids from the soil (Wan et al., 2015). Soil samples were
202 re-dissolved in 200 ml hexane containing 19:0 as an internal standard, the individual PLFAs were
203 determined and identified using a Hewlett-Packard 6890 Gas Chromatograph equipped with an
204 Ultra 2-methylpolysiloxane column. The fatty acid nomenclature described was defined by Zelles
205 (1999). Total lipid abundance was calculated as the sum of lipids of which chain length was from
206 C10 to C20 and could be measured as microbial biomass, and the valuable markers of soil PLFAs
207 were selected to categorize four groups: bacteria, fungi, arbuscular mycorrhizal fungi and
208 actinomycetes. Bacteria have two groups including Gram-positive (G^+) and Gram-negative (G^-)
209 bacteria (Wan et al., 2015). G^+ bacteria were represented by all iso and anteiso branch chain fatty
210 acids of the following PLFAs: 14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 iso, 17:0 anteiso
211 (Landesman and Dighton 2010; Wan et al., 2015), whereas G^- bacteria were represented by
212 monounsaturated and cyclopropane fatty acids such as PLFAs: 16:1 ω 9c, 16:1 ω 7c, 17:0 cyclo ω 7c,
213 18:1 ω 7c, 18:1 ω 5c, 19:0 cyclo ω 7c (Ushio et al. 2013; Wan et al., 2015). 18:2 ω 6c (Baath et al.
214 1995) and 18:1 ω 9c (Myers et al. 2001) were used as indicators of fungi biomass, while 16:1 ω 5 was
215 used to indicate arbuscular mycorrhizal fungi (Baath et al. 1995; Swallow et al. 2009; Wan et al.,
216 2015). The PLFAs 10 Me16:0, 10 Me17:0 and 10 Me18:0 were used to indicate soil actinomycetes

(Wan et al., 2015). The ratios of fungi/bacteria and G^+/G^- were calculated from the above PLFAs, respectively (Wan et al., 2015; Ma et al., 2021). Total microbial biomass was estimated from the sum of all the extracted PLFAs.

Soil enzyme activities and vector analysis

The activities of four extracellular enzymes in soils involved in C-acquiring enzyme (BG, β -1, 4-glucosidase), N-acquiring enzyme (NAG, β -1,4-N-acetyl-glucosaminidase; LAP, Leucine aminopeptidase) and P-acquiring enzyme (AP, acid phosphatase) acquisition were measured by their respective enzyme-linked immunosorbent assay (ELISA) kits (Jianglai Bio, Shanghai, China) per the kit's instructions (Liu et al., 2018; Zhang et al., 2021). We applied the vector analysis of enzymatic stoichiometry in soils which was proposed by Moorhead et al. (2013), to evaluate the relative microbial C limitation and nutrient limitation in pure *M. pauciflora* forest and two mixed forests. The method can provide measures of the relative and potential resource use limitation rather than the actual microbial resource limitation in soils. The vector length indicates the relative C limitation, and the vector degree indicates the relative P vs. N limitation. The two metrics were calculated using the following formulas (Jing et al., 2020; Ma et al., 2021):

$$\text{Vector length} = \sqrt{[\ln BG / \ln(NAG + LAP)]^2 + (\ln BG / \ln AP)^2}$$

$$\text{Vector degree} = \text{Degrees} \{ \text{ATAN2}[(\ln BG / \ln AP), (\ln BG / \ln (NAG + LAP))] \}$$

Relatively longer vector L indicated greater C-limitation; the vector $A < 45^\circ$ and $> 45^\circ$ indicated relative degrees of N- and P-limitation, respectively.

