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ARTICLE

Effects of Nitrogen Reduction Combined Microbial Fertilizers on Foliar Yield and Quality of *Ginkgo biloba*, Soil Properties and Microbial Community

Yichen Lu, Dong Cao, Ying Chen*, Yifan Wang, Jingjing Di and Na An

Co-Innovation Center for Sustainable Forestry in Southern China, College of Life Sciences, Nanjing Forestry University, Nanjing, China
*Corresponding Author: Ying Chen. Email: chynjfu@163.com

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ABSTRACT: The proportion of nitrogen (N), phosphorus (P), and potassium (K) in the soil could regulate plant yield and secondary metabolism. However, extensive chemical fertilizer utilization has reduced soil fertility and increased soil compaction, and even caused environmental pollution. Applying microbial fertilizers (MF) could improve soil structure and the environment. This work aimed to reveal the effects of N reduction and combined MF on *Ginkgo biloba* growth, soil properties, and microbial communities, and illustrate their interaction roles in improving Ginkgo leaf yield and flavonoid content. A field experiment with different fertilizer treatments, including low N, normal P-K (T1), low N, high P-K (T2), T1 + MF (T3), T2 + MF (T4), and MF (T5), was applied for Ginkgo trunk-cutting seedlings. Our data showed that the treatments of T2 and T4 both significantly increased the levels of net photosynthetic rate, flavonoid content, and yield in the leaves, and elevated the levels of nitrate-N, availability of P and K, and the activities of urease and acid phosphatase in the rhizosphere soil (RS). T2 treatment decreased bacterial diversity, increased the relative abundance (RB) of Nitrospirota and Gemmatimonadota in RS, and enhanced nitrate denitrification and nitrite respiration. However, T4 treatment significantly increased the RB of *Acidobacteria bacterium WWH8* and *delta-proteobacterium*, and improved microbial bacterial metabolism of nitrogen, thiosulfate, photosynthesis, etc. Therefore, the reasonable proportion of NPK fertilizer combined with MF could regulate the nutrient use efficiency, enhance flavonoid synthesis, and raise leaf yield in Ginkgo forests by improving the bacterial community composition. Our work provided evidence for the benefits of fine-regulating NPK proportion and integrating MF measures to promote plant growth and secondary metabolism. Meanwhile, this also provided references for the in-depth exploration of the mechanistic interactions between rhizosphere organisms and plants.

KEYWORDS: Nitrogen reduction; microbial fertilizers; flavonoids; soil properties; microbial community; *Ginkgo biloba*; trunk-cutting seedlings

1 Introduction

As an effective plant management practice, fertilizer application could improve soil fertility, microbial composition, and forest productivity [1,2]. Nitrogen (N), phosphorus (P), and potassium (K) are three essential nutrients in the process of plant growth [3–5]. Different proportions of NPK exert different influences on plant growth. Previous studies have demonstrated that N fertilizer has a greater impact on rice quality than P fertilizer [6]. Low soil P availability would limit plant growth [7,8]. An elevated K level might improve sweet potato production and quality [9]. Nevertheless, the extensive utilization of chemical fertilizers has depleted organic matter, reduced soil fertility, compacted soil particles, and even led to the soil being acidified and nitrous oxide emissions increasing [10–12]. Utilizing inorganic fertilizers over an

extended period has also inhibited the absorption of N and P elements, ultimately discharged into rivers and lakes or penetrating into underground water [10,13]. Therefore, the rational proportion of NPK fertilization could compensate for nutrient deficiencies and increase crop yield, as well as improve the soil environment.

Microbial fertilizers (MF) have received increasing attention in recent decades due to containing nitrogen fixers, phosphate solubiliers, sulfur oxidizers, or organic matter decomposers [14–16]. The application of MF improved the soil structure and changed soil aggregation and fertility [17,18]. For instance, adding exogenous biochar enhanced the retention of soil metal ions and modified the soil's humus structure [19,20]. MF also increased the dissolved organic matter, further enhanced the levels of available soil P or N, and enhanced microbial diversity, community composition, and function [21–23]. In addition, MF could raise the amount of carbon in the soil organic carbon (SOC) pool, reduce greenhouse gas emissions, and reduce environmental pollution and costs [10,24].

As a strategy to alleviate the agricultural problems caused by excessive chemical fertilization, applying MFs could also effectively increase both crop yield and quality. This improvement has been attributed to the beneficial microorganisms present in the MFs, which enhance soil fertility [11]. The utilization of microbial organic fertilizers improved grain yield and nutrient accumulation in winter wheat, as well as biomass accumulation in pakchoi [10,25,26]. Therefore, MFs hold considerable market potential and are highly economically efficient.

Ginkgo biloba L. is an ancient species of tree with significant medicinal and ecological value due to its high flavonoid content in leaves and with an elegant tree shape and strong stress resistance ability [27]. For example, *G. biloba* extract (GBE) has anti-asthmatic, cardiovascular, and neuroprotective effects, and could treat Parkinson's disease and Alzheimer's disease, etc., [28]. Traditional planting experience showed young *G. biloba* seedlings (aged 1–7 years) with high levels of flavonoids and lactones. Establishing a Ginkgo leaf-harvest forest has become the primary strategy to achieve high-efficiency production of Ginkgo leaves in China [29]. To date, fertilization remains an essential tool for increasing the yield and quality of the Ginkgo leaf-harvest forest. Applying fertilizers to promote bud and leaf growth has dramatically increased leaf dry weight and leaf flavonoid content [30]. NPK fertilizer improved the Ginkgo foliar nutritional and photosynthetic status, while excess N fertilization had adverse effects on tree growth [31]. N fertilizer increased foliar N and K concentrations in Ginkgo seedlings. However, the application of N fertilizer decreased the N and K retranslocation and increased P retranslocation in autumn [32]. The application of biochar and organic fertilizer could improve the growth of Ginkgo seedlings, such as by increasing total root length and shoot biomass in compacted soil [33]. Optimizing the rate and timing of N, P, and K fertilization increased the net income of Ginkgo and elevated the industrial benefits of leaf-harvest GInkgo plantations. Meanwhile, higher fertilizer rates increased nitrogen losses and greenhouse gas emissions [34].

Current research into the application of fertilizers to Ginkgo leaf-harvest forests mostly focuses on the use of inorganic fertilizers. Few studies have been conducted on the balance of N, P, and K, or the effects of biological combined fertilizer on the yield and quality. The interaction mechanism between plant growth and nutrient element absorption, and the rhizosphere microbial community, is still unclear. Soil metagenomics (soil microbial genome) analysis allows one to study the functional characteristics of soil microorganisms and assemble genomes [11,35].

In the present study, we utilized physiological, pedological, and microbiome approaches to focus on the effects of different fertilizer treatments on (i) growth, leaf yield, and flavonoid levels; (ii) soil physicochemical properties; and (iii) the soil microbial community in a Ginkgo forest field. These treatments included reducing N and increasing P-K fertilizer, applying microbial fertilizer plus chemical fertilizer, and applying a single microbial fertilizer. These effective measures will benefit the exploration of potential

relationships between soil microbial communities and Ginkgo growth. They will also provide novel insights into precision fertilization patterns for Ginkgo leaf harvesting plantations and bridge agronomic efficiency with environmental sustainability.

2 Materials and Methods

2.1 Experimental Sites and Seedling Management

The present work was conducted as a field experiment using trunk-cutting seedlings of *G. biloba* at Sihu Town, Pizhou City, Jiangsu Province, China. In November of the previous year, the four-year-old Ginkgo seedlings (from the same variety) were cut at the trunk, 40 cm from the ground. These trunk-cutting seedlings (subsequently, 'seedlings' will be used uniformly) were then transplanted to a Ginkgo field in Sihu Town (Fig. 1A). The following year, the truncated seedlings with consistent growth and height were routinely managed in the field, including watering and weeding.

2.2 Fertilizer Treatments

The experiment consisted of 21 plots, each with an area of approximately 40 m^2 (20 m long and 2 m wide, and there were about five rows of seedlings per plot. Three rows of unfertilized seedlings were used as boundary rows between plots. There were about 300 seedlings in each plot, with a spacing of 40 cm × 30 cm between seedlings and rows (about 5000 seedlings/667 m^2). Three types of fertilizers were used in the present work, containing urea (containing N ≥ 46%), superphosphate (containing P_2O_5 ≥ 12%), and potassium sulfate (containing K_2O ≥ 51%). Using the three chemical fertilizers in a particular proportion, three formula fertilizers were prepared, including N:P:K (18:18:18), N:P:K (9:18:18), and N:P:K (9:27:27). Microbial fertilizer (MF) used in this work was a complex microbial bacterium fertilizer (Shandong Junde Biotechnology Co., Ltd., China). The MF was a composite of various bacteria, including Bacillus, Lactobacillus, nitrogen-fixing bacteria, etc. It contained ≥20 billion effective active bacteria/g and ≥60% organic matter, and was free of N, P, and K. The microbial carrier was composed of materials derived from animals, aquatic and food sources, as well as decomposed organic matter.

