Coniferous-Broadleaf Mixture Increases Soil Microbial Biomass and Functions Accompanied by Improved Stand Biomass and Litter Production in Subtropical China

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Abstract: Although the advantages of multi-species plantations over single-species plantations have been widely recognized, the mechanisms driving these advantages remain unclear. In this study, we compared stand biomass, litter production and quality, soil properties, soil microbial community, and functions in a *Pinus massoniana* Lamb. and *Castanopsis hystrix* Miq. mixed plantation and their corresponding mono-specific plantations after 34 years afforestation in subtropical China. The results have shown that a coniferous-broadleaf mixture created significantly positive effects on stand biomass, litter production, soil microbial biomass, and activities. Firstly, the tree, shrub and herb biomass, and litter production were significantly higher in the coniferous-broadleaf mixed plantation. Secondly, although the concentrations of soil organic carbon (SOC) and total nitrogen (TN) were lower in the mixed stand, the concentrations of soil microbial biomass carbon (MBC), and nitrogen (MBN), along with MBC-to-SOC and MBN-to-TN ratio, were significantly higher in mixed stands with markedly positive admixing effects. We also found higher carbon source utilization ability and β-1,4-N-acetylglucosaminidase, urease and acid phosphatase activities in mixed stands compared with the mono-species stands. Our results highlight that establishment of coniferous-broadleaf mixed forests may be a good management practice as coniferous-broadleaf mixture could accumulate higher stand biomass and return more litter, resulting in increasing soil microbial biomass and related functions for the long term in subtropical China.

Keywords: coniferous-broadleaf mixture; soil microbial biomass; extracellular enzymes; phospholipid fatty acids (PLFA); carbon source utilization ability

1. Introduction

Planted forests have been rapidly expanding around the world to meet the ever-growing demand for timber and other ecosystem services while avoiding excessive harvesting of natural forests [1,2]. Most planted forests are monospecific plantations. In China, 85% of the country's planted forests are

monospecific plantations as reported [3]. However, there are serious concerns regarding monospecific plantations, including stand productivity decrease [4], soil quality degradation [5], and serious exotic plant invasion (e.g., *Eucalyptus* plantations) [6]. In contrast, multi-species plantations often have higher biodiversity, higher productivity, and better soil quality than single-species plantations [7–9]. Theoretically, higher productivity in multi-species compared to single-species plantations can only be expected when species interactions in the multi-species plantations increase the efficiency of resource use or one species increases the supply of a resource [10]. However, interactions between species growing in mixtures, e.g., competition, competitive reduction, and facilitation [11] are often dynamic, changing through space and time as resource availability or climatic conditions change [12,13]. Therefore, it is necessary to understand how tree species interact in mixed plantations and to further develop these systems to minimize competition while maximizing both wood production and other ecosystem services in the long term.

Masson pine (*Pinus massoniana* Lamb.) and *Castanopsis hystrix* Miq. are two afforestation tree species that are most widely planted in subtropical China, usually making up monoculture plantations. Such monospecific plantations have caused serious ecological concerns, including declines in stand productivity, litter quality, and soil fertility resulting from long-term single litter input, increased pest and disease attack, self-allelopathy, and site degradation [14–16]. To mitigate these disadvantages, mixed *P. massoniana* and *C. hystrix* plantations were established about 40 years ago at the Experimental Center of Tropical Forestry, Chinese Academy of Forestry, in subtropical China. At present, the area of these plantations is more than 5000 ha [1]. Previous studies have suggested that mixed plantations of these two genera could lead to more plant species diversity, more productivity, faster litter decomposition rate, and higher carbon stocks compared to monospecific stands [7,14,16,17]. Interactions between aboveground plant communities and belowground microbial communities may underlie some of these positive effects, while little is known about these interactions in mixed stands of *P. massoniana* and *C. hystrix*.

In general, there are enormously important interactions between the composition of aboveground plant communities and belowground microbial communities in forest ecosystems [18]. Management strategies such as tree species transformation, adding N_2-fixing species or broad-leaf species in coniferous monospecific stands may change the amount and chemical composition of litterfall, root mass, and exudates [14,19], then affect soil organic matter quantity and dynamics [8], along with microsite conditions [20]. As a result, these changes may control the composition and functions of the soil microorganisms which acquire carbon from primarily producer-derived organic resources [21]. At the same time, soil microbes are important regulators of plant productivity through the mineralization of, and competition for, nutrients that sustain plant productivity [22]. Moreover, soil microbial diversity and abundance/biomass play key roles in ecosystem sustainability by maintaining essential functions of soil health through carbon and nutrient turnover [23]. Meta-analysis has shown that plant mixtures have increased soil total microbial, bacterial, and fungal biomass across a diverse range of terrestrial ecosystems, and the increase in microbial biomass was more pronounced in older and more diverse mixtures [24]. This may be attributable to higher productivity inducing more carbon and nutrient inputs to the soil in mixtures, benefitting both fungi and bacteria [24,25]. Moreover, different tree species in mixtures produce litter of varying quality, interacting with the rhizosphere and causing microenvironmental changes that affect microbial activity and therefore soil nutrients [26–28]. However, the effects of tree species mixtures on soil microorganisms may change with ecosystem types, environment, and time. Whether coniferous-broadleaf mixture increases soil microbial biomass and activity in subtropical China in long-term afforestation remains unexplored.

In this study, we explored stand biomass, litter, soil physicochemical properties, and soil microbial community structure and function in a mixed *P. massoniana* and *C. hystrix* plantation and corresponding single species plantations at an age of 34 years in subtropical China. Our goal was to determine the effects of coniferous-broadleaf mixture on soil microbial communities and functions in long term. We hypothesized that (1) stand biomass, litter production, and quality are higher in the mixed stands

than those in corresponding monocultures in long term; (2) positive admixing effects on soil microbial biomass and functions are accompanied by improved stand biomass and litter production.

2. Materials and Methods

2.1. Study Sites and Experimental Design

This study was conducted at the Experimental Center of Tropical Forestry (22°10′ N, 106°50′ E), Chinese Academy of Forestry, Pingxiang City, Guangxi Zhuang Autonomous Region, P.R. China. This region belongs to a subtropical monsoon climate zone, and has a humid-to-semi-humid climate with dry-cool (from October to April) and wet-warm seasons (from May to September), according to the variation of precipitation and temperature [29]. The mean annual precipitation is about 1400 mm; most rainfall occurs from April to September. The mean annual temperature is 21 °C. The loamy-textured soil in this area was formed from granitic parent geological material, and is classified as red soil in the Chinese system of soil classification, which is equivalent to an oxisol in the USDA Soil Taxonomy [8,14].

Our study site consisted of a 33 ha plantation of pure *C. hystrix* (PCH), a 33 ha plantation of pure *P. massoniana* (PPM), and an 18 ha plantation of mixed *C. hystrix* and *P. massoniana* (MIX). Prior to the establishment of the current plantations, the area was covered by a *Cunninghamia lanceolata* monospecific plantation, which was established in 1958 on a historically deforested hill and clear harvested in 1983. After clearcutting the prior plantations, the PCH, PPM, and MIX plantations were established in 1983 with an initial planting density of 2500 tree ha^{-1} and a 1:1 mixed proportion in the MIX for comparison of tree productivity and the ecological benefits of mixed plantations. Buffer zones of 150–800 m were set up to separate these plantations. These stands had similar soil types, topographies, and management histories.

In June 2017, 34 years after the establishment of the plantations, we randomly established five sample plots (20 m × 20 m) in each plantation. The five sampling plots within a single experimental stand were well separated, with distances of at least 200 m between adjacent plots. Elevations of all plots were 530–650 m, and the degree of slopes was 30–39°. The stand density ranged from 355 to 400 tree ha^{-1}, and average height from 22 m to 25 m. The diameter at breast height (DBH) and basal area of MIX was significantly higher than that of PCH and PPM (Table 1).