Statistical analyses

237 All the data were tested for normality and homoscedasticity, and the original data were normalized
238 by log-transformation to meet the assumptions of parametric statistical tests prior to analysis when
239 necessary. Two-way ANOVA was used to detect the effects of mixed treatment, soil location
240 (rhizosphere soil and non-rhizosphere soil), and their interactions on soil properties, microbial
241 PLFAs, extracellular enzymatic activities and microbial metabolic limitation. Significant differences
242 among mixed treatments were then tested using Tukey's HSD post-hoc test at $P < 0.05$, and
243 significant differences between rhizosphere soil and non-rhizosphere soil were tested using
244 independent sample t-test. The Redundancy analysis (RDA) were performed to evaluate the
245 relationships between soil properties and soil microbial variables in rhizosphere and non-
246 rhizosphere soil separately. The Pearson correlation analysis of soil nutrient and microbial variables
247 were conducted for all soil samples (without distinguishing rhizosphere soil from non-rhizosphere
248 soil). RDA and correlation analysis was performed using CANOCO 5.0 software (Ithaca, NY, USA)
249 and OriginPro software (version 2022, Origin Laboratories, Massachusetts, USA), respectively.

250 **Results**

251 Soil physicochemical properties

252 In rhizosphere soil, MC has a significantly higher P concentration than the CK, and MC has a
253 significantly lower soil moisture than the CK. Soil C/N in MC was significantly lower than that in
254 CK and MM. In the non- rhizosphere soil, MC had significantly higher TC, SOC and DOC values
255 compared to MM. The higher soil N concentration and lower C/N were observed in MC compared
256 to CK and MM. The $\text{NH}_4^+\text{-N}$ showed significantly higher values in CK than that in MM. There

257 were no significant differences in the other physiochemical properties amongst different treatments.
 258 No significant difference was found between the rhizosphere soil and non-rhizosphere soil (Table
 259 2).

260

261 Table 2 Physicochemical characteristics of rhizosphere and non-rhizosphere soil in three forest types

Index	Soil location	CK	MC	MM
TC (g kg ⁻¹)	Rhizosphere soil	19.75±1.33Aa	22.27±2.93Aa	19.00±2.26Aa
	non-rhizosphere soil	19.35±0.80Aab	22.27±2.40Aa	15.48±1.96Ab
TN (g kg ⁻¹)	Rhizosphere soil	1.42±0.08Aa	1.82±0.21Aa	1.32±0.17Aa
	non-rhizosphere soil	1.38±0.05Ab	1.81±0.15Aa	1.13±0.18Ab
TP (g kg ⁻¹)	Rhizosphere soil	0.11±0.01Ab	0.17±0.03Aa	0.14±0.01Aab
	non-rhizosphere soil	0.12±0.02Aa	0.13±0.01Aa	0.12±0.01Aa
C/N	Rhizosphere soil	13.87±0.29Aa	12.15±0.26Ab	14.53±0.31Aa
	non-rhizosphere soil	14.03±0.38Aa	12.19±0.36Ab	13.93±0.69Aa
C/P	Rhizosphere soil	184.65±25.54Aa	132.93±4.95Aa	137.89±22.56Aa
	non-rhizosphere soil	183.77±44.08Aa	166.62±25.82Aa	123.17±13.23Aa
N/P	Rhizosphere soil	13.25±1.63Aa	10.96±0.55Aa	9.53±1.58Aa
	non-rhizosphere soil	13.04±3.09Aa	13.58±1.80Aa	9.00±1.29Aa
SOC (g kg ⁻¹)	Rhizosphere soil	17.98±2.16Aa	20.43±1.48Aa	15.88±2.28Aa
	non-rhizosphere soil	17.06±1.31Aab	20.73±1.95Aa	14.29±2.03Ab
DOC (mg kg ⁻¹)	Rhizosphere soil	31.22±4.24Aa	38.02±2.06Aa	29.15±5.25Aa
	non-rhizosphere soil	31.67±3.01Aab	36.42±3.27Aa	25.15±3.59Ab
NH ₄ ⁺ -N (mg kg ⁻¹)	Rhizosphere soil	54.72±5.23Aa	59.00±1.98Aa	49.11±2.09Aa
	non-rhizosphere soil	66.41±2.01Aa	60.05±4.32Aab	54.32±0.69Ab
NO ₃ ⁻ -N (mg kg ⁻¹)	Rhizosphere soil	5.94±1.49Aa	21.92±11.02Aa	10.57±5.15Aa
	non-rhizosphere soil	12.72±7.55Aa	17.56±7.40Aa	9.06±2.00Aa
pH	Rhizosphere soil	5.23±0.07Aa	5.35±0.09Aa	5.19±0.10Aa

	non-rhizosphere soil	5.23±0.03Aa	5.29±0.06Aa	5.24±0.06Aa
SM (%)	Rhizosphere soil	17.06±0.96Aa	13.03±1.38Ab	16.25±1.09Aab
	non-rhizosphere soil	16.88±1.41Aa	12.89±1.30Aa	16.02±1.62Aa