The experiment involved seven treatments, each with three replicates (plots) for each: (1) Control 1 (CK1: no fertilizer); (2) Control 2 (CK2: NPK (18:18:18), the local traditional fertilizer ratio); (3) T1 (NPK (9:18:18): low level of N, normal levels of P-K). (4) T2 (NPK (9:27:27): low level of N, high levels of P-K). (5) T3 (T1 + microbial fertilizer (MF)). (6) T4 (T2 + MF). (7) T5 (MF): microbial fertilizer. Compound NPK fertilizers were prepared according to the NPK proportion used for each treatment. The N, P, and K proportions refer to the conversion rates of urea, superphosphate, and potassium sulfate (1.0 g N corresponds to 1/0.46 = 2.17 g urea, 1.0 g P_2O_5 represents 1/0.12 = 8.33 g superphosphate, and 1.0 g K_2O represents 1/0.51 = 1.96 g potassium sulfate). According to local fertilization experience, the amount of fertilizer applied was 30 g per seedling. Therefore, 30 g of compound NPK fertilizer was applied to the CK2, T1, and T2 treatments, and 15 g of compound NPK fertilizer plus 15 g of microbial fertilizer was applied to the T3 and T4 treatments. The microbial fertilizer in T5 was 30 g per seedling. The experiment was carried out from November of the previous year to November of the following year, with fertilization taking place on 14–15 April of the following year.

2.3 Sample Collection and Determination

Prior to the fertilizer application, 30 seedlings (10 seedlings per plot, three replicates per treatment) were randomly selected (except for the seedlings in the border rows) from each treatment and marked for sampling and measurement. Surveying and sampling were carried out in the middle of each month

after fertilization. Growth indexes such as the number of new branches and new main shoot length were measured monthly. The number of lateral roots, main root length, root diameter, and biomass were measured in mid-September. The new main shoot length, root diameter, and main root length were measured with a vernier caliper. Photosynthesis parameters such as Pn were measured in mid-May and mid-July (on sunny days, 9:00–13:00) in the following year using the LI-COR-6400 portable photosynthesis system.

New branches/seedling = number per seedling branches (current month) − number of per seedling branches (previous month).

The length increment of the seedling main shoot (cm) = main shoot length (current month) − length of the main shoot (previous month).

Ginkgo leaves were collected in mid-September in the following year. For sampling, we randomly selected 30 seedlings of *G. biloba* and collected 5 mature leaves from each seedling. Portions of the leaf sample are rapidly frozen in liquid nitrogen, then stored at −80°C for enzyme analysis, and the remaining sample was dried at 65°C to constant weight for determination of flavonoid and lactone content analysis. In addition, all the leaves of the 30 seedlings from each treatment were removed for leaf yield statistics.

Ginkgo rhizosphere soil samples (collected mid-Sep in the following year) were taken randomly from the 15–20 cm rhizosphere soil layer (within 5 mm of the root surface), carefully removed from the roots, then collected with a brush and sieved. Portions of the soil samples were placed into sterilized centrifuge tubes, rapidly frozen in liquid nitrogen, and then stored in −80°C for bacterial microbiome analysis. A portion of the remaining soil samples was stored at 4°C for the determination of soil enzyme activities, and other samples were dried for analysis of soil chemical components. The rhizosphere soil was selected from three plots for each treatment. At each plot, three healthy plants were selected, and the rhizosphere root surfaces were sampled. The soil from these three plots was mixed to create one biological sample with a total of three such replicates. The experimental site and seedling status after fertilization are shown in Fig. 1.

2.4 Determination of Physiological Indexes

Total terpene lactone content was quantified according to Lu et al. [36]. Total flavonoids (TF) content and phenylalanine ammonia-lyase activity (PAL) were determined as Chen et al. [37]. Coenzyme A ligase (4CL) and cinnamyl alcohol dehydrogenase (CAD were determined using the Takshak and Agrawal [38] methods.

2.5 Soil Physical and Chemical Properties Analysis

The soil pH was determined using the extraction method. First, a soil-water suspension (1:5 w/v) was extracted, and then the pH was determined using an acidometer. The nitrate nitrogen (NO_3-N) and ammonium nitrogen (NH_4-N) in the rhizosphere soil were determined using the potassium chloride extraction and ultraviolet spectrophotometry method. Available phosphorus (AP) was determined by sodium bicarbonate leaching and the molybdenum-antimony resistance colorimetry method. Soil-available potassium (AK) was extracted using the ammonium acetate flame photometer method. All of the above methods were performed according to the methods described by Bao [39] and Vogt et al. [40].

The enzyme activities of urease, phosphatase activity, and catalase were determined using the sodium hypochlorite colorimetric method, the phenylene disodium phosphate colorimetric method, and the potassium permanganate titration-colorimetric method, respectively. Sucrase activity was determined using the method of 3–5 dinitrosalicylic acid colorimetric. The value is expressed as the number of mg of glucose produced in 1 g of soil. All of the above methods were described by Zhou [41].

2.6 Soil DNA Extraction and Quantification of 16S rRNA Gene Abundance

Rhizospheric soil samples (CK1, T2, T4, and T5, three repetitions each) used above for 16s full-length microbiome analysis. Total DNA was extracted from 0.5 g soil samples using the Soil DNA Kit (DP812, Tiangen, Beijing) according to the manufacturer's instructions. The primers 27 F_(5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492 R_(5′-RGYTACCTTGTTACGACTT-3′) was used to amplify the V1-V9 region of 16S rRNA [42,43]. The 16S full-length amplification reaction system: a final mixture volume of 20 μL containing 2 genomic μLDNA, 10 μL KOD OneTM PCR Master Mix reagent (KMM-101, Beijing BioMarker Technologies Co., Ltd., Beijing), 6.5 μL nuclease-free water, and 1.5 μL primer pair (barcode). PCR thermal cycling conditions: 95°C for 2 min; 98°C for 10 s, 55°C for 30 s, and 72°C for 90 s; followed by a final extension at 72°C for 2 min, total 22 cycles.

The PCR products were purified, quantified, and homogenized to create a sequencing library (SMRT Bell). Following library validation, sequencing was performed using the three generations of the PacBio Sequel IIe platform. The raw sequencing data generated were processed into circular consensus sequencing (CCS) files using the SMRT Link analysis software (v25.1). Sequences were then filtered through the CCS process to obtain the Clean CCS sequence without the primer sequence. The effective CCS sequence was obtained by identifying and removing the chimeric sequence. Operational taxonomic unit (OUT) clustering was performed using the optimized CCS data, followed by species annotation and abundance analysis to elucidate the taxonomic composition of the samples using R software (v4.0.2). Additionally, alpha diversity, beta diversity, and significant species difference analyses were conducted using the QIIME (v 2.0), Mothur (v1.0), and R software (v4.0.2). The DNA library sequencing and data analysis were performed by BioMarker (Biotech Ltd., Beijing, China) and BMKCloud (www.biocloud.net).

2.7 Statistical Analysis

Data were presented as the mean ± SD. SPSS 19.0 software was used for one-way analysis of variance (ANOVA). Duncan's multiple comparisons at $p < 0.05$ were used to determine significant differences between treatments.

3 Results and Analysis

3.1 Effects of Different Fertilizers on the Growth of Ginkgo Trunk-Cutting Seedlings

The growth of Ginkgo seedlings in the field was shown in Fig. 1B,C. Parameters such as the main shoot length and lateral branches are crucial indicators of leaf-harvesting forest yield and quality. The growth of new main shoots and lateral branches of *G. biloba* seedlings was mainly observed from April to June. However, the growth slowed down from July to September, finally stopped in the middle of September (Table 1). Compared to the CK1 (no fertilizer) and CK2 (NPK 18:18:18), the new main shoot length increment was significantly increased in T1, T2, T4, and T5 treatments. For example, the April-June values for these treatments were increased by 34.7%, 39.1%, 94.3%, and 87.9% compared to CK2, respectively. Moreover, the increment of new branche numbers (NB) of T2–T4 in April-May was significantly increased by 127.2%, 206.3%, 241.5%, and 271.8% respectively compared to CK2. Leaf yield per seedling was also observed in T2 and T4 treatments, and increased by 49.6% and 70.9%, respectively, over than in CK1. The leaf yield per mu (667 m^2) in T4 treatment reached 873.5 Kg (Table 1), and the leaf area was observed to be larger in the T2 and T4 treatments than in the others in September (Fig. 1D).