Table 1. Characteristics of pure *Castanopsis hystrix* Miq. (PCH) plantation, pure *Pinus massoniana* Lamb. (PPM) plantation, and mixed *C. hystrix* and *P. massoniana* (MIX) plantation (mean ± standard error; *n* = 5).

Stand	Density (Tree ha^{-1})	Height (m)	DBH (cm)	Basal Area (m^2 ha^{-1})
PCH	355 ± 15 [a]	23.95 ± 0.80 [a]	28.24 ± 0.97 [b]	23.15 ± 1.50 [b]
MIX	380 ± 10 [a]	24.52 ± 0.37 [a]	34.31 ± 1.02 [a]	39.67 ± 2.27 [a]
P. massoniana	95 ± 9	22.01 ± 0.67	26.16 ± 0.75	5.35 ± 0.85
C. hystrix	285 ± 15	25.33 ± 0.39	36.95 ± 1.63	34.32 ± 2.91
PPM	400 ± 24 [a]	23.32 ± 0.71 [a]	28.16 ± 1.04 [b]	26.60 ± 2.57 [b]

Different superscripts indicate significant differences among stand types (*p* < 0.05). DBH, diameter at breast height.

2.2. Stand Biomass Measurements

Three 2 m × 2 m subplots and three 1 m × 1 m subplots were randomly placed in each plot for shrub and herb biomass measurements, respectively, in the wet-warm (July 2017) and dry-cool seasons (January 2018). In each season, shrubs and herbs were destructively harvested and divided into aboveground and belowground parts. All understory vegetation samples were transported to the laboratory and oven-dried at 75 °C for dry biomass determination (Mg ha^{-1}) [30].

In addition, we measured tree height with a digital clinometer (Vertex IV, Haglöf Inc., Langsele, Sweden) and DBH with a diameter tape of each tree in each plot and in each season. Tree aboveground

biomass (TAB) and tree belowground biomass (TBB) were calculated by the allometric equations described by You et al. (2018) [7].

2.3. Litter Production and Quality Measurements

A litter collector with an effective collection area of 4 m^2 was randomly set in each plot. Each litter collector was made with nylon net, with a mesh size of 1 mm, and set 0.5 m above the ground with PVC (polyvinyl chloride) tube. Litter was collected once a month from August 2017 to July 2018. Litter samples were transported to the laboratory and oven-dried at 75 °C for production determination (Mg ha^{-1}). Litter organic carbon (OC$_{litter}$) concentration was determined by the K$_2$Cr$_2$O$_7$–H$_2$SO$_4$ calefaction procedure [30]. Litter total nitrogen (TN$_{litter}$), total phosphorus (TP$_{litter}$), and total potassium (TK$_{litter}$) concentrations were measured after heat digestion with 98% H$_2$SO$_4$ [31–33].

2.4. Soil Sampling and Analysis

Soil samples were also collected in the wet-warm (July, 2017) and dry-cool seasons (January, 2018). In each season, we took eight soil cores systematically arranged at a distance of 5 m from the plot center in the directions of 0°, 45°, 90°, 135°, 180°, 215°, 270°, and 315°. Each core was taken using an 8.7 cm inner diameter steel tube at a depth of 0–10 cm below the soil surface. The eight cores of each plot were mixed to form a composite sample. Each soil sample was divided into two subsamples. One subsample was air-dried to a constant weight and sieved using a 2 mm sieve for measuring soil properties. The other subsample was sieved to 2 mm and immediately stored at −4 °C for measuring moisture content, soil ammonium nitrogen (NH$_4^+$-N), nitrate nitrogen (NO$_3^-$-N), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) concentration, phospholipid fatty acid analysis (PLFA) assays, carbon source utilization ability of the soil microbial communities, and enzyme activity. At the same time, three soil cores (100 cm^3) from 0–10 cm soil layer were sampled from each plot to measure bulk density by the cutting ring method [34].

The soil water content (WC) was measured using a sub-sample dried in oven at 105 °C until the sample reached a constant weight. The soil temperatures were measured with an LI-8100A Automated Soil Gas Flux System (Li–Cor, Lincoln, NE, USA). Soil pH was measured in a 1:2.5 soil/water suspension using a pH meter [8,35]. Soil organic carbon (SOC) concentration was determined by the K$_2$Cr$_2$O$_7$–H$_2$SO$_4$ calefaction procedure [31]. Total nitrogen (TN), total phosphorus (TP), and total potassium (TK) concentration were measured after heat digestion with 98% H$_2$SO$_4$. TN and TK were measured using the liquid supernatant with a continuous flow analytical system (AA3; SEAL Analytical, Norderstedt, Germany) [31,33] and an atomic absorption spectrometer (ZEENIT 700P; Analytik, Jena, Germany) [32], respectively. TP and AP were determined by Mo–Sb–Vc colorimetry with the liquid supernatant after heat digestion and HCl (0.05 M)–H$_2$SO$_4$ (0.025 M), respectively. And available potassium (AK) was determined by atomic absorption spectrometry with 1 M NH$_4$OA$_C$. Soil ammonium nitrogen (NH$_4^+$-N) and nitrate nitrogen (NO$_3^-$-N) were calorimetrically determined in a continuous flow analyzer after 10 g fresh mass of soil had been extracted in 50 mL of 2 M KCl [8,36]. The cation exchange capacity (CEC) was determined by [Co(NH3)$_6$]Cl$_3$ extraction spectrophotometry [37,38].

Soil microbial biomass carbon (MBC) and nitrogen (MBN) were measured by fumigation-extraction, using 0.5 M K$_2$SO$_4$ as the extraction agent [39,40], with a total organic carbon analyzer (1020A; OI, College Station, TX, USA).

Phospholipid fatty acid analysis (PLFA) assays were employed to analyze soil microbial community structure with freeze-dried soil samples using the procedure developed by Bossio et al. (1998) [41]. The PLFA markers 14:0, 15:0, 17:0, 18:0, 14:1 ω5c, 15:0 3OH, 15:1 ω6c, 16:0 2OH, 16:1 2OH, 16:1 ω9c, 16:1 ω7c, 18:1 ω7c, 18:0 2OH, 18:1 ω5c, a15:0, a16:0, a17:0, cy17:0, cy19:0, i14:0, i15:0, i16:0, i17:0, i18:0 and i19:0 were chosen to represent bacteria. Gram-positive (GP) bacteria were represented by a15:0, a16:0, a17:0, i14:0, i15:0, i16:0, i17:0, i18:0, and i19:0; and gram-negative (GN) bacteria were represented by 14:1 ω5c, 15:0 3OH, 15:1 ω6c, 16:0 2OH, 16:1 2OH, 16:1 ω7c, 16:1 ω9c, 18:0 2OH, 18:1 ω5c, cy17:0,

cy19:0, and 18:1 ω7c [36,42,43]. Actinobacteria were represented by 10-Me17:0, 10-Me18:0, TBSA, 11-Me18:1 ω7c, and 10-Me16:0 [12,43,44]. Arbuscular mycorrhizal fungi (AMF) were represented by 16:1 ω11c, and 16:1 ω5c [35]; and fungi were represented by 18:1 ω9c, 18:3 ω6c (6,9,12), and 18:2 ω6,9c [12,44]. Other PLFAs, such as 15:0 2OH, 16:0, 17:1 ω8c, 18:0 iso, and i20:0, were also used for the analysis of the composition of microbial communities.

Carbon source utilization ability of the soil microbial communities was analyzed using BIOLOG® Eco plates (Biolog, Hayward, CA, USA) with fresh soil samples. Each Biolog Eco–plate comprised 96 wells containing triplicates of 31 different carbon sources and a blank [45]. We added 10 g dry weight equivalent of fresh soil to 90 mL of 0.85% sterile saline physiological solution, and shook the mixture for 1 h on a rotary shaker (100 rpm) at 25 °C. After 10^{-3} dilution, we transferred samples containing 150 μL of liquid into the 96 wells, and read the optical density (OD) at 590 and 750 nm with a Microstation™ reader (Biolog) after 24, 48,72, 96, 120, 144, 168 and 192 h of incubation [46,47].

