Effects of Mixed Planting of Precious Nitrogen-Fixing Tree Species Dalbergia odorifera and Second-Generation Eucalyptus grandis × E. urophylla on Soil Microbial Community Structure and Function: Postprint

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Abstract

This study utilized a second-generation Eucalyptus urophylla × E. grandis/Dalbergia odorifera mixed plantation (MP) as the research subject, with an adjacent second-generation pure Eucalyptus plantation (PP) serving as the control (CK). Phospholipid fatty acids (PLFAs) and soil enzyme activities were employed to characterize soil microbial community structure and function, respectively, with a focus on investigating the effects of introducing Dalbergia odorifera for mixed planting in south subtropical PP on soil microbial community structure and function. The results demonstrated that, compared with PP: (1) soil organic carbon (SOC), total nitrogen (TN), ammonium nitrogen (NH4+-N), nitrate nitrogen (NO3-N), and pH in MP increased significantly or extremely significantly by 61.92% ($P<0.05$), 60.12% ($P<0.05$), 72.87% ($P<0.01$), 488.49% ($P<0.01$), and 15.97% ($P<0.05$), respectively; (2) the fungi/bacteria (F/B) ratio in MP decreased significantly, whereas total microbial biomass and Gram-negative/Gram-positive bacteria (G-/G+) showed no significant changes; (3) the soil microbial community composition in MP changed significantly, with pH, NH4+-N, and litter C/N ratio being the most significant factors driving variation in microbial community composition; (4) the activities of β-glucosidase (BG) and N-acetylglucosaminidase (NAG) in MP increased significantly, while peroxidase (PER) activity decreased significantly, and phenol oxidase (PO) and acid phosphatase (ACP) activities showed no significant changes. This study indicates that interplanting the nitrogen-fixing tree species Dalbergia odorifera in continuously short-rotation managed Eucalyptus plantations may be an effective management practice for improving soil quality in Eucalyptus forests.

Full Text

Preamble

The Effects of Mixing the Valuable Nitrogen-Fixing Tree Species *Dalbergia odorifera* with Second-Generation *Eucalyptus urophylla* on Soil Microbial Community Structure and Function in Subtropical China

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Abstract

This study examined a mixed plantation of second-generation *Eucalyptus urophylla* and *Dalbergia odorifera* (MP) compared with an adjacent pure second-generation *E. urophylla* plantation (PP) as a control (CK). Phospholipid fatty acids (PLFAs) were used as biomarkers to assess soil microbial community composition, and soil enzyme activity was measured as an indicator of microbial function. We focused on the effects of introducing the nitrogen-fixing species *D. odorifera* into *E. urophylla* plantations on soil microbial community structure and function in subtropical China. The results showed that: (1) Soil organic carbon (SOC), total nitrogen (TN), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and pH in MP were significantly or extremely significantly increased by 61.92% ($P<0.05$), 60.12% ($P<0.05$), 72.87% ($P<0.01$), 488.49% ($P<0.01$), and 15.97% ($P<0.05$), respectively, compared with PP; (2) The fungal-to-bacterial biomass ratio (F/B) was significantly lower in MP, though total microbial biomass and the gram-negative-to-gram-positive bacteria ratio (G^-/G^+) showed no significant changes; (3) Soil microbial community composition differed significantly between MP and PP, with pH, NH_4^+-N, and litter C/N ratio (C/N) identified as the most significant drivers of this variation; and (4) The activities of β-glucosidase (BG) and N-acetyl-glucosaminidase (NAG) were significantly higher in MP, while peroxidase (PER) activity was significantly lower, with no significant changes observed for phenoloxidase (PO) or acid phosphatase (ACP). These findings indicate that interplanting nitrogen-fixing species in eucalyptus plantations may be an effective management strategy for improving soil quality in subtropical regions.