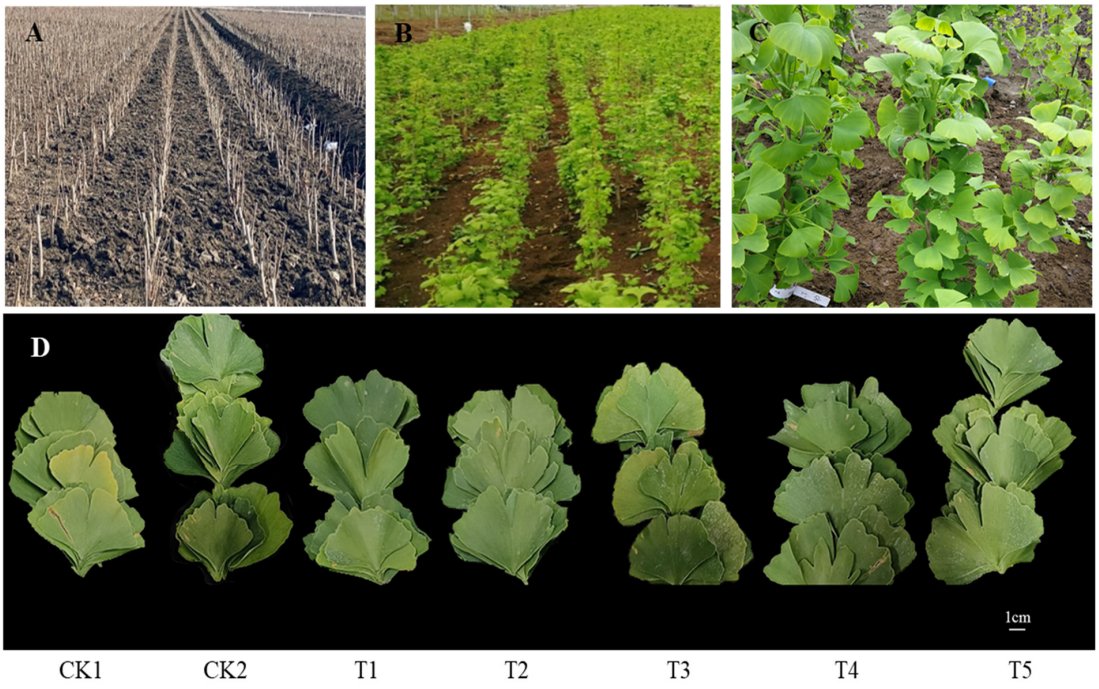


Figure 1: The fertilization site of the *G. biloba* trunk-cutting forest and the growth changes of Ginkgo seedlings after fertilization. (**A**) Trunk-cutting seedlings before fertilization (November of the previous year); (**B**): Trunk-cutting seedlings growth in May of the following year after fertilization; (**C**) Trunk-cutting seedlings growth in early June of the following year after fertilization. (**D**) The seedling leaves in September of the following year after different fertilizations. CK1 (no fertilizers); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K); T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF); T5 (MF).

The NPK fertilizer combination with microbial fertilizer also significantly promoted the root growth of *G. biloba*, such as root diameter, main root length, and lateral roots (LR). Treatments T1 and T3 were more effective in improving main root growth, while treatments T2, T4, and T5 were more effective in improving lateral root growth. For example, the main root length in treatment T3 or the number of LR in treatment T4 were 58.6% and 115.9%, or 21.3% and 45.0% higher than CK1 and CK2, respectively (Table 2).

Table 1: Effect of different fertilizer treatments on shoot and branche growth of *Ginkgo biloba* seedlings.

Treatment	Mid-April to Mid-June	Mid-June to Mid-Sep.	Mid-April to Mid-May	Mid-May to Mid-June	Mid-June to Mid-Sep.	Leaf Fresh Yield (LFY)	
	Increment of New Main Shoot Length/cm		Increment of New Branches (NB)/Seedling			g FW/ Seedling	kg FW/Mu (667 m^2)
CK1	9.0 ± 1.2d	4.4 ± 1.3d	5.4 ± 1.0c	1.8 ± 0.2ab	0	97.5 ± 8.37d	487.5
CK2	21.5 ± 0.3c	7.4 ± 1.2cd	3.2 ± 0.6d	0.3 ± 0.1c	0	102.2 ± 9.75d	511.2
T1	29.0 ± 4.7b	6.6 ± 1.2cd	5.4 ± 1.0c	1.3 ± 0.2b	0	106.9 ± 8.62d	534.6
T2	29.9 ± 5.6b	8.6 ± 0.7bcd	7.2 ± 1.4b	1.0 ± 0.1b	0	152.9 ± 5.90b	764.3
T3	21.9 ± 5.8c	10.5 ± 1.6bc	9.8 ± 1.9ab	0.7 ± 0.1b	0	127.7 ± 5.74c	638.5
T4	41.8 ± 1.1a	16.2 ± 5.5a	10.9 ± 2.1a	2.1 ± 0.3a	0	174.7 ± 7.83a	873.3
T5	40.4 ± 4.4a	12.0 ± 1.8ab	11.9 ± 2.3a	0.4 ± 0.1c	0	124.7 ± 4.29c	623.6

Values represent the mean ± standard deviation. Lowercase letters indicate the significance analysis results of treatments, different letters in the same column represent significant differences ($p < 0.05$). The leaf fresh yield was determined in the middle of September. CK1 (no fertilizers); CK2 (the local traditional fertilizer ratio); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K); T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF); T5 (MF); FW: fresh weight; DW: dry weight.

Table 2: Effect of different fertilizer treatments on the root growth and soil pH of *G. biloba* seedlings (determined on the middle of September).

Treatment	Root Diameter /mm	Main Root Length /cm	Lateral Roots (LR) /Strips	Soil pH
CK1	8.5 ± 0.7b	16.3 ± 1.5d	20.0 ± 1.6c	6.11 ± 0.24bc
CK2	9.0 ± 0.5b	22.2 ± 2.0bc	24.0 ± 2.2b	5.99 ± 0.17c
T1	11.0 ± 0.7ab	32.0 ± 4.6a	26.0 ± 2.8b	6.46 ± 0.14a
T2	11.3 ± 0.5ab	25.8 ± 1.3b	27.0 ± 1.4ab	6.48 ± 0.10a
T3	13.5 ± 3.1a	35.2 ± 1.6a	25.0 ± 1.7b	6.17 ± 0.11bc
T4	11.2 ± 1.5ab	30.9 ± 2.8a	29.0 ± 1.1a	6.10 ± 0.11bc
T5	9.4 ± 0.2b	30.9 ± 1.2a	27.0 ± 1.3ab	6.27 ± 0.05ab

Values represent the mean ± standard deviation. Lowercase letters indicate the results of the significance analysis of the treatments, different letters in the same column represent significant differences ($p < 0.05$). CK1 (no fertilizers), CK2 (the local traditional fertilizer ratio); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K); T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF), T5 (MF).

3.2 Changes in Physiological Characteristics of Seedling Leaves

The NPK fertilizer combination with MF also enhanced the net photosynthetic rate (Pn) of *Ginkgo biloba* leaves. Notably, Pn in T2 (NPK9:27:27), T3 (NPK9:18:18 + MF), T4 (NPK9:27:27 + MF), and T5 (MF) treatments was significantly increased by 4.9%, 27.2%, 23.0%, and 22.1% at mid-May, and by 19.2%, 15.9%, 17.2%, and 25.0% at mid-July compared to CK2, respectively (Fig. 2). There was no significant difference in PAL activity between the different fertilizer treatments. In contrast, the activities of 4CL and CAD were significantly enhanced by 23.4%, 24.6%, and 18.5% in T2, T4, and T5 treatments (4CL), and 24.0%, 31.1%, 23.3%, and 29.8% in T2–T5 treatments (CAD), respectively. In addition, no significant difference was found between the T1 treatment and CK2 (Fig. 2A–C). Fertilization also improved the soluble protein content in T2–T5 compared to CK1 (Fig. 3A–D).

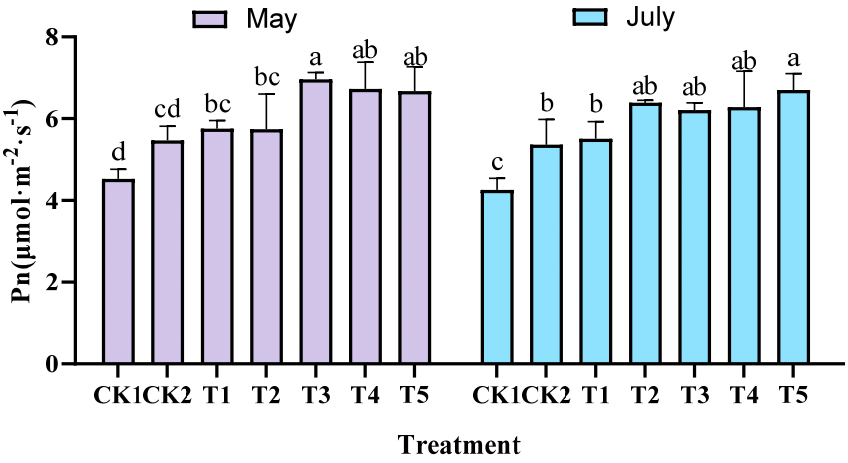


Figure 2: Effects of fertilization on photosynthesis parameters of *G. biloba* seedlings. Values represent the mean ± standard deviation. Different letter(s) on top of each bar indicate significant differences among treatments ($p < 0.05$). CK1 (no fertilizers); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K), T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF), T5 (MF).