Enzyme assays were performed with a fully automatic full-wavelength enzyme labeling apparatus (Infinite M200 Pro; Tecan Inc., Männedorf, Switzerland) using the procedure describe in detail by Saiya-Cork et al. (2002) [48] and Steinauer et al. (2015) [49]. The phenol oxidase and peroxidase assays were colorimetric, using L3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. The colorimetric urease assay used urea as the substrate. The α-Glucosidase (AG), β-1,4-glucosidase (BG), β-Cellobiosidase (CB), acid phosphatase (APH) and β-1,4-N-acetylglucosaminidase (NAG) activities were fluorometric with substrate 4-methylumbelliferyl-α-D-glucoside, 4-methylumbelliferyl-β-D-glucoside, 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-phosphate, and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively.

2.5. Statistical Analysis

One-way analyses of variances (ANOVAs) were used to examine the differences of stand biomass, litter production and quality, soil physicochemical properties, soil microbial biomass, soil microbial PLFAs content and functions among PPM, PCH, and MIX plantations in each season. Two-way ANOVAs were used to determine the interaction effects between stand type and season on the above variables. Statistical analyses were performed with SPSS software (ver. 25.0; IBM Corp., Armonk, NY, USA). Significant differences between means were tested by least significant difference (LSD) at the 0.05 level.

In order to evaluate admixing effects on soil microbial biomass, we calculated the relative effects (RE) of mixing species by comparing the observed value (OV) of one variable in mixed plantation with the predicted value (PE) based on the respective mono-specific plantations. RE was calculated by following Wardle et al. (1997) [50]:

$$RE = [(OV - PE)/PE] \times 100, \tag{1}$$

where PE is the mean of the observed value of one variable in respective mono-specific plantations. If RE differs from zero, it would indicate non-additive effects (NAE) of mixing species on soil microbial biomass. Negative and positive deviations from zero are referred to as antagonistic and synergistic effects, respectively. One-sample Student's *t*-tests with 95% confidence intervals were employed to test whether RE differed significantly from zero.

Redundancy analysis (RDA) was used to determine the relationships among biotic and abiotic factors, and soil microbial composition and functions by CANOCO software for Windows 5 (Biometris-Plant Research international, Wageningen, The Netherlands). In RDA, the relative abundance of individual PLFAs (mole present of the total PLFAs) was used as the response variables, and soil physicochemical properties, litter and stand biomass were used as the explanatory variables [35,51]. We also used RDA to determine the linkage between soil microbial community and functions, including carbon source metabolic function and soil extracellular enzyme activity involved in biogeochemical circulation of carbon, nitrogen, and phosphorus. All data were log-transformed before in the analyses.

The forward selection procedure in RDA, based on Monte Carlo permutation with 499 iterations, was performed to determine the dominant variables that influenced the composition of individual PLFAs and microbial functions. A significance level of $p < 0.05$ was used in all analyses.

3. Results

3.1. Stand Biomass

Stand type had significant effects on the -above- and below-ground biomass of trees, shrubs and herbs (Table S1; Figure 1). TAB, TBB, shrub aboveground biomass (SAB), shrub belowground biomass (SBB), and herb aboveground biomass (HAB) in MIX were significantly higher than those in PCH and PPM in both the dry-cool and wet-warm seasons (Figure 1a–e). Except for HAB, season had no significant effects on the tested biomass variables. Moreover, significant interaction effects between stand type and season only found on HAB and HBB. These results supported our first hypothesis that tree species mixture increased stand biomass.
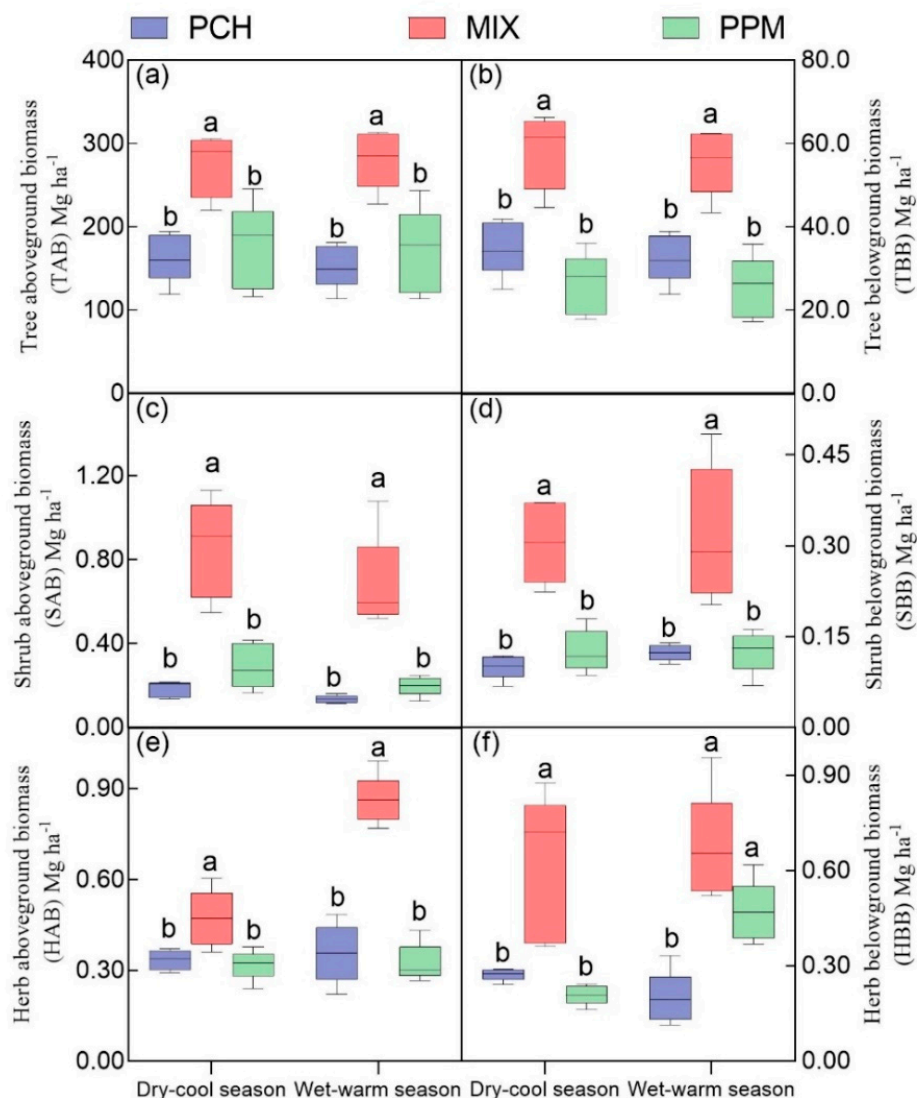
Figure 1. Biomass of trees (**a**,**b**), shrubs (**c**,**d**) and herbs (**e**,**f**) in three plantations in different seasons. Different letters above the bars denote significant differences at $p < 0.05$ level by LSD (least significant difference) multiple comparisons among stand types ($n = 5$).

3.2. Litter Production and Quality

Stand type, the interaction between stand type, and season significantly influenced litter production at our study sites (Table 2). The MIX had significantly higher litter production than PCH and PPM in both seasons. OC_{litter} and CN_{litter} were significantly affected by both stand type and season with higher value in the wet-warm season than in the dry-cool season in all plantations. An opposite trend was observed in TN_{litter}. TP_{litter} was significantly affected by stand type, season, and their interaction with MIX had significantly lower concentration than PCH and PPM in the dry-cool season. TK_{litter} was significantly affected by stand type with MIX had significantly higher concentration than PCH and PPM in the wet-warm season. These results partially supported our first hypothesis that tree species mixture increased litter production, although the increases of litter quality in mixed plantations were not significant.