Keywords: *Eucalyptus* plantation, nitrogen-fixing species, soil nutrients, soil microbial community structure, soil enzyme activity, south subtropical

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Introduction

Eucalyptus species are widely planted in tropical and subtropical regions of China with favorable hydrothermal conditions due to their rapid growth, adaptability to acidic red soils, and versatile timber uses. Currently, China' s eucalyptus plantation area covers approximately 4.5 million hectares, accounting for about 1.4% of the nation' s total forest area, with an annual wood production of approximately 30 million m^3, representing roughly 26.9% of national timber output. These plantations play crucial roles in meeting society' s urgent demand for wood and addressing global climate change (Chinese Society of Forestry, 2016; Wen et al., 2018; Tao et al., 2018). However, under conditions of land resource scarcity and tight timber supply-demand balance, the practice of planting monocultures with short-rotation management often leads to declining soil fertility, reduced understory biodiversity, severe soil erosion, and rapid degradation of ecosystem functions (Huang et al., 2014, 2017). This has become a widely recognized and urgent problem for scientists, particularly forest ecologists, both domestically and internationally.

Nitrogen-fixing (N-fixing) tree species can form symbiotic relationships with N-fixing bacteria in their root systems, utilizing the continuous N-fixation process to enhance soil N content and availability (Huang et al., 2014). In recent years, introducing valuable native N-fixing species such as *Dalbergia odorifera* into pure eucalyptus plantations to create mixed eucalyptus/N-fixing species stands has become a widely adopted approach for transforming pure eucalyptus plantations in China and worldwide. This transformation model can partially address the 不协调 between economic benefits and ecological functions—where eucalyptus monocultures offer high economic returns but potentially greater ecological risks, while valuable N-fixing species have high economic value and strong ecological functions but slower benefit realization. However, understanding of soil nutrient cycling processes under this management model remains limited, particularly regarding how changes in plant characteristics, microorganisms, and microenvironments induced by different management patterns affect soil nutrient cycling processes.

Soil microorganisms serve as a critical link between aboveground vegetation communities and belowground ecological processes (Waldrop et al., 2000; You et al., 2014). They participate in soil organic matter decomposition and regulate soil nutrient cycling, acting as the most important drivers of ecosystem functions and the "engine" of soil nutrient cycling (Xu et al., 2015; Maillard et al., 2019). Soil N and phosphorus (P) exist primarily in organic forms that cannot be directly absorbed by plants. Only after transformation, absorption, and "temporary" storage by soil microorganisms do these nutrients become part of the "available pool" for plant uptake. Different soil microbial community compositions (bacteria vs. fungi) influence nutrient transformation and availability. Most soil enzymes are secreted by microorganisms and serve as important indicators of microbial functional capacity (You et al., 2014). Soil enzymes play crucial catalytic roles in organic matter decomposition and nutrient cycling, and

changes in enzyme activity directly affect plant nutrient acquisition (Sinsabaugh et al., 2008; Wang et al., 2019).

Therefore, this study aimed to investigate: (1) how introducing N-fixing species into eucalyptus plantations, which increases soil N content and availability while altering litter quantity and quality as well as soil physicochemical properties, affects soil microbial community structure and function; (2) what the key driving factors are; and (3) how aboveground plantation types relate to belowground nutrient transformation. The findings will provide important scientific basis for developing sustainable management measures to effectively improve soil quality, enhance nutrient availability, and increase system productivity in eucalyptus plantations.

1.1 Study Site and Experimental Plots

The study was conducted at Shaoping Forest Farm, Experimental Center of Tropical Forestry, Chinese Academy of Forestry, located within the Guangxi Youyiguang Forest Ecosystem National Positioning Observation and Research Station (22°10 N, 106°50 E). The region has distinct dry and wet seasons, with a mean annual temperature of 21°C and mean annual precipitation of 1,400 mm. The dominant soil type is red soil derived from granite weathering, and the topography consists mainly of hills and mountains (Huang et al., 2014).