CK, pure plantation of *M. pauhoi*; MC, mixed plantattion of *M. pauhoi* and *C. serrulata*; MM, mixed plantattion of *M. pauhoi* and *M. glauca*; TC, soil total carbon concentration; TN, soil total nitrogen concentration; TP, soil total phosphorus concentration; C/N, soil carbon-nitrogen ratio; C/P, soil carbon-phosphorus ratio; N/P, soil nitrogen-phosphorus ratio; SOC, soil organic carbon concentration; DOC, dissolved organic carbon concentration; $\text{NH}_4^+\text{-N}$, soil ammonium nitrogen concentration; $\text{NO}_3^-\text{-N}$, soil nitrate nitrogen concentration; SM, soil moisture. Values are mean $\pm$ standard error. Different uppercase letters indicate significant differences ($P < 0.05$) between the rhizosphere soil and non-rhizosphere soil, and different lowercase letters indicate significant differences ($P < 0.05$) amongst the three forest types in the rhizosphere soil or non-rhizosphere soil.

Soil microbial community structure

Two-way ANOVAs showed that different mixed treatment had significant effects on G^+/G^- ratio and rhizosphere had no significant effects on soil microbial community structure (Fig. 1). Only in the non-rhizosphere soils, soil G^+/G^- ratio value of the mixed forests of MC were significantly lower than that of MM ($P < 0.05$, Fig. 1), and the other variables were not affected obviously by treatments neither in rhizosphere soil nor in non-rhizosphere soil.

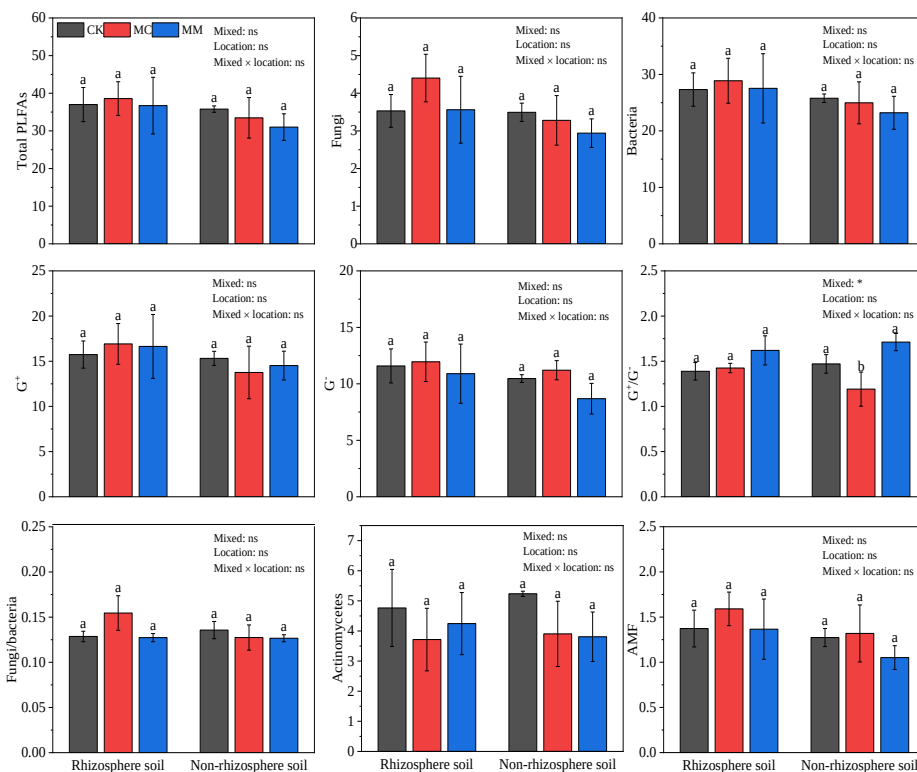


Fig. 1. Results of two-ANOVAs for the effects of different plantation treatments and soil location on Soil microbial PLFA. Value is mean ± standard error. Soil location indicate the difference between rhizosphere soil and non-rhizosphere soil. Different letters denote significant differences ($P < 0.05$) amongst different stands in the rhizosphere or non-rhizosphere soil. CK, pure plantation of *M. pauhoi*; MC, mixed plantation of *M. pauhoi* and *C. serrulata*; MM, mixed plantation of *M. pauhoi* and *M. glauca*.