3.3. Soil Physical and Chemical Properties

In the dry-cool season, the mixed plantations had the highest soil bulk density (BD) and soil water content (WC), and the lowest soil temperature and medium pH compared to the pure plantations (Table 3). In the wet-warm season, MIX had the medium BD, WC and soil temperature, and the highest pH compared to PPM and PCH.

The PPM plantations had the highest SOC, TN, TP, SOC-to-TN ratio, NH_4^+-N and CEC, while MIX had the highest AP, TK and AK, and PCH had the highest NO_3^--N and AN in the dry-cool season (Table 3). On the other hand, the PCH had the highest SOC, TN and AP, while MIX had the highest TP, TK, AK, NO_3^--N and AN, and PPM had the highest SOC-to-TN ratio, NO_3^--N, and CEC in the wet-warm season.

Two-way ANOVAs indicated that season significantly affected all the tested soil variables (Table 3). In addition, the stand type influenced nearly all soil variables except BD, TN, NH_4^+-N and AN. Interactions between stand type and season significantly influenced WC, temperature, pH, SOC, TN, TP, AP, AK, NO_3^--N and CEC.

Table 2. The quantity and quality of litter in pure *Castanopsis hystrix* (PCH) plantation, pure *Pinus massoniana* (PPM) plantation, and mixed *C. hystrix* and *P. massoniana* (MIX) plantation (mean ± standard error; *n* = 5).

Factors	Dry-Cool Season			Wet-Warm Season			Stand Type		Season		Stand Type × Season	
	PCH	MIX	PPM	PCH	MIX	PPM	*F*	*p*	*F*	*p*	*F*	*p*
LP	6.68 ± 0.19 [b]	8.36 ± 0.69 [a]	4.84 ± 0.19 [c]	5.53 ± 0.43 [b]	9.21 ± 0.40 [a]	6.16 ± 0.23 [b]	37.52	<0.001	0.93	0.343	5.27	0.013
OC_{litter}	190.64 ± 10.63 [b]	248.59 ± 18.48 [a]	255.05 ± 18.00 [a]	277.57 ± 18.85 [b]	274.3 ± 11.42 [b]	321.75 ± 5.44 [a]	6.83	0.004	24.86	<0.001	2.26	0.127
TN_{litter}	17.38 ± 0.78 [a]	13.53 ± 1.06 [b]	14.96 ± 0.62 [ab]	12.95 ± 0.59 [a]	13.57 ± 0.90 [a]	11.89 ± 0.50 [a]	3.21	0.058	15.79	0.001	4.45	0.023
CN_{litter}	11.12 ± 0.95 [b]	18.50 ± 0.81 [a]	17.33 ± 1.81 [a]	21.82 ± 2.23 [b]	20.56 ± 1.56 [b]	27.22 ± 0.96 [a]	7.67	0.003	38.90	<0.001	5.18	0.013
TP_{litter}	1.34 ± 0.08 [a]	0.50 ± 0.07 [b]	1.16 ± 0.05 [a]	1.19 ± 0.15 [a]	1.38 ± 0.15 [a]	1.31 ± 0.05 [a]	6.67	0.005	12.81	0.002	14.17	<0.001
TK_{litter}	1.16 ± 0.16 [a]	1.52 ± 0.10 [a]	0.82 ± 0.09 [b]	1.07 ± 0.02 [b]	1.49 ± 0.13 [a]	0.88 ± 0.07 [c]	20.84	<0.001	0.07	0.796	0.29	0.751

Different superscripts indicate significant differences among stand types in each season ($p < 0.05$). LP (Mg ha^{-1}), litter production; OC_{litter} (g kg^{-1}), litter organic carbon; TN_{litter} (g kg^{-1}), litter total nitrogen; CN_{litter}, C/N ratio of litter; TP_{litter} (g kg^{-1}), litter total phosphorus; TK_{litter} (g kg^{-1}), litter total potassium.

Table 3. Soil physical and chemical properties (0–10 cm soil depth) in pure *Castanopsis hystrix* (PCH) plantation, pure *Pinus massoniana* (PPM) plantation, and mixed *C. hystrix* and *P. massoniana* (MIX) plantation (mean ± standard error; *n* = 5).

Factors	Dry-Cool Season			Wet-Warm Season			Stand Type		Season		Stand Type × Season	
	PCH	Mixed	PPM	PCH	Mixed	PPM	*F*	*p*	*F*	*p*	*F*	*p*
BD	1.09 ± 0.03 [b]	1.25 ± 0.04 [a]	1.19 ± 0.02 [a]	1.03 ± 0.01 [a]	1.03 ± 0.03 [a]	1.04 ± 0.05 [a]	3.11	0.063	30.44	<0.001	2.97	0.070
WC	39.66 ± 1.36 [b]	43.59 ± 0.61 [a]	40.17 ± 1.58 [ab]	40.53 ± 1.07 [c]	44.40 ± 0.84 [b]	50.48 ± 1.41 [a]	10.35	0.001	16.81	<0.001	10.48	0.001
Tem.	16.82 ± 0.21 [a]	15.50 ± 0.09 [c]	16.17 ± 0.05 [b]	26.28 ± 0.43 [a]	25.98 ± 0.22 [a]	25.41 ± 0.08 [a]	8.21	0.002	2833.34	<0.001	4.31	0.025
pH	4.32 ± 0.02 [a]	4.19 ± 0.03 [b]	4.04 ± 0.05 [c]	4.02 ± 0.02 [b]	4.21 ± 0.03 [a]	3.90 ± 0.09 [b]	14.40	<0.001	13.70	0.001	6.23	0.007
SOC	37.42 ± 1.08 [b]	40.82 ± 0.22 [b]	56.98 ± 1.89 [a]	53.31 ± 1.94 [a]	48.39 ± 2.00 [a]	50.83 ± 0.54 [a]	24.60	<0.001	23.69	<0.001	28.64	<0.001
TN	1.63 ± 0.05 [b]	1.60 ± 0.06 [b]	2.16 ± 0.22 [a]	2.48 ± 0.11 [a]	2.13 ± 0.13 [a]	2.17 ± 0.14 [a]	2.48	0.105	44.08	<0.001	4.80	0.018
SOC/TN	22.98 ± 0.59 [b]	25.67 ± 1.03 [b]	29.49 ± 1.59 [a]	21.67 ± 1.33 [a]	23.04 ± 1.62 [a]	23.90 ± 2.02 [a]	4.57	0.021	7.22	0.013	1.14	0.338
TP	0.31 ± 0.04 [c]	0.42 ± 0.02 [b]	0.60 ± 0.04 [a]	0.52 ± 0.06 [b]	0.78 ± 0.04 [a]	0.54 ± 0.04 [b]	21.86	<0.001	28.89	<0.001	17.95	<0.001
AP	9.19 ± 0.58 [b]	12.52 ± 0.69 [a]	10.02 ± 0.30 [b]	13.30 ± 0.40 [a]	13.15 ± 0.57 [a]	9.98 ± 0.27 [b]	16.70	<0.001	15.25	0.001	10.29	0.001
TK	4.14 ± 0.15 [b]	5.45 ± 0.49 [a]	2.42 ± 0.16 [c]	5.40 ± 0.50 [a]	6.94 ± 0.82 [a]	3.38 ± 0.13 [b]	32.26	<0.001	10.34	0.004	0.39	0.680
AK	29.90 ± 1.49 [b]	46.29 ± 0.96 [a]	28.46 ± 1.08 [b]	47.19 ± 2.28 [a]	52.79 ± 3.65 [a]	28.37 ± 1.44 [b]	53.75	<0.001	22.57	<0.001	9.27	0.001
NO_3^--N	3.55 ± 0.24 [a]	1.85 ± 0.04 [c]	2.86 ± 0.22 [b]	4.06 ± 0.19 [c]	5.30 ± 0.09 [b]	5.79 ± 0.09 [a]	10.96	<0.001	291.06	<0.001	45.47	<0.001
NH_4^+-N	21.29 ± 1.01 [a]	23.34 ± 1.13 [a]	24.47 ± 1.37 [a]	42.24 ± 1.62 [a]	43.50 ± 1.66 [a]	38.83 ± 2.47 [a]	0.75	0.482	196.16	<0.001	2.47	0.105
AN	27.34 ± 1.57 [a]	25.19 ± 1.14 [a]	24.85 ± 0.90 [a]	46.3 ± 1.55 [a]	48.8 ± 1.69 [a]	44.62 ± 2.46 [a]	1.20	0.319	245.55	<0.001	1.17	0.328
CEC	45.53 ± 3.27 [c]	57.58 ± 4.11 [b]	82.33 ± 1.94 [a]	55.43 ± 2.02 [a]	54.46 ± 1.19 [a]	62.24 ± 2.91 [a]	45.14	<0.001	12.16	0.002	21.68	<0.001