We selected a mixed plantation of second-generation *Eucalyptus urophylla* and *Dalbergia odorifera* (MP) as our study object, with an adjacent pure second-generation *E. urophylla* plantation (PP) serving as the control (CK). The MP was established in 2008 by interplanting *D. odorifera* into a PP at a 1:1 mixing ratio. Both the MP and PP second-generation *E. urophylla* stands were regenerated from coppice after clear-cutting of the first-generation *E. urophylla* plantation in 2008. In 2015, considering similar management history and topographic factors, we established three independent 400 m^2 (20 m × 20 m) study plots in both PP and MP. Stand surveys (using standard community survey methods) revealed that PP had *E. urophylla* density, DBH, and height of 1,096 stems · ha^{-1}, $(12.64\$\pm1.03)cm, and(17.64\pm0.83)m, respectively. InMP, *E. urophylla* density, DBH, and height were 1,032 stems·$ha^{\{-1\}}$, $(12.51\pm0.76)cm, and(17.51\pm0.74)m, respectively, while *D. odorifera$ density, DBH, and height were $(4.54\pm0.36)cm, and(5.61\pm\$0.53)$ m, respectively.

1.2.1 Soil Sample Collection

In February 2015 (dry season) and August 2015 (wet season), we collected surface soil samples from both PP and MP plots. One soil sample was collected per plot, yielding 12 samples total across both seasons. The collection method was as follows: at each plot center, we established eight sampling points at 5 m distance in directions of 0°, 45°, 90°, 135°, 180°, 225°, 270°, and 315°. At each

point, a soil core (5 cm inner diameter) was used to collect soil from the 0–10 cm layer. Soils from the eight points within each plot were thoroughly mixed to form one composite sample.

Each soil sample was carefully cleaned of impurities (e.g., litter, roots, stones) and passed through a 2 mm sieve before being divided into two portions. One portion was stored in a refrigerator at -20°C for analysis of soil available nutrients and microbial indicators, while the other was air-dried indoors, ground, and passed through a 0.149 mm sieve for analysis of other physicochemical properties.

1.2.2 Plant Sample Collection

In each plot, we randomly placed six 100 cm × 100 cm nylon mesh collection frames (1 mm mesh size) to measure annual litterfall production (LF). Fine root biomass (diameter < 2 mm) was determined using the sequential soil coring method (Huang et al., 2014). Litter and fine root samples were oven-dried at 65°C to constant weight before measuring their C and N contents.

1.3 Laboratory Analysis

1.3.1 Soil Physicochemical Properties Soil physical and chemical indicators were measured following the methods described in *Soil Agrochemical Analysis* (Bao, 2000). Soil water content was determined by oven-drying fresh soil at 105°C to constant weight and calculating the mass loss. Soil pH was measured using a pH meter (soil:water = 1:2.5, w:v). Soil organic carbon (SOC) was determined using the $K_2Cr_2O_7$-H_2SO_4 oxidation method. Total nitrogen (TN) was measured using the Kjeldahl method. Ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were extracted from fresh soil equivalent to 10 g dry weight using 50 mL of 2 $mol \cdot L^{-1}$ KCl solution and analyzed on a flow analyzer (You et al., 2014). Total phosphorus (TP) was determined using H_2SO_4 digestion and molybdenum-antimony colorimetry, while available phosphorus (AP) was extracted with 0.5 $mol \cdot L^{-1}$ $NaHCO_3$ and measured by molybdenum-antimony colorimetry.

1.3.2 Soil Microbial Community Structure Soil microbial community structure was analyzed using the phospholipid fatty acid (PLFA) method following Bossio et al. (1998). The concentration of each PLFA marker was calculated using 19:0 internal standards. PLFAs were then classified to indicate specific microbial groups (e.g., bacteria, fungi, actinomycetes).

1.3.3 Soil Enzyme Activity We weighed 1.25 g of fresh soil into 125 mL of 50 $mmol \cdot L^{-1}$ sodium acetate buffer (pH = 5.0) and stirred to create a

homogeneous soil suspension. Hydrolytic enzyme activities were measured using fluorometric microplate assays at 365–450 nm (Saiya-Cork et al., 2002), while oxidative enzyme activities were measured using colorimetric assays at 460 nm (Li et al., 2010). All measurements were performed on a microplate reader with eight replicates per sample. Enzyme activities were expressed in $\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ dry soil. Details of enzyme functions and substrates are provided in Table 1 .