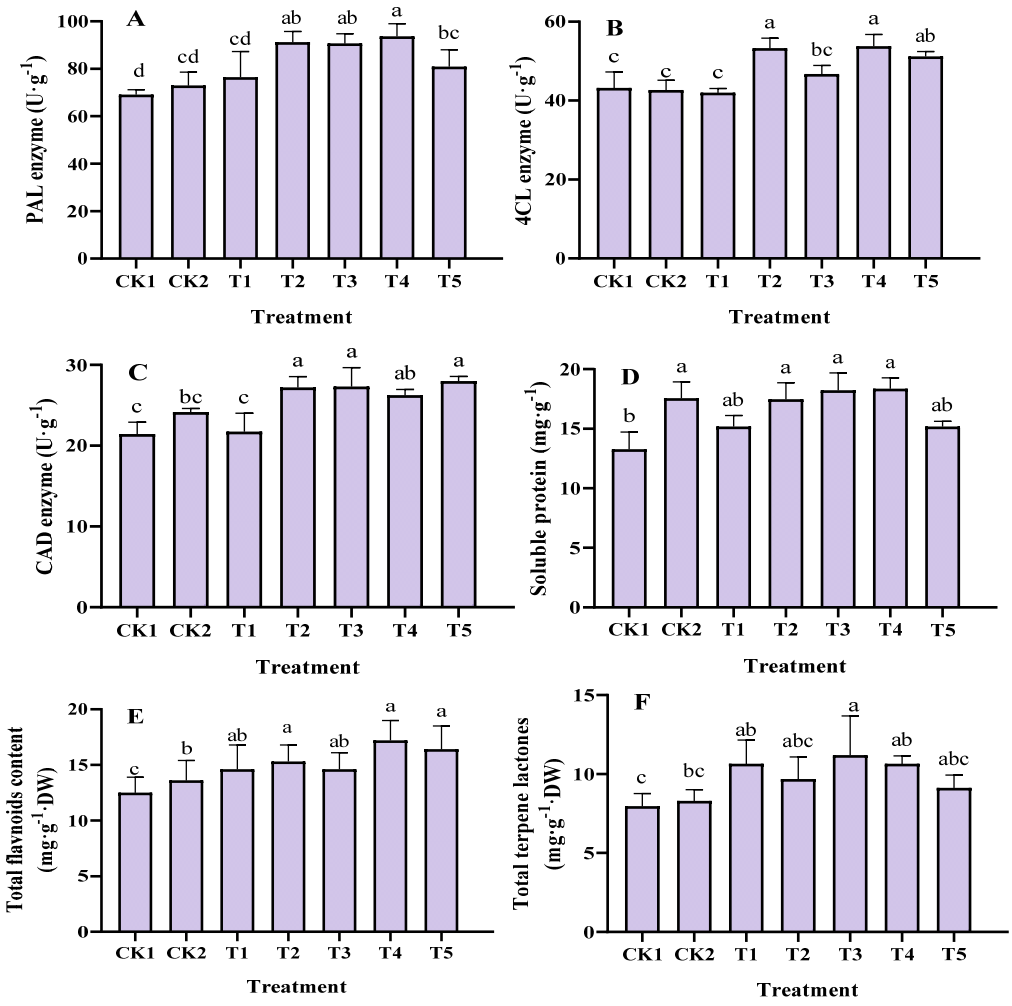


Figure 3: Effects of fertilization on key enzymes of flavonoids such as PAL (**A**), 4CL (**B**), CAD (**C**) and contents of soluble protein (**D**) total flavonoids (**E**) and terpene lactones (**F**) in leaves of *G. biloba* seedlings. Values represent the mean ± standard deviation. Different letter(s) on top of each bar indicate significant differences among treatments ($p < 0.05$). CK1 (no fertilizers); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K); T3 (T1 + Microbial fertilizer (MF)); T4 (T2 + MF), T5 (MF).

After treatment with the NPK formula fertilizer and MF, the total flavonoids (TF) and terpene lactones (TP) content of Ginkgo leaves were significantly increased compared to the CK1 (no fertilizer). In September, the total flavonoid content of T2–T4 treatment were increased significantly by 44.5%, 35.3%, and 39.5%, respectively, compared to control (CK1). Total terpene lactones (TP) content in the T2–T4 treatments increased by 33.7%, 36.4%, and 33.6% compared to control (CK1), respectively. There was no significant difference in TF and TP levels between T1 and CK2 (Fig. 3E,F).

3.3 Changes in Rhizosphere Soil Physicochemical Properties

After fertilization treatment, the pH value of the rhizosphere soil was between 6.10–6.48 for T1–T5 treatments and was higher than for CK1 and CK2 (5.99). Soil pH increased when formula fertilizer was applied alone (T1–T2). Conversely, there was no significant change in soil pH after the application of formula fertilizer with MF fertilizer (T3–T4) (Table 2). Compared to the control group (CK1), the nitrate N

(NO_3-N) content in the rhizosphere soil increased significantly under five treatments and CK2. Specifically, the nitrate N content in the T2–T5 treatments was 1.48-, 1.43-, 1.78- and 1.06-fold higher than that in CK1; in CK2, it was 0.53-fold higher than in CK1. Fertilizer also improved the ammonium N (NH_4-N) content in the rhizosphere soil compared to CK1. The highest content was found in the T3–T4 treatments, which were 84.5% and 118.4% higher than that in CK1, respectively. The level of ammonium nitrogen in CK2 also increased by 48.2% compared to CK1 (Fig. 4A,B). The available P (AP) or available K (AK) in the T2–T5 treatments was also significantly higher than in the CK1 treatment. For example, the levels of AP or AK in the T2–T4 treatments were 62.1%, 93.0% and 72.1%, or 75.3%, 92.1% and 109.7% higher than in CK1, respectively (Fig. 4C,D).

The formula fertilizer and its combination with MF also enhanced the soil enzymes related to soil fertility. In the present work, the urease activity in the rhizosphere soil was improved in the T2–T5 treatments increased by 28.3%, 25.6%, 36.9.1%, and 37.0% compared to CK1, respectively (Fig. 5A). Phosphatase activity in the rhizosphere soil under T2–T5 treatments was also higher than in the two CKs, at 32.6%, 28.0%, 82.0%, and 69.9% higher than CK1, respectively (Fig. 5B). On the contrary, no significant differences were observed in the sucrose activity in rhizosphere soil under T1–T3 treatments. In contrast, this enzyme activity enhanced in T4–T5 treatments by 45.9% and 37.8% compared to CK1, respectively (Fig. 5C). The activities of catalase in the rhizosphere soil under the T1–T5 treatments were all higher than in the two CKs, and with no significant differences between the T1–T4 treatments (Fig. 5D).

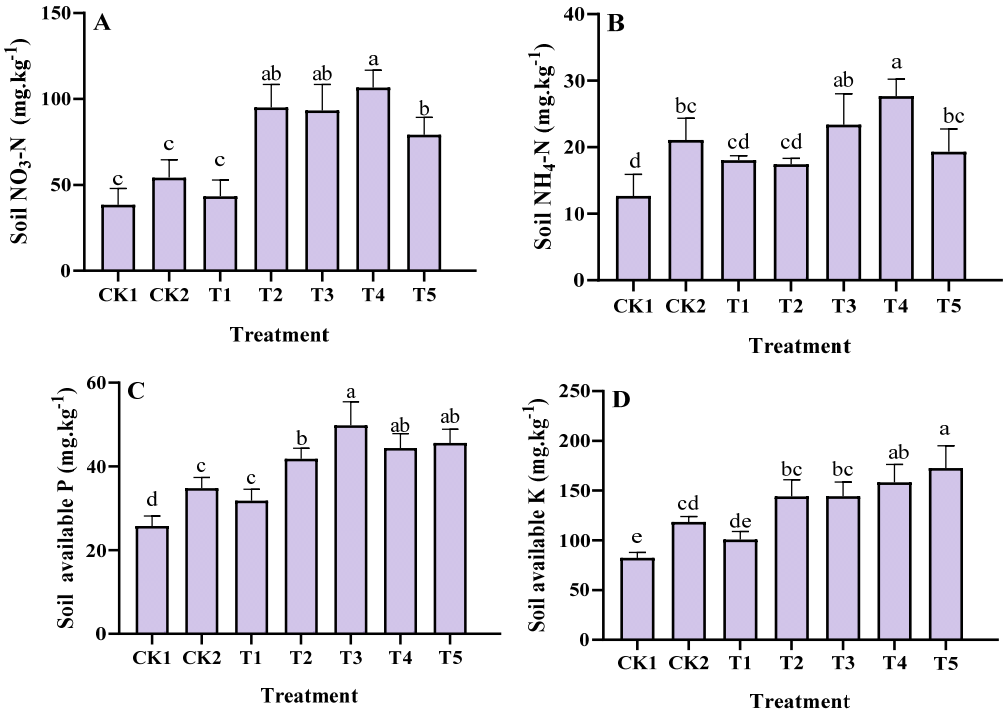


Figure 4: Effects of different fertilization on the nitrate N (NO_3-N) (**A**), ammonium N (NH_4-N) (**B**), available P (AP) (**C**), Available K (AK) (**D**), in rhizosphere soil of *G. biloba*. Values represent the mean ± standard deviation. Different letter(s) on top of each bar indicate significant differences among treatments ($p < 0.05$). CK1 (no fertilizers); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K), T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF), T5 (MF).