Extracellular enzyme activities

Different mixed plantation treatments had no significant effects on the extracellular enzyme activities (Fig. 2). No significant difference was found in the extracellular enzyme activities among

the three studied plantations. Two-way ANOVAs showed that soil location (rhizosphere soil and non-rhizosphere soil) had significant effects on AP, and AP of rhizosphere soil was higher than that of non-rhizosphere soil, this led to difference in $\text{Ln}(\text{NAG}+\text{LAP})/\text{LnAP}$.

Mixed stands and soil location did not change significantly vector L (C limitation). All points were above the 1:1 line and vector angles were greater than 45° across all observations, indicating strong P limitation in the microbial community in this study (Fig. 3). The vector angle of rhizosphere soil was lower than that in non-rhizosphere soils in MM, indicating the microbial metabolism was more limited by P in non-rhizosphere soil.

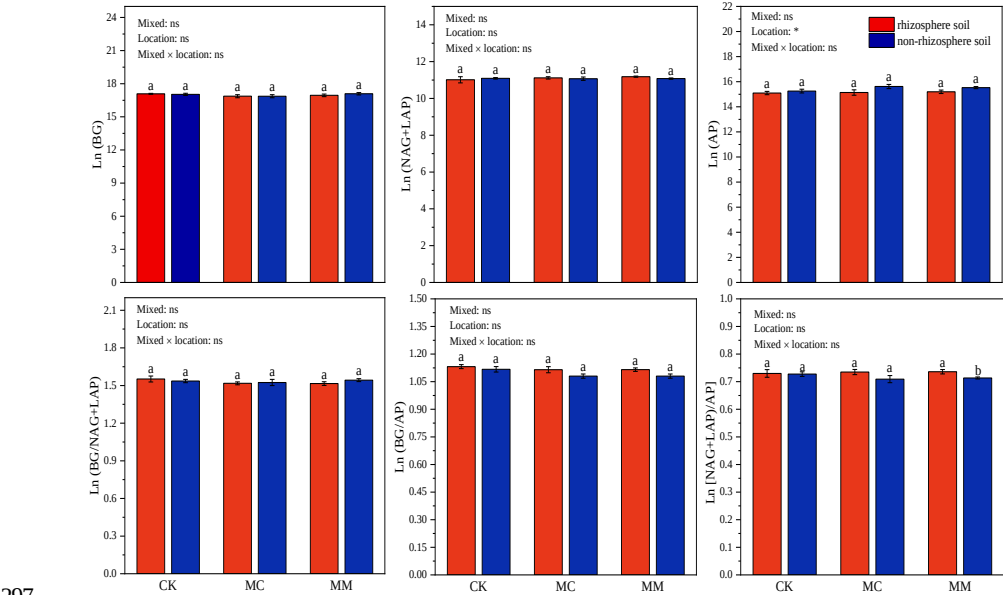


Fig.2 Results of two-way ANOVAs for the effects of different plantation treatments and soil location on extracellular enzymatic stoichiometry. Value is mean ± standard error. Soil location indicate the difference between rhizosphere soil and non-rhizosphere soil. The different letters denote significant difference ($P < 0.05$) between rhizosphere

soil and non-rhizosphere soil. CK, pure plantation of *M. pauhoi*; MC, mixed plantation of *M. pauhoi* and *C.serrulata*; MM, mixed plantattion of *M. pauhoi* and *M.glauca*.