Different superscripts indicate significant differences among stand types in each season ($p < 0.05$). BD (g cm^{-1}), bulk density; WC (%), soil water content; Tem. (°C), temperature; SOC (g kg^{-1}), soil organic carbon; TN (g kg^{-1}), total nitrogen; TP (g kg^{-1}), total phosphorus; AP (mg kg^{-1}), available phosphorus; TK (g kg^{-1}), total potassium; AK (mg kg^{-1}), available potassium; NO_3^--N (mg kg^{-1}), nitrate nitrogen; NH_4^+-N (mg kg^{-1}), ammonium nitrogen; AN (mg kg^{-1}), Available nitrogen; CEC (cmol + kg^{-1}), cation exchange capacity.

3.4. Soil Microbial Biomass and Community Structure

Two-way ANOVAs indicated that MBC, MBN, MBC-to-SOC and MBN-to-TN were significantly affected by stand type, and MBN was also significantly affected by season and interaction between stand type and season (Table S2).

In the dry-cool season, MBC was significantly higher in MIX than in PCH and PPM (Figure 2a). In the wet-warm season, MBC was significantly higher in MIX than in PPM, and MBN was significantly higher in MIX than in PCH and PPM (Figure 2b). The coniferous-broadleaf mixed stand had the highest MBC-to-SOC and MBN-to-TN ratio in both seasons (Figure 2c,d). The differences of the MBC-to-SOC and MBN-to-TN ratio between the mixed stand and the corresponding pure stands reached a significant level in the wet season. Non-additive effects (NAE) of species mixing on MBC, MBN, MBC-to-SOC, and MBN-to-TN ratio were recorded. Synergistic effects on MBC and MBC-to-SOC ratio in the dry-cool season (Figure 3a), and on MBC, MBN, MBC-to-SOC, and MBN-to-TN ratio in the wet-warm season (Figure 3b) were significant.
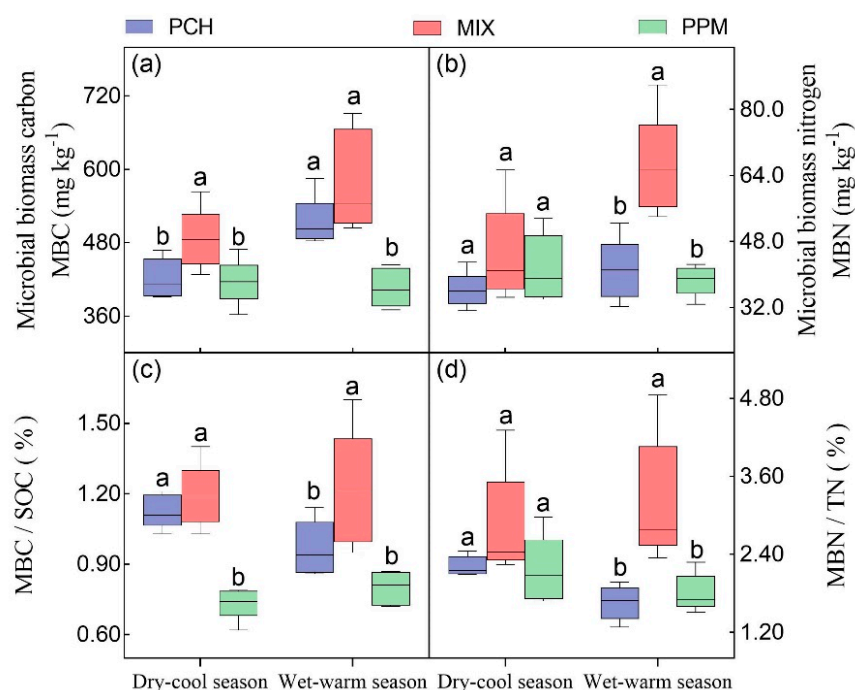

Figure 2. Soil microbial biomass in three plantations in different seasons ((**a**–**d**) refer to MBC, MBN, MBC-to-SOC and MBN-to-TN). Different letters above the bars denote significant differences at $p < 0.05$ level by LSD multiple comparisons among stand types ($n = 5$).
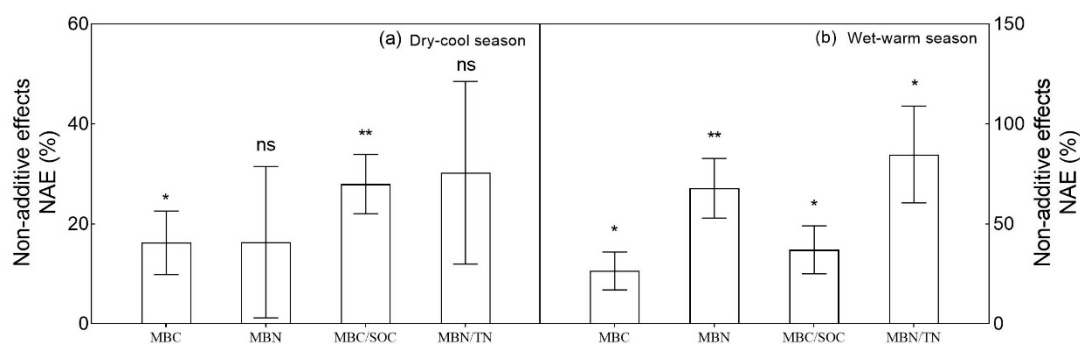
Figure 3. Not-additive effects of mixed plantation on soil microbial biomass in the dry-cool season (**a**) and wet-warm season (**b**).

In the dry-cool season, total PLFAs, AMF PLFAs, actinobacteria PLFAs, bacteria PLFAs, fungi PLFAs, GP bacteria PLFAs, and GP-to-GN PLFAs ratio were smaller in MIX than those in PPM and PCH. On the other hand, in the wet-warm season, these PLFAs were smaller in PPM than those in PCH and MIX (Table 4). Specifically, the ratios of bacteria-to-fungi PLFA and GP-to-GN PLFA were significantly higher in MIX than those in PPM and PCH in the wet-warm season. These results indicated that the response of the soil microbial community structure to tree species mixture varied in different seasons. Two-way ANOVAs showed that both stand type and season significantly affected all the tested microbial variables, while the interaction between stand type and season significantly influenced total PLFAs, bacteria PLFAs, GP bacteria PLFAs, Acti PLFAs, bacteria-to-fungi PLFA, and GP-to-GN PLFA (Table 4).