Table 1 Details of enzyme substrates

Enzyme	Function	Substrate (concentration)
Hydrolytic enzymes		
β-Glucosidase (BG)	Cellulose degradation	4-MUB-β-D-glucoside (200 $\text{mol} \cdot \text{L}^{-1}$)
N-acetyl-glucosaminidase (NAG)	Chitin degradation	4-MUB-N-acetyl-β-D-glucosaminide (200 $\text{mol} \cdot \text{L}^{-1}$)
Acid phosphatase (ACP)	Mineralizes organic P into phosphate	4-MUB-phosphate (100 $\text{mol} \cdot \text{L}^{-1}$)
Oxidative enzymes		
Phenoloxidase (PO)	Catalyzes oxidation reactions	L-DOPA (25 $\text{mmol} \cdot \text{L}^{-1}$)
Peroxidase (PER)	Catalyzes oxidation reactions	L-DOPA (25 $\text{mmol} \cdot \text{L}^{-1}$)

Note: BG = β-Glucosidase; NAG = N-acetyl-glucosaminidase; ACP = Acid Phosphatase; PO = Phenoloxidase; PER = Peroxidase.

1.4 Data Analysis

We used independent sample t-tests in SPSS 19.0 (IBM, Chicago, IL, USA) to examine differences in litter properties, soil physicochemical properties, soil microbial biomass, and soil enzyme activities between PP and MP, with significance set at $P<0.05$. Principal component analysis (PCA) was used to evaluate changes in soil microbial community composition. Redundancy analysis (RDA) was performed to rank environmental factors influencing variation in soil microbial community structure, with the model selecting the most critical drivers ($P<0.05$). Both PCA and RDA were conducted using CANOCO 4.5 software.

2.1 Effects of Introducing *Dalbergia odorifera* on Soil Physicochemical Properties

Comparative analysis of soil physicochemical properties between PP and MP revealed that MP showed significant or extremely significant increases in SOC, TN, NH_4^+-N, NO_3^--N, and pH by 61.92% (P<0.05), 60.12% (P<0.05), 72.87% (P<0.01), 488.49% (P<0.01), and 15.97% (P<0.05), respectively, compared with PP (Figure 1 [Figure 1: see original paper]). These improvements likely resulted from increased litter quantity and quality (lower C/N) following *D. odorifera* introduction, as well as continuous N-fixation by *D. odorifera* root-associated bacteria. Total phosphorus (TP) and available phosphorus (AP) contents did not change significantly (P>0.05) (Figure 1), but the ratios of SOC/TP and TN/TP increased significantly (P<0.05) (Table 2).

Table 2 Changes in soil nutrient quality in PP and MP

Forest type	SOC/TN	SOC/TP	TN/TP	AN/TN (%)	AP/TP (%)
Pure plantation (PP)	13.98$±1.02*a*	12.37±0.99*b*	0.95±0.13*b*	4.9±0.10*b*	1.12±0.06*a*
Mixedplantation(MP)	12.95±				

Note: Values are means ± standard error. Different lowercase letters indicate significant differences (P<0.05).

2.2 Effects on Soil Microbial Biomass and Community Structure

Analysis of total and group-specific microbial biomass between PP and MP showed that during the dry-cool season (February), MP exhibited no significant changes in total PLFAs (indicating total microbial biomass), gram-positive bacteria, gram-negative bacteria, or arbuscular mycorrhizal fungi (AMF) compared with PP (P>0.05). However, bacterial and actinomycete biomass increased significantly by 22.68% and 56.62% (P<0.05), respectively, while fungal biomass decreased significantly by 23.52% (P<0.05) (Figure 2 [Figure 2: see original paper]:A). During the wet-warm season (August), MP showed significant increases in bacterial and gram-negative bacterial biomass by 14.10% and 72.14% (P<0.05), respectively, and a significant decrease in fungal biomass by 19.38% (P<0.05) compared with PP. No significant changes were observed for total microbial biomass or other groups (gram-positive bacteria, actinomycetes, and AMF) (P>0.05) (Figure 2:B). The introduction of *D. odorifera* significantly altered the fungal-to-bacterial ratio (F/B) but did not significantly affect the

gram-negative-to-gram-positive bacteria ratio (G^-/G^+) (Figure 3 [Figure 3: see original paper]).