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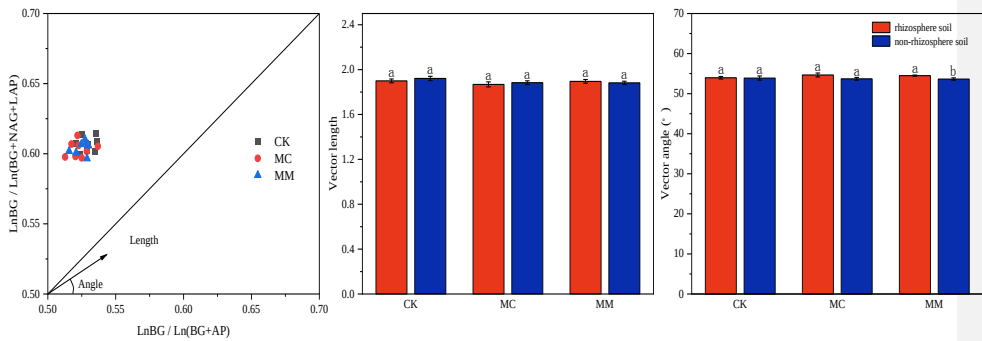
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Fig.3 Extracellular enzyme stoichiometry of the relative proportions of C to N acquisition versus C to P acquisition, the variation of vector length and angle. vector length represents soil C limitation for microbes, vector angle represents soil N or P limitation for microbes. Values are the means $\pm$ standard error. Different letters indicate significant differences ($P < 0.05$) between rhizosphere soil and non-rhizosphere soil. CK, pure plantation of *M. pauhoi*; MC, mixed plantattion of *M. pauhoi* and *C.serrulata*; MM, mixed plantattion of *M. pauhoi* and *M.glauca*.

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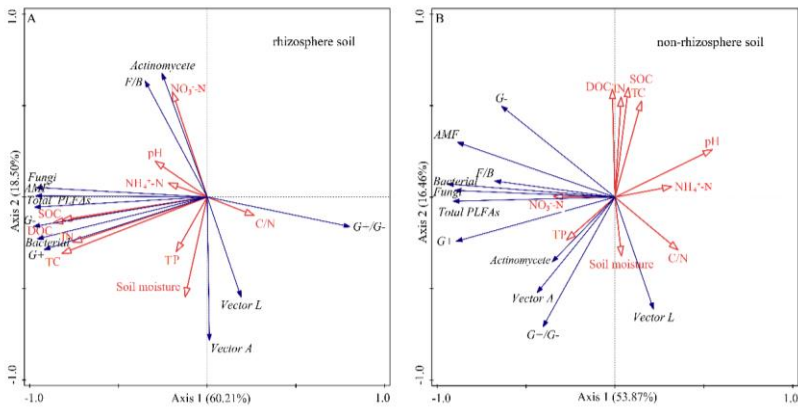
Soil properties linked to microbial variables

Redundancy analysis (RDA) showed that the first two axes accounted for 78.71% of the total variation in rhizosphere soil (Fig. 4A). The first RDA axis accounted for 60.21% of the total variation, with the strong loadings of TC, TN, SOC and DOC, and the second RDA axis accounted for 18.50% of the total variation. DOC ($F=8.3$, $P=0.002$), TC ($F=6.9$, $P=0.004$), SOC ($F=7.0$, $P=0.004$) and TN ($F=5.5$, $P=0.016$) were identified as the dominant factors influencing soil



microbial variables, and they were positively associated with total PLFAs, G⁺, G⁻, fungi, AMF and bacterial, while negatively associated with G⁺/G⁻. However, no significant factor was found that significantly affected the microbial variables in non-rhizosphere soil (Fig. 4B). Then, the correlation analysis of soil physicochemical properties and microbial variables was made without distinguishing rhizosphere soil from non-rhizosphere soil, we also found that soil moisture was positively related to microbial C limitation, and TP was positively related to microbial P limitation. There was no significant correlation between microbial C (vector L), P limitation (vector A) and PLFAs (Fig 5).

Fig. 4. Redundancy analysis on soil properties and soil microbial variables. Figure A shows rhizosphere soil; Figure B shows non-rhizosphere soil. TC, total carbon content; TN, total nitrogen content; TP, total phosphorus content; NO₃⁻-N, nitrate nitrogen content; NH₄⁺-N, ammonium nitrogen content; SOC, soil organic carbon content; DOC, Soil dissolved organic carbon content; G⁺, Gram-positive bacteria; G⁻, Gram-negative bacteria; AMF, arbuscular mycorrhizal fungi; F/B, the ratio of fungi to bacteria; Vector L, vector length present relative microbial C limitation; Vector A, Vector length present microbial N or P limitation. The same below.