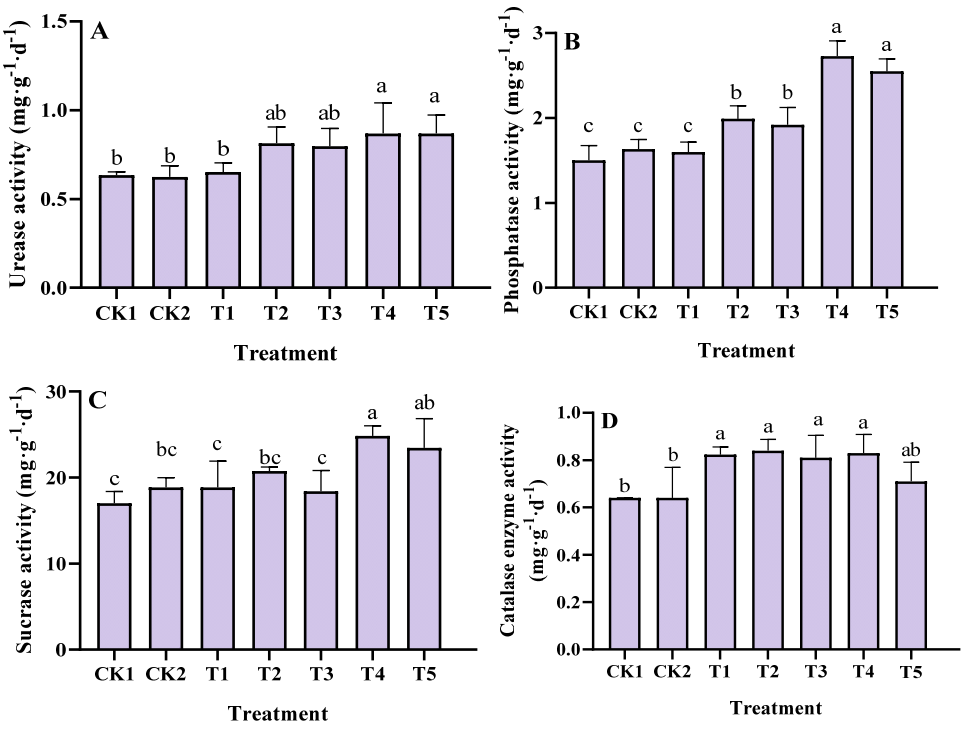


Figure 5: Effects of different fertilization treatment on urease activity (**A**), phosphatase activity (**B**), sucrase activity (**C**) and catalase activity (**D**) in rhizosphere and non-rhizosphere soils of *G. biloba* trunk-cutting seedlings. Values represent the mean ± standard deviation. Different letter(s) on top of each bar indicate significant differences among treatments ($p < 0.05$). CK1 (no fertilizers); T1 (low level of N, normal levels of P-K); T2 (low level of N, high levels of P-K), T3 (T1+ Microbial fertilizer (MF)); T4 (T2 + MF), T5 (MF).

3.4 Changes of Microbial Bacterial Diversity and Community Abundance in Rhizosphere Soil

PacBio Sequel IIe sequence platform were performed on 12 samples from four groups, including CK1 (no fertilizer, group 1), T2 (NPK 9:27:27, group 2), T4 (NPK 9:27:27 + MF, group 3), and T5 (MF, group 4) treatments. A total of 132,780 high-quality CCS reads were acquired with an average of 12,981 compelling reads per sample (ranging from 12,848 to 13,195) of the V1–V9 regions of the 16S rRNA obtained (Table 3). The Shannon and Chao 1 indexes were used to indicate the alpha diversity and richness of the soil bacteria at OUT level. In this work, the sequencing library coverage rate of all 12 samples was approximately 95%, suggesting that the sequencing results accurately represent the species richness and diversity of the samples. CK1 (no fertilizers) had the highest ACE (abundance-based coverage estimator) index and Chao1 index values, followed by the T4 and T5 treatments, with the lowest values observed in the T2 (NPK 9:27:27) treatment. Significant differences were observed between T2 and T4, CK1 treatments ($p < 0.05$). Similarly, the levels of the Simpson and Shannon indices of the rhizosphere soil in the T2 treatment were also the lowest, and differed significantly from those in the T4, T5 and CK1 treatments (there were no significant differences between the T4, T5 and CK1 treatments) ($p < 0.05$). The results indicated the T2 treatment decreased the abundance of the rhizosphere soil bacterial community (Table 3).

Table 3: Index of the bacterial microbial diversity and sequence abundance in the soil rhizosphere of *G. biloba* under different fertilizer treatments.

Treatment	Clean CCS	ACE	Chao1	Simpson	Shannon	PD Whole Tree	Cover-r Age
CK1	13,124 ± 688.3a	2325.9 ± 38.1a	2241.0 ± 78.7a	0.995 ± 0.00a	9.11 ± 0.09a	94.99 ± 1.99	0.95
T2	12,848 ± 137.2a	2148.8 ± 70.1c	2101.6 ± 14.0b	0.994 ± 0.00b	8.78 ± 0.05b	87.17 ± 3.78	0.95
T4	12,928 ± 1544.2a	2277.5 ± 47.0ab	2204.4 ± 55.3ab	0.995 ± 0.00a	9.07 ± 0.05a	94.47 ± 2.79	0.95
T5	13,195 ± 603.8a	2203.9 ± 23.2bc	2115.7 ± 26.7b	0.995 ± 0.00a	9.01 ± 0.09a	93.37 ± 3.10	0.95

Values represent the mean ± standard deviation. Lowercase letters indicate the significance analysis results of treatments, different letters in the same column represent significant differences among treatments ($p < 0.05$). PD: phylogenetic diversity. ACE: abundance-based coverage estimator. CK1 (no fertilizers), T2 (low level of N, high levels of P-K), T4 (T2 + MF), T5 (MF), MF: microbial fertilizer.

The rhizosphere soil microorganisms in the 12 samples included 29 phylum, 64 classes, 174 orders, 307 families, 535 genera, and 870 species (Table 4). There was no significant difference in the phylum, class, order, and family of rhizosphere soil microbial diversity between three fertilizer treatments and CK1, only the levels of T2 treatment in genus and species were lower than those of the other treatments and the CK1 with a significantly difference (Table 4). Total of 2850 soil bacterial OTUs were annotated (Table 4), with an average of 1624 OTUs for treatments (range: 1546–1672). The Venn diagram provided a visual representation of the OTU overlap. Formula fertilizer and its combination with MF affected the number of OTUs in the rhizosphere soil of Ginkgo seedlings. The Venn diagram shows that 1980 OTUs were overlapping in the four groups, while the unique OTUs in the CK1 (no fertiliser), T2, T4 and T5 treatments were 57, 10, 48 and 57, respectively (Supplemental Fig. S1).

In the PCoA graph, which was based on beta diversity analysis, the closer two point was, the more similar the microbial composition of the samples was. The cumulative contribution rates of the first two axes in the PC2 was 13.53%, and PC1 was 41.81% (Supplemental Fig. S2A). The multi-group rarefaction curves of the observed species for the bacterial communities indicated that when the number of sequences reached 10,000, the curve tended to flatten out. This suggested that the number of sequencing samples was sufficient to represent microbial diversity (Supplemental Fig. S2B).

The co-occurrence network analysis of rhizosphere microbial phylums (top 43) differences between treatments and CK1 was shown in Supplementary Fig. S3A ($p < 0.01$). The three strongest positive correlations were found between unclassified_Bacteria and RB41, and the delta proteobacterium, while the three strongest negative correlations were observed between Bradyrhizobium, Chthoniobacter and Vicinami-Bacteraceae.

Table 4: The relative abundance of microbial community in soil rhizosphere of *G. biloba* under different fertilizer treatments.

Treatment	Phylum	Class	Order	Family	Genus	Species	OUTs
CK1	26.0 ± 0.0	55.7 ± 1.1	147.7 ± 3.2a	240.0 ± 7.5a	399.0 ± 1.7a	610.3 ± 1.15a	1672 ± 41
T2	25.5 ± 0.7	53.5 ± 0.7	136.0 ± 12.7a	220.0 ± 15.6a	359.0 ± 10.6b	532.5 ± 27.6b	1546 ± 53
T4	25.0 ± 1.0	52.7 ± 1.5	141.3 ± 2.1a	231.7 ± 5.13a	392.0 ± 4.35ab	599.0 ± 12.16a	1654 ± 60
T5	25.3 ± 0.6	55.3 ± 3.8	145.3 ± 8.1a	230.0 ± 16.1a	381.3 ± 29.1ab	573.3 ± 28.04a	1616 ± 23
Total	29	64	174	307	535	870	2850

Values represent the mean ± standard deviation. Different letter(s) on top of each bar indicate significant differences among treatments ($p < 0.05$). CK1 (no fertilizers); T2 (low level of N, high levels of P-K); T4 (T2 + MF); T5 (MF); MF: microbial fertilizer.

The Phylogenetic tree is showed in Supplementary Fig. S3B. From the UPGMA phylogenetic tree, the species diversity, abundance similarity, and dominant species of each sample were the same as those identified in the genus network analysis. The species with the highest relative abundance in CK1 was

Niastella gongiuensis (OTU136), in the T2 treatment was Vicinamibacterales (OTU26); in the T4 treatment, it was *preudomonas frederikabergensis* (OTU99), and *Priestia aryabhattai* (OTU22), and in the T5 treatment, it was Diplorickettsiaceae (OTU16) and *cytophagaceae bacterium* (OUT 10).