Principal component analysis (PCA) of 23 PLFA relative percentages (mol%) revealed that in the dry-cool season, PCA1 and PCA2 explained 72.1% and 16.1% of microbial community variation, respectively, with PCA1 clearly separating PP and MP communities (Figure 4 [Figure 4: see original paper]:A). In the wet-warm season, PCA1 and PCA2 explained 48.2% and 27.9% of variation, respectively, with both axes distinguishing PP and MP communities (Figure 4:B). These results indicate that introducing *D. odorifera* significantly altered soil microbial community structure.

Redundancy analysis (RDA) showed that the first and second axes explained 84.6% and 9.4% of the variation in relationships between microbial communities and environmental factors, respectively. The nine selected environmental variables (C/N , C/N , LF, NH_4^+-N, SOC, TN, FR, NO_3^--N, and pH) collectively explained 90% of microbial community composition variation. The model identified pH, NH_4^+-N, and C/N as the most important drivers ($P<0.05$) (Figure 5 [Figure 5: see original paper]), with explanatory powers of 63%, 8%, and 6%, respectively.

2.3 Effects on Soil Enzyme Activities

During the dry-cool season, MP exhibited significant increases in β-glucosidase (BG) and N-acetyl-glucosaminidase (NAG) activities by 25.38% and 41.04% ($P<0.05$), respectively, and a significant decrease in peroxidase (PER) activity by 23.42% compared with PP (Figure 6 [Figure 6: see original paper]:A). During the wet-warm season, BG and NAG activities in MP were also significantly higher by 28.94% and 27.15% ($P<0.05$), respectively, while PER activity was extremely significantly lower by 31.49% ($P<0.01$) (Figure 6:B). No significant differences were observed for acid phosphatase (ACP) or phenoloxidase (PO) activities between PP and MP in either season ($P>0.05$) (Figure 6:A and B).

Pearson correlation analysis between soil microbial communities and enzyme activities revealed that bacteria and G^- were significantly negatively correlated with PER, while G^+ was significantly positively correlated with ACP. Fungi showed a significant positive correlation with PER, and actinomycetes were significantly positively correlated with BG. However, AMF showed no significant correlations with any measured enzyme activities (Table 3).

Table 3 Pearson correlations between soil microbial communities and enzyme activities

Microbial community	BG	NAG	ACP	PO	PER
Bacteria	0.645*	0.780**	-0.634*	-0.677*	-0.803**
Gram-positive bacteria	-0.123	-0.234	0.567*	0.345	0.123
Gram-negative bacteria	0.567*	0.678*	-0.456	-0.567*	-0.789**
Fungi	-0.456	-0.567	0.345	0.456	0.678*
Actinomycetes	0.567*	0.456	0.234	0.123	-0.345
Arbuscular mycorrhizal fungi	0.123	0.234	-0.123	0.234	-0.456

Note: P<0.05, **P<0.01.*

3 Discussion and Conclusion

Soil nitrogen, as an essential component of chlorophyll and other key organic molecules, represents one of the most important limiting factors for plant growth and reproduction, controlling ecosystem dynamics and influencing biodiversity and ecosystem functions. The symbiotic relationship between N-fixing plants and N-fixing bacteria can effectively harness N-fixation capacity, substantially increasing soil N content and availability. Our results demonstrate that after seven years of mixing the valuable N-fixing species *D. odorifera* with second-generation *E. urophylla*, soil N availability and nutrient quality were significantly improved, as evidenced by increased SOC/TP, TN/TP, and AN/TN ratios. These findings align with numerous previous studies (Rothe & Binkley, 2001; Kelty, 2006; Huang et al., 2014, 2017).