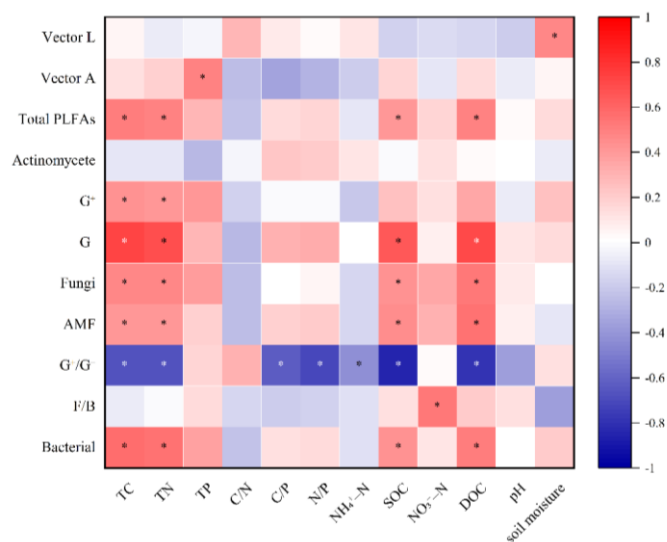


Fig. 5. The correlations among soil properties and microbial variables. * present significant differences ($P < 0.05$). See Fig 4 for abbreviations. Red and blue indicated positive and negative correlations, respectively, and the darker the color, the more significant the correlation.

Discussion

Responses of microbial community structure

The microbial biomass in soil is the final result of all microbial strains transformations in soil. Different groups of microorganisms have different functions. In the current study, G^+/G^- was significantly lower in MC than that in MM, which indicates that the soil in MC forest tended to have higher carbon availability. The possible explanation for this is that the soil microbial community structure was related to the soil nutrient. The shifts in G^+ and G^- relative proportion could be assess energy limitation in soils (Fanin et al., 2019). G^- as a group tend to grow more

349 rapidly than G^+ in high nutrient environments ([Kelly et al., 2007](#)), G^+/G^- as an indicator of carbon
350 availability can represent the soil nutrient status ([Hammesfahr et al., 2008](#)), the higher the value, the
351 more intense the nutrient stress. G^+/G^- decreases with increasing ecosystem productivity ([Fanin et](#)
352 [al., 2019](#)). Diverse mixtures of leaf litter and various root system interaction, on account of mixed
353 tree configuration, may play a key role in influencing the composition of microbial communities in
354 soils ([Bardgett, 2005](#)). It is intuitive that broad-leaved deciduous forest in MC produces large
355 quantities of litter that also decompose at a faster rate than evergreen broad-leaved forests
356 ([Cornwell et al., 2008](#)), and is evident from significantly suitable soil nutrient status in the mixed
357 evergreen and deciduous broad-leaved MC forest than in other forests ([Table 2](#)), the same as the
358 study results of [Wang et al. \(2009\)](#) and [Cornwell et al., \(2008\)](#).

359 However, we detected no significant change in other indexes of soil total microbial biomass and
360 community structure under mixed treatments ([Fig. 1](#)), indicating that microorganisms' communities
361 in this forest were similar in the three different afforested studied communities. The identity or
362 composition of plant species in forests could play vital roles in shaping soil microbial community
363 composition in distinct form at medium-long term by influencing access to nutrients and modifying
364 localized microenvironment ([Prober et al., 2015](#); [Guo et al., 2016](#)), but the main reason may be that
365 the change in environmental factors has not yet triggered the threshold of microbial variations,
366 given the short-term of plantations. Our results also may be due to multitrophic effects of mixed
367 plantations on soil microbial communities, or also because the differences in litter traits are not
368 enough to imply great and fast changes in microbial composition during their first couple of years.

369 So the effects of mixed tree species treatment on soil microbial community structure in the
370 subtropical artificial forest require further research. Therefore, the mixed tree species configuration
371 induced a relative equilibrium stability in microbial community including total PLFAs,
372 actinomycetes, G⁺, G⁻, fungal, AMF, F/B and bacterial in the subtropical artificial forest in the first
373 several years.