Across plantations and seasons, bacteria PLFAs were the most abundant, followed by fungi and actinobacteria PLFAs (Figure 4 and Table S3). It was worth noting that the relative abundance of GN bacteria PLFAs was significantly higher in MIX than in other plantations in the dry-cool season (Figure 4). Furthermore, the relative abundance of GP bacteria PLFAs was significantly higher, and that of fungi PLFAs was significantly lower in MIX than in other plantations in the wet-warm season.
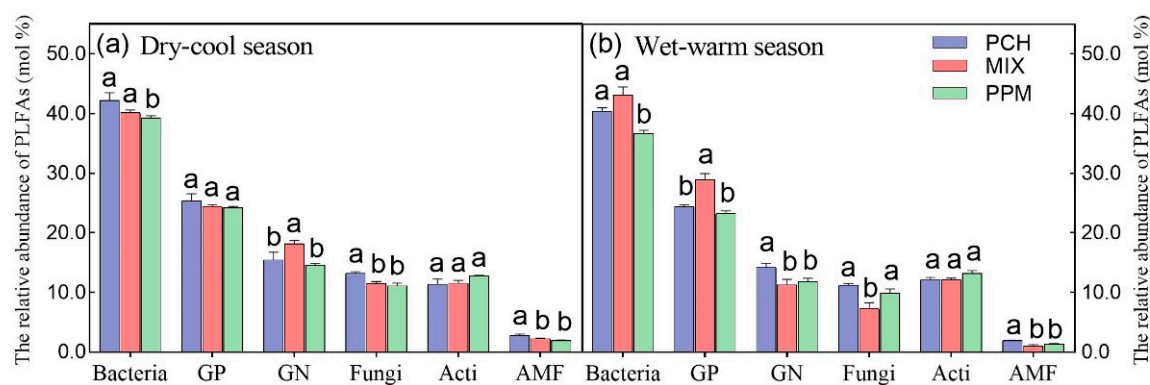
Figure 4. The relative abundance of PLFAs (mol %) in three plantations in the dry-cool season (**a**) and wet-warm season (**b**). Different letters above the bars denote significant differences at $p < 0.05$ level by LSD multiple comparisons among stand types ($n = 5$).

Table 4. Soil microbial community PLFAs content and structure (0–10 cm soil depth) in pure *Castanopsis hystrix* (PCH) plantation, pure *Pinus massoniana* (PPM) plantation, and mixed *C. hystrix* and *P. massoniana* (MIX) plantation (mean ± standard error; *n* = 5).

Factors	Dry-Cool Season			Wet-Warm Season			Stand Type		Season		Stand Type × Season	
	PCH	MIX	PPM	PCH	MIX	PPM	*F*	*p*	*F*	*p*	*F*	*p*
TPLFAs	37.67 ± 2.71 [a]	32.55 ± 1.24 [a]	37.96 ± 1.28 [a]	43.73 ± 2.08 [a]	40.01 ± 0.7 [a]	34.78 ± 1.11 [b]	4.64	0.020	6.46	0.018	6.07	0.007
B	15.79 ± 0.81 [a]	13.06 ± 0.43 [b]	14.90 ± 0.51 [ab]	17.64 ± 0.72 [a]	17.23 ± 0.42 [a]	12.76 ± 0.41 [b]	12.74	<0.001	7.68	0.011	15.56	<0.001
GP	9.45 ± 0.43 [a]	7.94 ± 0.29 [b]	9.18 ± 0.31 [a]	10.66 ± 0.51 [a]	11.55 ± 0.31 [a]	8.10 ± 0.31 [b]	8.17	0.002	17.09	<0.001	20.30	<0.001
GN	5.91 ± 0.76 [a]	5.90 ± 0.16 [a]	5.53 ± 0.22 [a]	6.19 ± 0.25 [a]	4.55 ± 0.37 [b]	4.11 ± 0.20 [b]	5.26	0.013	7.02	0.014	3.13	0.062
F	4.98 ± 0.34 [a]	3.75 ± 0.22 [b]	4.24 ± 0.28 [ab]	4.92 ± 0.35 [a]	2.96 ± 0.43 [b]	3.45 ± 0.21 [b]	13.56	<0.001	4.48	0.045	0.91	0.416
AMF	1.04 ± 0.03 [a]	0.75 ± 0.03 [b]	0.75 ± 0.04 [b]	0.85 ± 0.07 [a]	0.44 ± 0.08 [b]	0.48 ± 0.03 [b]	28.22	<0.001	34.83	<0.001	0.56	0.577
Acti	4.26 ± 0.37 [ab]	3.77 ± 0.21 [b]	4.86 ± 0.20 [a]	5.31 ± 0.19 [a]	4.87 ± 0.12 [b]	4.58 ± 0.10 [b]	2.73	0.085	12.36	0.002	6.50	0.006
B/F	3.19 ± 0.11 [a]	3.51 ± 0.13 [a]	3.56 ± 0.17 [a]	3.62 ± 0.14 [b]	6.35 ± 0.94 [a]	3.77 ± 0.33 [b]	7.50	0.003	11.31	0.003	5.98	0.008
GP/GN	1.74 ± 0.29 [b]	1.35 ± 0.03 [b]	1.66 ± 0.04 [a]	1.73 ± 0.08 [b]	2.63 ± 0.28 [a]	1.98 ± 0.11 [b]	1.07	0.359	13.76	0.001	7.33	0.003

Different superscripts indicate significant differences among stand types in each season ($p < 0.05$). TPLFAs (nmol g^{-1} soil), total PLFAs; B (nmol g^{-1} soil), bacteria PLFAs; GP (nmol g^{-1} soil), gram-positive bacteria PLFAs; GN (nmol g^{-1} soil), gram-negative bacteria PLFAs; F (nmol g^{-1} soil), fungi PLFAs; Acti (nmol g^{-1} soil), actinobacteria PLFAs; AMF (nmol g^{-1} soil), arbuscular mycorrhizal fungi PLFAs; B/F, bacteria-to-fungi PLFA ratio; GP/GN, GP-to-GN ratio.