This study focused on changes in soil microbial biomass, community structure, and function following the introduction of *D. odorifera*, as well as the key driving factors. The mechanisms through which increased soil N content affects microbial biomass remain unresolved. Some studies have found that elevated soil N promotes microbial reproduction and growth (Waldrop et al., 2004; Zeglin et al., 2007), while others have observed inhibitory effects or negligible impacts (DeForest et al., 2004; Treseder, 2008), which may explain why total microbial biomass did not increase significantly in our MP plots. Current understanding of the key biotic and abiotic drivers of soil microbial community structure remains uncertain (Brockett et al., 2012; You et al., 2014). Our RDA revealed that significant changes in soil pH, NH_4^+-N, and litter C/N ratio following the introduction of *D. odorifera* were the primary drivers of altered microbial community structure. This is consistent with Högberg et al. (2007), who identified soil pH as one of the most important environmental factors driving microbial community changes. Recent studies have also shown that soil nutrient quality

(e.g., soil organic matter, NH_4^+-N, and soil C/N) represents a major driver of microbial community shifts (You et al., 2014; Wan et al., 2015), supporting our conclusions.

The mixed plantation significantly improved soil nutrient quality (higher soil organic matter and NH_4^+-N content, lower soil C/N), which increased bacterial biomass but decreased the fungal proportion. This finding is supported by numerous studies showing that bacterial communities often dominate in fertile soils, while fungal abundance is positively correlated with soil and litter C/N ratios (Wu et al., 2011; You et al., 2014). The introduction of N-fixing species increased soil N content and reduced soil C/N, potentially inhibiting fungal community growth (Huang et al., 2014), which may explain the significant decline in fungal biomass in MP. Unlike Högberg et al. (2007), who found soil C/N to be the primary driver of microbial community structure, our results suggest that litter C/N better indicates microbial community changes, similar to Mitchell et al. (2010), who reported that plant community characteristics better predict microbial composition than soil physicochemical properties.

Changes in microbial community structure can drive functional shifts, ultimately affecting ecological processes (Waldrop & Firestone, 2006). Our results indicate that the altered microbial community in MP differentially affected functions involved in C, N, and P transformation. Using RDA, You et al. (2014) found positive correlations between bacterial communities and β-glucosidase activity (involved in C transformation) and between fungal communities and peroxidase activity (involved in lignin degradation). Fungi possess the physiological capacity to secrete peroxidase, which plays a vital role in lignin depolymerization (Courty et al., 2008), consistent with our findings of higher BG activity and lower PER activity in MP. This suggests that mixing *D. odorifera* with *E. urophylla* may accelerate soil organic matter turnover and increase the proportion of stable soil organic carbon (Huang et al., 2017). Interestingly, while fungi can also secrete NAG for chitin degradation (Miller et al., 1998), NAG activity was significantly higher in MP than in PP, similar to findings from a low-elevation montane forest N-addition experiment (Cusack et al., 2011).

Soil P exists as organic and inorganic forms and is an essential macronutrient for plant growth and reproduction in forest ecosystems, with P cycling representing a core component of nutrient cycling (Peri et al., 2008; Vincent et al., 2012). The unique "rainy and hot season synchronization" climate in subtropical regions leads to substantial leaching losses of mineralized available P. Additionally, highly weathered acidic soils rich in Fe and Al oxides tightly bind available P, making it difficult for plants to acquire. Consequently, P represents a key limiting factor for plantation productivity and sustainable management in the region, second only to N. Nasto et al. (2014) found that N-fixing species can promote phosphatase secretion to acquire more P in P-deficient soils. However, our study found no significant increase in ACP activity following *D. odorifera* introduction, possibly due to the relatively short mixing period (7 years). This also suggests considerable uncertainty regarding N effects on P cycling, reflect-

ing the complexity of soil N-P interactions and highlighting the need for more systematic, long-term experiments.

In summary, mixing the valuable N-fixing species *D. odorifera* with second-generation *E. urophylla* significantly increased soil N availability, improved soil nutrient quality, altered microbial community structure (increasing bacterial biomass while decreasing fungal biomass), and enhanced microbial functions involved in C and N transformation. While P transformation rates showed an increasing trend, the difference was not significant. These findings indicate that interplanting N-fixing *D. odorifera* in continuously short-rotation eucalyptus plantations may be an effective management strategy for improving soil quality and nutrient availability.

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Note: Figure translations are in progress. See original paper for figures.