374 Responses of enzyme activities

375 The resource allocation theory predicts that microorganisms maximize their productivity by
376 optimizing their allocation of resources to the synthesis of extracellular C-, N-, and P-acquiring
377 enzymes ([Ma et al., 2021](#)). We found that non-rhizosphere soil had significant higher the AP activity
378 than that in rhizosphere soil, indicating P limitation of microorganisms is spread in non-rhizosphere
379 soil in the study site. The increase of AP activity was a direct response of microorganisms to low P
380 to get more available phosphorus ([Lin et al., 2022](#)). However, the mixed plantation treatment did
381 not significantly alter the enzymatic activity, this finding is not consistent with previous studies
382 indicating tree diversity could promote soil enzyme activities ([Kooch et al., 2017](#)). This could be
383 because this study was conducted in young forests during early stages of tree growth.

384 In the present study, the mean enzyme ratio of C, N, and P acquisition was close to 1: 0.7: 0.9,
385 this ratio value was inconsistent with the 1:1:1 at a global scale ([Sinsabaugh et al., 2009](#)). And C-
386 acquiring enzyme/ P-acquiring enzyme (1.11) and N-acquiring enzyme/ P-acquiring enzyme (0.72)
387 in this study were higher than the values (0.62; 0.44) in global scale ([Waring et al., 2014](#)), indicating
388 a strong P limitation in the microbial metabolisms in our study area. Vector A in this study, which is

a direct indicator of P limitation, all showed a performance greater than 45 degrees in the all treatment. The possible reason is that soil P comes from rock weathering and organic matter decomposition, high rainfall and strong soil weathering declined the soil nutrient availability through leaching of nutrient elements from soils in hot and humid regions, and P are the main limiting factors of forest ecosystem productivity and stability in these habitats in a subtropical region. Meanwhile, P is an essential nutrient for biological growth and metabolism, plant growth intensifies the competition of plants and microorganisms for soil P (Mo et al 2015; Zhang et al., 2017).

Our results revealed the mixed plantation treatment did not exhibit significantly influenced on vector L and vector A with respect mono-species plantation. It's quite possible that this is due to time-lag effects or multitrophic effects of mixed plantations on microbial metabolic limitation. In addition, P limitation existed in all plantation treatments, and P limitation differed significantly between rhizosphere soil and non-rhizosphere soil especially in MM plantation. P is important element required in large quantities for the plant growth (Fang et al., 2019), and the higher demand for absorbable phosphorus in rhizosphere soil.

Soil properties mediate microbial variable

Previous studies showed that differences in leaf, root and the quantity and quality of litter inputs in different stand types lead to the difference in soil properties and nutrient availability, which changes the quantity and quality of substrate and nutrient available to soil microorganisms, and affects the extracellular enzyme activity and ecological enzyme stoichiometry ratio (Zhang et al., 2018). Our

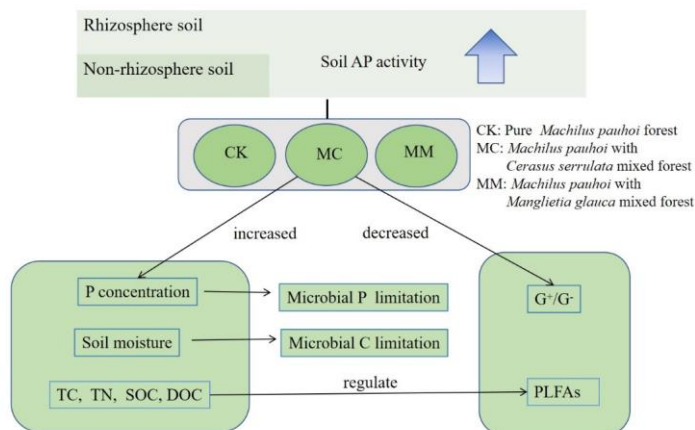
409 study showed that soil nutrient concentrations in MC was higher than those in the other two
410 plantation treatments. The differences in this study may be caused by differences in tree species, the
411 litter yield in MC was higher because in the mixed community one species was broadleaf.