3.5 Response of Mcrobial Composition to Different Fertilization Treatments

As the difference in the relative abundance of fertilizer treatments and CK1, the microbial communities were classified according to phylum, family, genus, and species. At the phylum level, the relative abundance proportions of ten phyla including Acidobacteriota, Proteobacteria, Bacteroidota, unclassified_Bacteria, Verrucomicrobiota, Nitrospirota, Bdellovibrionota, Gemmatimonadota, Planctomycetota, and Myxococcota, reached 92.3%, 91.3%, 91.0% and 91.3% of relative abundance in the CK1, T2, T4 and T5 treatment, respectively. The levels of Acidobacteriota (35.8%, 15.8%, and 9.5%) and Bdellovibrionota (54.3%, 24.4%, and 19.0%) in the chemical fertilizer treatments or the combination with MF treatments (T2, T4, and T5) were higher than the CK1 group, respectively. The levels of unclassified Bacteria in T2, T4, Gemmatimonadota and Nitrospirota in T2, and Myxococcota in T4 and T5 were obviously higher than in the CK1. However, the levels of Proteobacteria, Verrucomicrobiota and Bacteroidota in T2 treatment were significantly lower than in the CK1 (Fig. 6A).

At the family level, the following accounted for a high proportion of the soil microbial communities in the CK1, T2, T4 and T5 treatments: Vicinamibacteraceae (33.2%), Chitinophagaceae (38.8%), Vicinamibacterales (34.8%) and Pyrinomonadaceae (33.1%). Vicinamibacteraceae had the highest level of relative abundance among the five families and was higher in the T2 and T5 treatments than in the CK1 and T4 treatments (Fig. 6B).

At genus level, the five genera with the largest proportions were *Vicinamibacteraceae*, *RB41*, *Bacteria*, *Vicina-mibacterales*, and *Nitrospira*. The relative abundance of the above five bacterial genera in the T2 treatment was the highest among the four groups. The levels of the first four genera in the three fertilizer treatments were all higher than CK1 (Fig. 6C).

The combination chemical fertilizer and MF also affected the bacterial relative abundance of bacterial species in the rhizosphere soil. The proportions of *Vicinamibacteraceae*, and *Vicinamibacterales* increased after fertilization treatments (T2, T4, and T5) compared to CK1, rising by 18.9%, 41.3%, 23.3% in the former and by 9.0% 39.0%, and 12.2% in the latter, respectively. Compared to CK1, the relative abundance in the T4 treatment also increased in *Acidobacteria bacterium* WWH8 (14.9%), *delta proteobacterium* (27.1%), and decreased in MND1 (−25.1%). In the T2 treatment, it increased in *Acidobacteria bacterium* WWH8 (29.3%), and *Nitrospira_japonica* (17.7%), *Acidobacteria bacterium* (28.2%), and *delta proteobacterium* (57.5%). However, the relative abundance of *Acidobacteria bacterium* WWH8 (−24.2%), *Nitrospira_japonica* (−33.6%), *Acidobacteria bacterium* (−17.2%), *delta proteobacterium* (−29.4%) decreased, while unclassified MND1 (21.7%) in T5 treatment increased compared to CK1 (Fig. 6D).

3.6 Microbial-Related Predictive Functions under Different Fertilization Treatments

By Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, a total of 45 functions were detected in the rhizosphere soil bacterial sequences ($p < 0.05$). The dominant functions of bacteria (average of 87.44% in total) were the functional groups named Global and overview maps (44.81%), carbohydrate metabolism (7.98%), amino acid metabolism (6.69%), cofactors and vitamins metabolism of (4.74%), energy metabolism (4.43%), nucleotide metabolism (3.96%), and translation 4.58%) (Fig. 7).

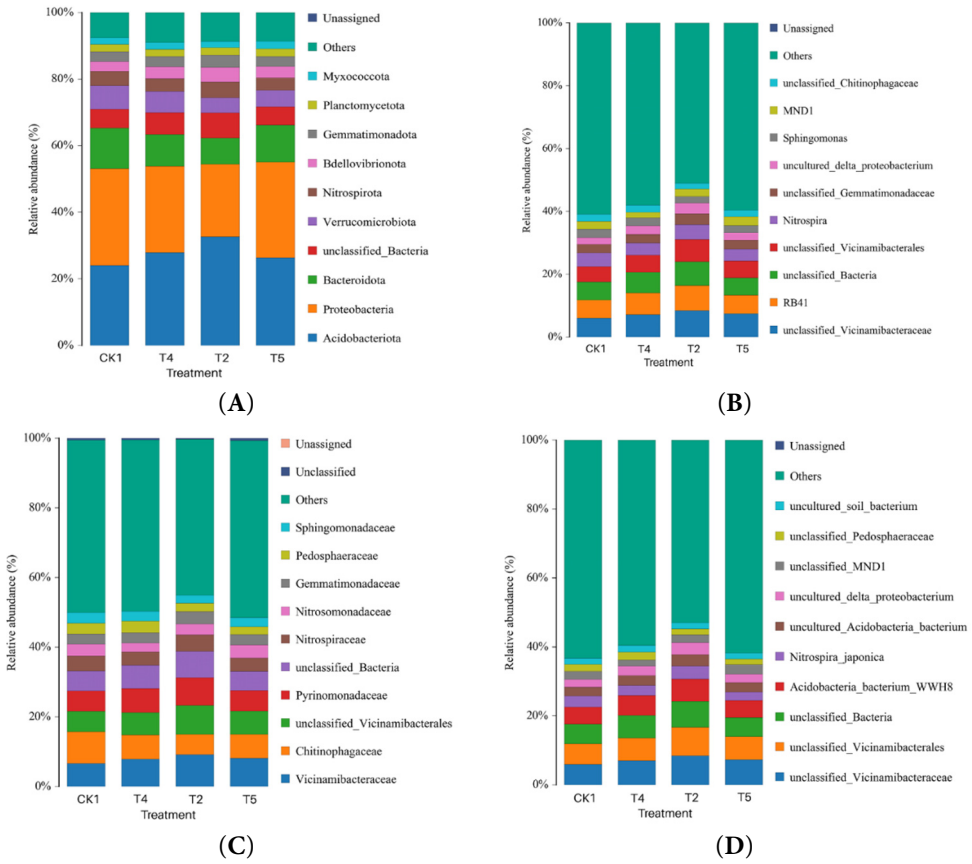


Figure 6: Relative abundances of the top 12 dominant bacterial phylum (**A**), family (**B**), genus (**C**), and species (**D**) in Ginkgo rhizosphere bacterial community. CK1 (no fertilizers); T2 (low level of N, high levels of P-K); T4 (T2 + MF); T5 (MF).

Among them, the phyla of Actinobacteriota, and Bacteroidota were involved in amino acid metabolism; Acidobacteriota and Actinobacteriota in carbohydrate metabolism; and Actinobacteriota, Proteobacteria and Myxococcota in xenobiotics biodegradation and metabolism; Campylobacterota, and Nitrospirota in energy metabolism. The three largest bacterial phyla including Firmicutes, Fusobacteriota, and Deinococcota were involved in membrane transport (Fig. 7).

A total of 57 functional groups were identified in the FAPROTAX (Functional Annotation of Prokaryotic Taxa) database in response to different fertilizer treatments. The relative abundance levels were significantly increased in nitrate ammonification (131.9%, 221.0%, and 294.5%), denitrification (11.8%, 58.9%, and 33.9%), aerobic chemoheterotrophy (12.6%, 8.8%, and 47.6%), and oxygenic photoautotrophy, photoautotrophy, aerobic anoxygenic phototrophy, photoheterotrophy, and phototrophy (73.6%, 103.2%, and 104.9%) in the T2, T4 and T5 treatments compared to the CK2, respectively; whereas, all the relative levels decreased by −13.2%, −20.8%, and −14.4% in nitrate respiration, by −23.3%, −28.0%, and −31.3% in nitrate reduction, by −35.4%, −47.5%, and −12.1% in chitinolysis, and by −64.5%, −34.5%, and −57.1% in plant pathogenesis. In addition, T2 and T5 treatments increased by 48.4% and 21.9% in nitrate denitrification, by 32.3% and 20.6% in nitrite respiration, and by 20.1% and 14.0%in xylanolysis; T4 treatment increased nitrogen respiration (29.8%), methanol_oxidation (49.8%), methylotrophy, ureolysis (29.5%), and aliphatic non-methane hydrocarbon degradation (57.3%), manganese respiration (220.3%), compared to the CK1, respectively. Similarly, T4 and T5 treatments increased dark thiosulfate oxidation (41.7%, 49.4%), dark oxidation of sulfur compounds

(34.8%, 42.0%), dark hydrogen oxidation (72.7%, 19.5%), iron respiration (220.4%, 38.4%), arsenite oxidation detoxification (87.4%, 47.6%), and aromatic hydrocarbon degradation (19.7%, 30.7%). At the same time, T2 decreased above these metabolism levels, compared to the CK1 (Fig. 8).