3.5. Soil Microbial Functions

Soil enzyme activity was lower in the pure coniferous plantation (PPM) than in the pure broadleaf plantation in both seasons (Figure 5 and Table S4). The mixture of tree species significantly increased APH and NAG activity in the dry-cool season. Furthermore, mixed tree species significantly increased urease and PO activity but significantly decreased APH activity in the wet-warm season (Figure 5d,f,g). Carbon source utilization ability of the soil microbial community, indicated by average well color development (AWCD), was significantly higher in MIX than in PCH and PPM after 144 h of incubation (Figure 6).
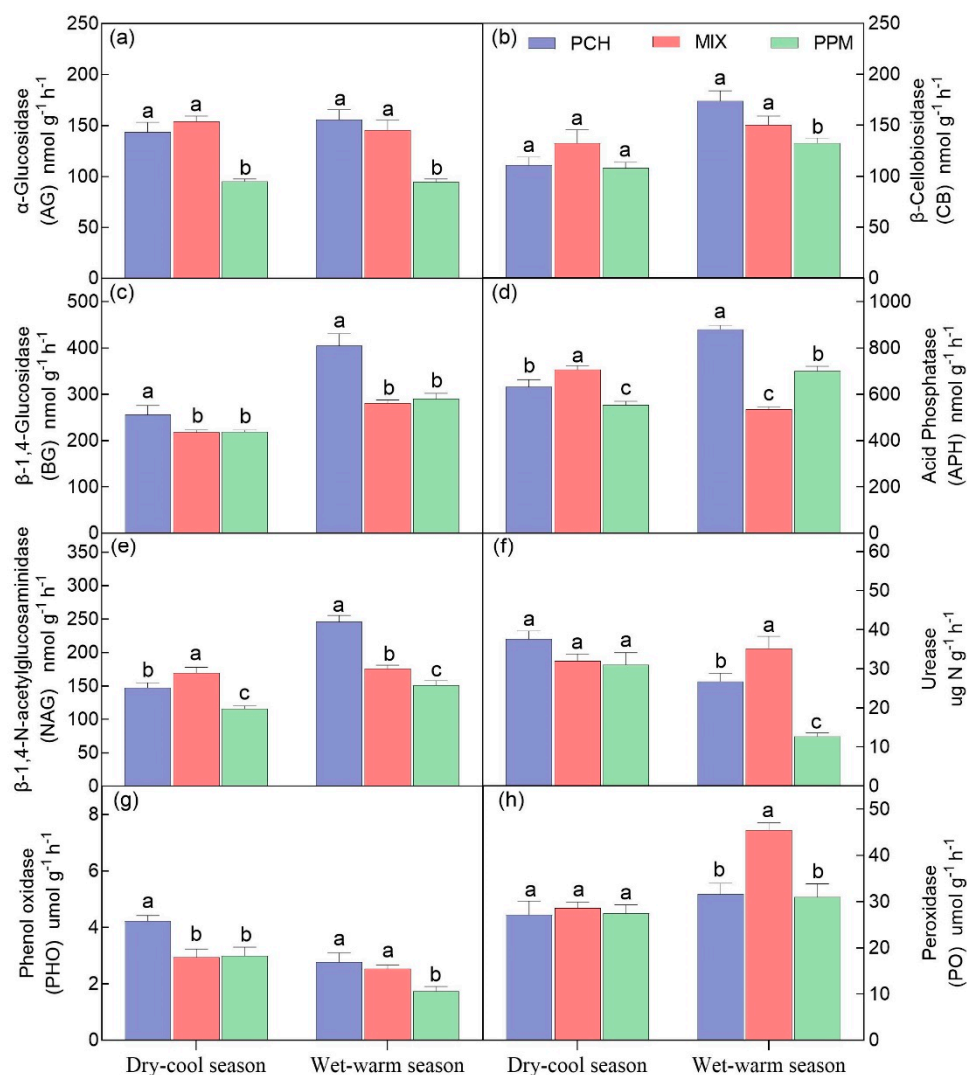
Figure 5. Soil extracellular enzyme activities ((**a**–**f**) refer to hydrolases, (**g**,**h**) refer to oxidases) in three plantations in different seasons. Error bars are standard error. Different letters above the bars denote significant differences at $p < 0.05$ level by LSD multiple comparisons among stand types ($n = 5$).
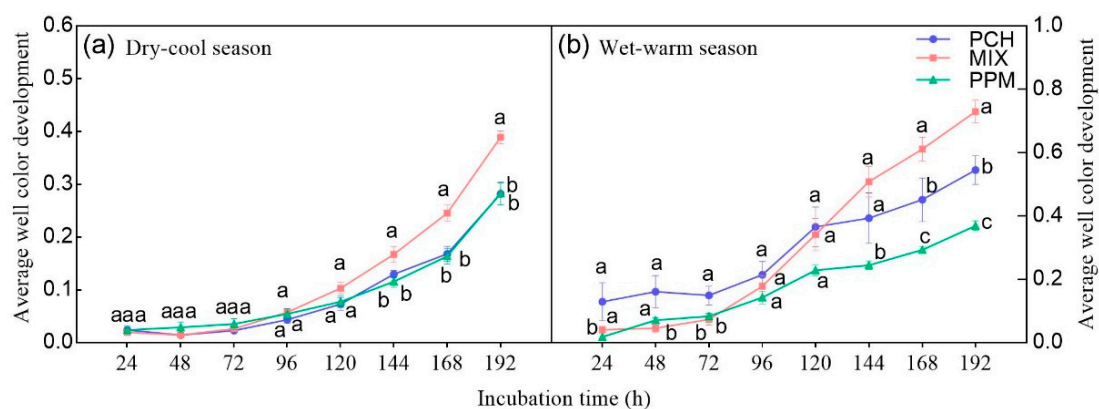
Figure 6. Soil microbial activity represented by average well color development (AWCD) in three plantations in the dry-cool season (**a**) and wet-warm season (**b**). Different letters above the bars denote significant differences at $p < 0.05$ level by LSD multiple comparisons among stand types ($n = 5$).

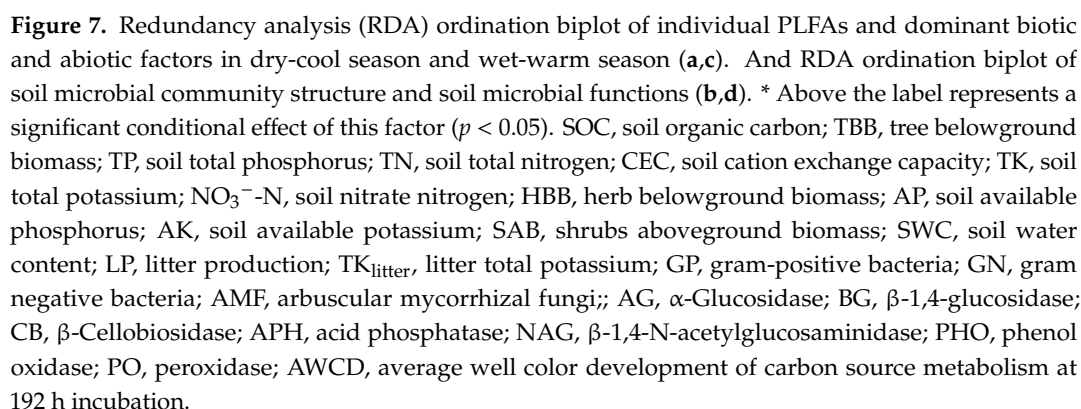### 3.6. Relationship between Biotic and Abiotic Factors and Soil Microbial Composition

The first two RDA axes explained 64.60% of the variation in soil microbial composition in the dry-cool season ($F = 2.0$, $p = 0.044$, Figure 7a) and 65.87% of the variation in the wet-warm seasons ($F = 2.3$, $p = 0.036$, Figure 7c). Nine biotic and abiotic factors, including SOC, TBB, TN, CEC, pH, TK, LP, TP, and TK_{litter}, were significantly related to the soil microbial composition in the dry-cool season, in which SOC and TBB were the major correlates of changes in soil microbial composition (Table S5). A total of 11 biotic and abiotic factors, including NO_3^--N, TBB, pH, TK_{litter}, HBB, AP, AK, TP, SAB, LP, and SWC were significantly related to the soil microbial composition in the wet-warm season, in which NO_3^--N, TBB, pH and TK_{litter} were the major correlates of changes in soil microbial composition (Table S5). Specifically, the Gram-positive bacteria, a15:0, a17:0, i17:0, i14:0, i15:0 and i16:0, were positively related to TBB, pH, and TK_{litter} in MIX during the wet-warm season (Figure 7c).

3.7. Relationship between Soil Microbial Structure and Functions

Soil enzyme activity and soil microbial carbon source utilization ability are both important soil microbial functions. The first two RDA axes explained 49.48% of the variation in soil enzyme activity and carbon utilization ability in the dry-cool season ($F = 2.4$, $p = 0.006$, Figure 7b) and 68.91% of the variation in the wet-warm season ($F = 6.4$, $p = 0.002$, Figure 7d). AMF and GP were significantly correlated with soil microbial functions in the dry-cool season (Table S6). Total bacteria, GP bacteria, GN bacteria, and actinobacteria were significantly correlated with soil microbial functions in the wet-warm season, in which total bacteria, GP bacteria, and GN bacteria were the major correlates of changes of soil enzyme activity (Table S6).

Figure 7. Redundancy analysis (RDA) ordination biplot of individual PLFAs and dominant biotic and abiotic factors in dry-cool season and wet-warm season (**a**,**c**). And RDA ordination biplot of soil microbial community structure and soil microbial functions (**b**,**d**). * Above the label represents a significant conditional effect of this factor ($p < 0.05$). SOC, soil organic carbon; TBB, tree belowground biomass; TP, soil total phosphorus; TN, soil total nitrogen; CEC, soil cation exchange capacity; TK, soil total potassium; NO_3^--N, soil nitrate nitrogen; HBB, herb belowground biomass; AP, soil available phosphorus; AK, soil available potassium; SAB, shrubs aboveground biomass; SWC, soil water content; LP, litter production; TK_{litter}, litter total potassium; GP, gram-positive bacteria; GN, gram negative bacteria; AMF, arbuscular mycorrhizal fungi;; AG, α-Glucosidase; BG, β-1,4-glucosidase; CB, β-Cellobiosidase; APH, acid phosphatase; NAG, β-1,4-N-acetylglucosaminidase; PHO, phenol oxidase; PO, peroxidase; AWCD, average well color development of carbon source metabolism at 192 h incubation.