412 Key environmental factors affected microbial community structure. RDA and Correlation
413 analysis indicated that DOC, TC, TN and SOC concentrations were the key factors affecting
414 microbial community structure. Carbon and nitrogen, as the major source of energy and cell
415 materials for microorganisms, are closely related to their maintenance and growth (Bai et al., 2020).
416 Nitrogen is involved in the synthesis of protein, nucleic acid and other macromolecules, and it is an
417 essential mass element for the growth and metabolism of microorganisms. Soil carbon and nutrient
418 availability, as determined by the quantity and quality of substrates by litter inputs and root
419 exudations, were crucial factors that affected microbial biomass. Similar to previous studies, this
420 result showed that soil total PLFAs, fungi, bacterial, G^+ , G^- and AMF were significantly positively
421 correlated with SOC, TC, TN and DOC (Fig. 6), as also reported by several studies (Han et al.,
422 2015; Bai et al., 2020).

423 Soil moisture is a major factor affecting enzymatic stoichiometry and is imperative for soil
424 microbial activity and function (Zhang et al., 2021). It plays an essential role in substrate
425 dissolution and transformation and controls the rate of all biochemical reactions, including enzyme
426 production (Shi et al., 2020). The study showed microbial relative C limitation were significantly
427 positively affected by soil moisture. The effect suggested that higher soil moisture content could
428 promote nutrient cycling and microbial metabolism, which increasing microbial demand for C

429 sources (Cui et al., 2020). Zhang et al., (2021) have also found that soil moisture can affect soil
430 nutrient availability and control decomposer community activity, and constrain litter decomposition
431 nutrient release, and aggravate microbial nutrient limitations. Our study found that soil TP was
432 correlated positively with vector A, which is accordance with Cui et al., (2021), suggesting different
433 effects of soil total nutrient on microbial nutrient limitation. In addition, we found that microbial
434 nutrient limitation was no correlated with microbial community structure. Similar to previous study,
435 Tan et al., (2021) suggested soil chemical properties rather than the abundance of active and
436 potentially active microorganisms control soil enzyme kinetics.

437 However, this study only focus on the young forest stage of *Machilus pauhoi*, the soil microbial
438 community and enzymatic stoichiometry may be change significantly due to a long-term impact of
439 different species mixed on leaf litter and root interaction. Therefore, further research of mixed
440 species composition should be carried out for longer and more continuous observation. Our study
441 only focused on the rhizosphere soil and non-rhizosphere soil of *Machilus pauhoi*, not study the soil
442 of broad-leaved tree species that interbred with it, studies evaluating more tree species will help
443 improve our understand of interspecific relationship among mixed species.



444
 445 Fig. 6. A conceptual framework showing the response of soil microbial variables to mixed broad-
 446 leaved plantations in a subtropical forest ecosystems.

447 Conclusions

448 We examined the effects of different native tree species mixed plantations on soil properties,
 449 microbial community structure and enzyme activity. Our study revealed that the soil total P
 450 concentration was higher in MC than that in CK, showing that adequate species mixing can
 451 improve the soil concentrations of the limiting nutrient with respect mono-species plantations. Total
 452 soil C, TN, SOC and DOC concentrations were higher in MC compared to MM, which mainly
 453 attribute to the presence of deciduous species in MC. Woody deciduous species (such as, *Cerasus*
 454 *serrulata*) usually return faster and have shorter individual leaf spans and have faster litter
 455 decomposition rates than evergreen species. Different mixed species plantations had significant
 456 effects on G^+/G^- ratio. P limitation of rhizosphere soil were higher than those of non-rhizosphere

soil. Soil TC, TN, SOC, DOC were the key factors affecting microbial variables. Thus, in this subtropical environments adequate mixing plantations with native tree species, by improving soil P concentrations, can improve soil microbial community structure and enhance the bio-availability of the limiting soil nutrients. Mixing with *Cerasus campanulata* may be an effective management measure to improve soil environment in future afforestation models. Our study suggest that we still have a long way to go to explore microbial PLFAs and metabolic nutrient limitation in subtropical plantation ecosystems under longer different species composition.

Declaration of competing inte rest

The authors declare no competing interests.

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