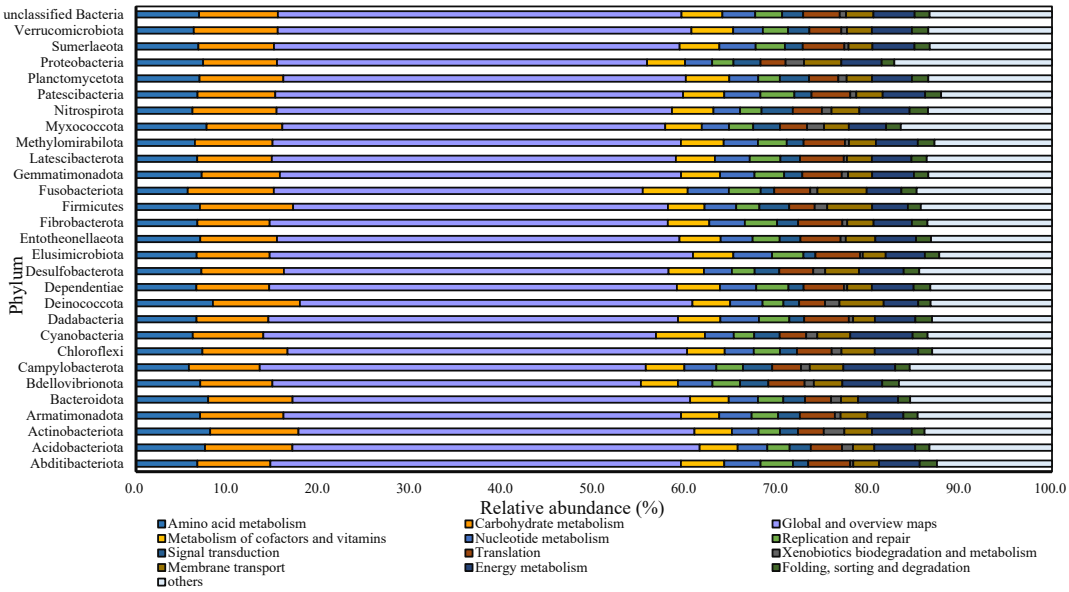


Figure 7: Relative abundances of the top 29 bacterial phylums in domain functions under the different fertilizations in Ginkgo rhizosphere soil.

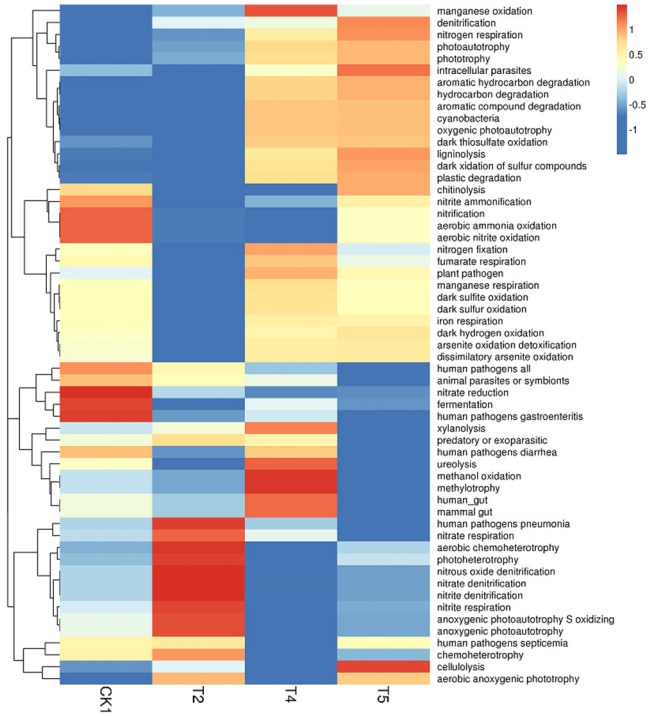


Figure 8: The heatmap of micro-ecologically significant functions of bacteria phylums by Faprotax analysis under the different fertilizations in Ginkgo rhizosphere soil. CK1 (no fertilizers); T2 (low level of N, high levels of P-K); T4 (T2 + MF); T5 (MF). The color intensity in heatmap represents the relative abundance within the treatments and microbes.

3.7 Correlation of Microbiological Communities with Seedling Growth, Soil Physicochemical Characteristics

Cluster analysis was used to explain the relationship between the treatments and soil bacterial functions through dimensionality reduction. Compared to the control (CK1), the relative abundance of rhizosphere soil bacteria such as Gemmatimonadota, Bdellovibrionota, Acidobacteriota, and Nitrospirota increased in the T2 treatment, while Bacteroidota, Proteobacteria, *Verrucomicrobiota* declined at phylum level (Fig. 9A). However, the relative abundance of the species-level such as *Acidobacteria bacterium* WWH8 and *delta proteobacterium* in the T4 treatment was high compared to CK1 (Fig. 9B).

A PCA (principal component analysis) diagram was used to quantify the relative influence of important physicochemical properties on microbial community structure. Twenty-two parameters accounted for 86.2% of the variation, with axis 1 explaining 58.2%, and axis 2 explaining 27.7%. The Gemmatimonadota, Acidobacteriota Bdellovibrionota, and Nitrospirota were classified in the fourth quadrant. Parameters such as NO_3-N, NH_4N, NB, LR, AK, TF and LFY were classified in the second quadrant (Fig. 10A). A heatmap was generated using Spearman's analysis to investigate the correlation between physiological parameters and soil physicochemical parameters, and microbial abundance at the phylum level. NO_3-N, NH_4N, AP, AK, TF, NB, LR, and LFY showed extremely significant positive pairwise correlations ($p < 0.01$). NO_3-N showed a significantly positive correlation with *Acidobacteriota*, *Acidobacteria bacterium WWH8*, and *Nitrospira japonica*, and a negatively correlation with *Pedosphaeraceae* ($p < 0.05$). A significantly positive correlation was found between *Acidobacteria*, *bacterium WWH8*, *Acidobacteria bacterium*, *deltaproteobacterium*, and AP, AK TF, and LYF ($p < 0.05$). A significant negative correlation was found between *Vicinamibacteraceae* and TF (Fig. 10B).

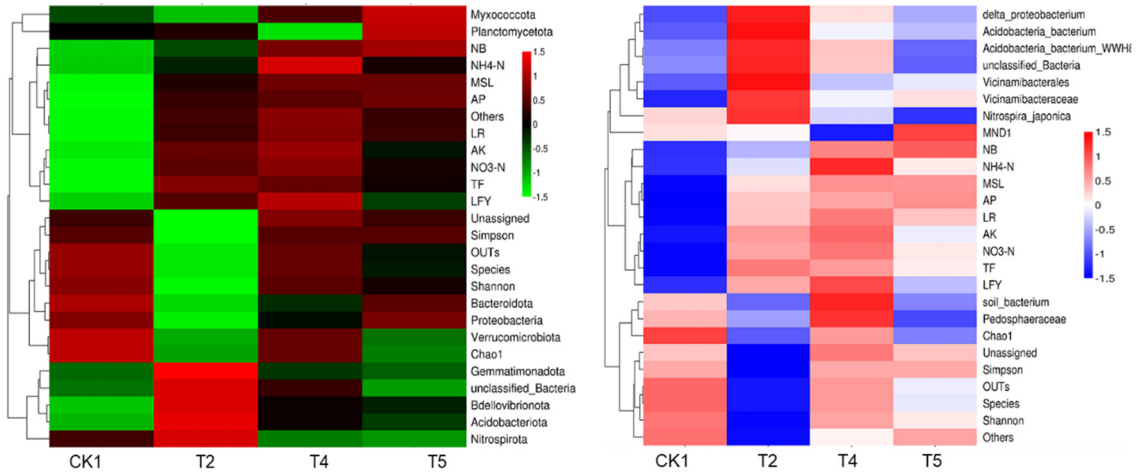
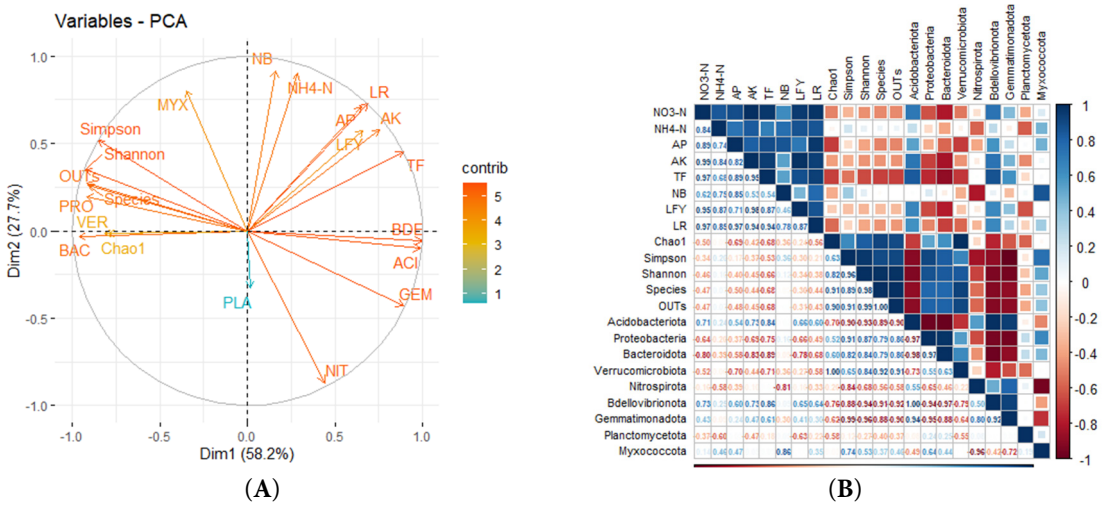


Figure 9: The heatmaps of physiological parameters and the relative abundance of bacteria phylums level (**A**) and species level (**B**) under the different fertilizing treatments. NO3-N: soil nitrate nitrogen; NH4-N: ammonium nitrogen. AP: available phosphorus AK: Soil-available potassium (AK); TF: flavonoids; NB: new branches; LFY: Leaf fresh yield; LR: lateral roots. CK1 (no fertilizers); T2 (low level of N, high levels of P-K); T4 (T2 + MF); T5 (MF). The color intensity in heatmap represents the relative abundance within the treatments and indexes.