4. Discussion

4.1. Coniferous-Broadleaf Mixture Significantly Increase Stand Biomass and Litter Production

The results of this study indicated that admixing of coniferous and broadleaf tree species significantly increased stand biomass and litter production after 34 years of afforestation in both dry-cool and wet-warm seasons (Figure 1, Table 2). This may be a consequence of a greater growth of

C. hystrix in the mixed stands. These results confirmed previous findings conducted in our experiment site measured when the plantations were 26-year-old and 28-year-old, respectively [7,16]. A bulk of studies revealed that mixed-species management could affect tree growth, stand structure and environment [14,52,53]. Mixed forests can develop stratified canopies due to niche differences among different species. In the present study, trees of the faster growing broad-leaf species (*C. hystrix*) were taller, had a larger DBH, and thus formed an upper canopy stratum in the mixed stands (Table 1). While trees of the shorter species (*P. massoniana*) formed a lower stratum with lower DBH and tree height, and the reduced amount of light transmitted through the upper canopy contributed to their slower growth. The dominant tree species within the stand can absorb more sunlight and use it more efficiently for growth [54], and thus contribute to higher tree biomass and litter production in mixed stands. In addition, stratified canopies in mixed plantations can help to maintain levels of understory species richness and biomass compared with pure plantations due to relatively high light availabilities [55,56]. This may be why the mixed stand had higher shrub and herb biomass in our study.

Specifically, in the present study, the broad-leaf species attained greater heights and diameters in mixed stands, while masson pine had reduced diameter and height growth in mixed-species compared to mono-species stands (Table 1). The severe suppression of *P. massoniana* by the faster growing *C. hystrix* may be attributed to differences in the light requirements of the two species [7]. The growth pattern of *C. hystrix* and *P. massoniana* in mixed stands in our study (34-year-old) were differed from when they were 26-years-old, in which the growth of *C. hystrix* and *P. massoniana* in mixed stands were both greater than in the corresponding mono-species stands [7]. This suggests that effects of the tree species mixture could change over time, and long-term investigation of mixed forests is of great significance to reveal mixed effects.

4.2. Coniferous-Broadleaf Mixture Significantly Increase Soil Microbial Biomass

The changes of tree species and composition may significantly affect the amount of organic matter input in soil [57]. As the most active part in soil organic matter (SOM), soil microbial biomass is important in nutrient cycling and release, participating in the circulation of critical elements such as carbon and nitrogen [23]. Because of the quantity and quality of the bulk of SOM changes slowly and the changes of SOM are difficult to measure due to the spatial heterogeneity of soils, soil microbial biomass and, in particular, MBC-to-SOC and MBN-to-TN ratio have been suggested as indicators of the state and modification of SOM [58–60] and as an indicator for carbon (nitrogen) availability in soil [61,62]. The concentrations of SOC, TN and MBC and MBN measured in our study were affected by stand type and seasons (Table 3 and Table S2, Figure 2). In the dry cool season, although the SOC content in mixed stands did not significantly differ with that in PCH, but significantly lower than that in PPM, the MBC concentration in mixed stands was markedly higher than that in the corresponding mono-species stands. Likewise, although the TN content in mixed stands was smaller than that in PCH and PPM stands, the MBN content in mixed stands was markedly higher than that in the corresponding mono-species stands in the wet-warn season. Previous studies found that higher soil microbial biomass correlated with higher SOC and TN content that sustain microbial metabolism [21,63]. However, in our study, the lower SOC and TN content in MIX was able to maintain a higher microbial biomass than sites where SOC was more abundant. Presumably, the quality of SOC could explain such a trend [64] and merit further investigation.

In this study, MBC-to-SOC and MBN-to-TN ratio were significantly higher in mixed stands (Figure 2), suggesting higher carbon and nitrogen availability in soil and higher soil quality for the growth of soil microorganisms by coniferous-broadleaf mixture [21]. In addition, the effects of admixing on soil microbial biomass, MBC-to-SOC, and MBN-to-TN ratio were non-additive, suggesting the interaction of *P. massoniana* and *C. hystrix* can have a synergistic effect on improving soil carbon and nitrogen availability (Figure 3). These findings are consistent with many previous studies that have determined the effects of tree species mixture on soil microbial biomass [21,65]. These results may be

contributed to (1) greater amount and more diverse organic matter input via litter and rhizodeposition products in the mixture of two contrasting tree species [21]; (2) the higher use efficiency of soil organic carbon and nitrogen by microorganisms in mixed stands [66].

We also sought to determine the admixing effects on composition and abundance of soil microbial community with community-level PLFA profiles. Surprisingly, there were not significant admixing effects on PLFA content of the major microbial functional groups, such as bacteria, fungi, AMF, and actinobacteria (Table 4). However, tree species mixture significantly increased the abundance of GN bacteria in the dry-cool season, while markedly increased the abundance of GP bacteria but decreased the abundance of fungi in the wet-warn season (Figure 4). It seemed that the dominated GN bacteria in the dry-cool season and GP bacteria in the wet-warn season would correlate with the higher availability of soil organic carbon and nitrogen in mixed stands [67,68]. These correlations are complicated and further investigation is required to reveal the underlying mechanisms.

4.3. Effects of Coniferous-Broadleaf Mixture on Soil Microbial Functions

Soil microorganisms excrete soil enzymes to drive nutrient mineralization and litter decomposition [64]. Therefore, soil extracellular enzyme activities may be related to the carbon, nitrogen, and phosphorus cycling in soil [69,70]. In the present study, we found positive admixing effects on the activities of APH and NAG in the dry-cool season (Figure 5d,e). On the other hand, positive admixing effects on the activities of urease and peroxidase while negative admixing effects on APH was detected in the wet-warn season (Figure 5d,f,g). This result suggests that the mixture of *C. hystrix* and *P. massoniana* can accelerate the mineralization rates of soil organic phosphorus and nitrogen. These findings were consistent with previous study [67].

The community-level physiological profiling (CLPP) is widely used to characterize microbial communities [71–73]. The average well color development (AWCD) in the CLPP approach reflects the ability of soil microorganisms to utilize carbon sources and their metabolic activity. The higher AWCD represents the stronger metabolic activity of soil microorganisms [71]. In this present study, we detected a significantly positive admixing effect on carbon source utilization ability of the soil microbial communities in both dry-cool and wet-warn seasons after 144 h of incubation (Figure 6). Moreover, the RDA analysis revealed that carbon source utilization ability of the soil microbial communities were positive related with APH in the dry-cool season, while related with urease and peroxidase activities in the wet-warn season (Figure 7). As a whole, the higher soil microbial functions in mixed stands are likely the results of the effects of AMF and GP bacteria in the dry-cool season and the effects of bacteria, including GP and GN bacteria, the wet-warn season (Table S6).

5. Conclusions

In our study, we measured significantly higher tree, shrub, and herb biomass and litter production in the coniferous-broadleaf mixed plantation after 34 years of afforestation. As expected, MBC, MBN, MBC-to-SOC, and MBN-to-TN ratio were significantly higher in mixed stands, suggesting higher carbon and nitrogen availability in soil and higher soil quality for the growth of soil microorganisms in our coniferous-broadleaf mixed stand. In addition, coniferous-broadleaf mixture can increase carbon source utilization ability of soil microbial community and accelerate the mineralization rates of soil organic phosphorus and nitrogen by increasing acid phosphatase, β-1,4-N-acetylglucosaminidase, and urease activities. The higher soil microbial biomass and activities are likely the result of greater amount and more diverse organic matter input via litter and rhizodeposition products, and the higher use efficiency of soil organic carbon and nitrogen by microorganisms in mixed stands. However, the correlations between soil dominated microbial functional groups and the higher availability of soil organic carbon and nitrogen, along with the quality of SOC, in mixed stands merit further investigation. Our results highlight that establishment of coniferous-broadleaf mixed forests may be a good management practice as coniferous-broadleaf mixture could accumulate higher stand

biomass, return more litter, and increase soil microbial biomass and functions over the long term in subtropical China.

Conflicts of Interest: The authors declare no conflicts of interest.

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