(**A**) (**B**)

Figure 10: The Principal component analysis (**A**), and the correlation of parameters and the relative of abundance microbial bacterium (**B**). ACI: Acidobacteriota; PRO: Proteobacteria; BAC:Bacteroidota; VER:Verrucomicrobiota; NIT: Nitrospirota; BDE: Bdellovibrionota; GEM: Gemmatimonadota; PLA: Planctomycetota; MYX: Myxococcota. Bar color in (**B**) denotes difference significance between parameters and the microbial bacterium.

4 Discussion

Fertilization serves as a critical step in the forest production process, effectively enhancing both crop yield and quality [44,45]. Appropriate levels of N, P, and K fertilizer have distinct effects on promoting plant growth. The most effective proportions for promoting the growth and leaf yield of Ginkgo plantation were N:P:K = 40:20:9 for 10-year-old seedlings and N:P:K = 6:5:3 for four-year-old seedlings. However, excessive fertilization and higher fertilization rates increased greenhouse gas emissions and nitrogen losses [31,34]. Meanwhile, the long-term excess N fertilizer decreased plant growth and secondary metabolism, and also made the microbial community more sensitive to environmental changes [46–48]. In the present study, reducing nitrogen fertilizer and maintaining a normal level of P-K (T1, N:P: K = 9:18:18, low N) slightly improved shoot and root growth, and significantly increased the content of flavonoids and terpene-lactones compared to CK2 and CK1. Previous reports have also shown that appropriately reducing N fertilizer did not reduce total N uptake or dry matter production [49].

P and K are the main limiting elements for crop growth and yield due to their low solubility in soil and low availability to plants [10,50]. Increasing the proportion of P and K in fertilizer is also an effective measure to improve fertilizer efficiency. In the present work, treatment T2 (low N, high P-K) significantly enhanced the growth, flavonoids, and terpene lactones of trunk-cutting G. biloba seedlings (Tables 1 and 2, Figs. 2 and 3). For example, new branches, new shoot length, biomass, Pn, flavonoids, terpene-lactones, and key enzyme activities increased in T2 than in T1, CK2, or CK1. Previous reports have also indicated that elevated P availability could improve nutrient uptake efficiency, coordinate the nitrogen-phosphorus balance, and promote the synthesis of secondary metabolites under low N conditions [51]. The application of K fertilizer increased crop yield, starch properties, and total soil K uptake [9,52]. On the other hand, K fertilization increased the biomass of *Cynanchum taiwanianum* and sweet potato (*Ipomoea batatas* L.), while high N fertilization decreased the total phenolic and total flavonoid contents [53,54]. Therefore, reducing the N rate or increasing the P-K rate is a beneficial measure for the growth and the accumulation of secondary metabolites of *G. biloba* trunk-cutting seedlings under the existing fertilizer management.

Previous studies have shown that prolonged use of chemical fertilizers alone could decrease crop yield, reduce soil fertility, and even increase soil pollution [11,55]. However, the combined application of organic and chemical fertilizers has been shown to significantly increase crop yield and quality, while improving N and P balance [6,56,57]. In our work, treatments T3 (low N, normal level P-K + MF), T4 (low N, high P-K + MF), and T5 (MF) all significantly increased the main shoot length, biomass and flavonoid levels, which were the most relevant for the yield and quality of Ginkgo leaf-harvest forest, especially treatment T4, it was the best fertilization measures among the five treatments. The results of our work were in agreement with previous reports. This indicates that reducing N fertilizer and increasing P-K plus microbial fertilizer could promote the synthesis of plant secondary metabolites and regulate the balance of N, P, and K fertilizer in the soil.

Integrating chemical and microbial fertilizers might enhance bio-fertilization-mediated nutrient solubilisation, improving soil fertility and fertilizer utilization [12,58]. In our work, the appropriate levels of N, P, and K fertilizer combined with microbial fertilizer also improved the physicochemical properties of the rhizosphere soil of Ginkgo seedlings. For example, the T2 treatment (low N, high P-K) enhanced nitrate-N, AP, and AK uptake in the rhizosphere soil compared to the control (CK1). These results suggest that N limitation may induce plants to prioritize nitrate assimilation, while compromising the mobilization of other nutrients [5,59]. However, the combination of chemical (N, P, and K) with MF (T3–T4) increased the levels of nitrate-N, ammonium-N, AP, and AK in the soil compared to CK1, and was more effective than the T2 treatment. These results were consistent with studies showing manure/straw application could improve soil fertility [60], and that microbial, organic fertilizer could improve the soil physicochemical properties by enhancing the levels of AP, AN, and AK [12,21,61].

Our work also showed the synergistic effects of MF and optimized P-K ratios (T2–T4), which became more apparent when four rhizosphere enzyme activities were modulated. Specifically, treatments T4 and T5 elevated all four enzyme activities, including urease, phosphatases, sucrose, and catalase (Fig. 5A–D). However, the sucrose and phosphatase activities in the T2–T3 treatments were lower than in the T4 and T5 treatments. The increase in urease might reflect microbial-driven hydrolysis of urea to ammonium N and elevated soil nitrogen supply capacity [62]. The increase in sucrase activity indicates improved microbial carbon metabolism, which promotes rhizosphere symbiosis and nutrient turnover [7]. Applying organic or inorganic fertilization also enhanced the activities of urease and sucrase in soil [62,63]. The results of our work were consistent with previous reports and indicated that chemical (N, P, and K) with MF might accelerate the transformation of available P and K nutrients and the N-supply capacity in the soil rhizosphere. Phosphatases could release bioavailable inorganic P from the organic form of P in soil [64,65]. Previous studies have shown that the phosphatase activity is inhibited by the addition of inorganic P to the soil, whereas P deficiency promotes root secretion of P to mobilize and utilize organic P under intercropping systems [66–68]. However, the opposite effect was observed when applying NPK and NP fertilisers to the root zone, which enhanced phosphatase activity and increased fertiliser use efficiency [69]. This was in agreement with our work and indicated that the appropriate proportion of NPK, rather than simply adding P fertilizer, was beneficial in improving phosphatase activity. The T4–T5 treatment had significantly increased the phosphatase activity in the rhizosphere soil. This suggested that functional bacteria in the MF might enhance soil P availability through inorganic P solubilization and organic P mineralization, thereby improving bioavailability and soil physical properties [10,69].

Soil fertility, planting patterns, plant species and age, and crop intercropping could all influence soil bacterial diversity [70]. Applying microbial fertilizer effectively improved microbial community structure and ecological function [10,12]. In the present study, T2 treatment decreased the Chao1, Simpson, and

Shannon indices compared to no fertilizer (CK1). This indicated that a single application of chemical fertilizer reduced the abundance of the rhizosphere soil bacterial community in Ginkgo seedlings. These results were consistent with reports that the addition of N fertilization significantly decreased soil bacterial diversity [10,71]. However, the above indices did not differ significantly between T4 and T5 treatment (except for Chao1). This showed that the effect on the bacterial abundance was minimal when applying chemical and microbial fertilizers. Previous studies also showed that microbial and organic fertilizer or intercropping increased or had no significant change in the alpha richness and diversity of soil microbial communities [12,21,23,70]. Therefore, our findings confirmed that microbial fertilization did not significantly alter bacterial diversity.

In our work, the relative abundance of *Acidobacteria bacterium* WWH8 (Acidobacteriota) and *Bdellovibrionota* and *Vicinamibacteraceae*, *Vicinamibacterales* (Proteobacteria) in T2 and T4 treatments was all increased. Moreover, the level in the application chemical alone (T2) was higher than application chemical plus MF (T4) and MF alone (T5). In addition, the abundance of Gemmatimonadota and Nitrospirota was also increased significantly in T2 compared to CK1. Previous reports showed that under N limitation, the abundance of Acidobacteria increased, and that of Actinobacteria and Proteobacteria declined [72], while under N enrichment, the abundance of the two bacteria increased in the soil [46,73]. Applying organic fertilizer also promoted Proteobacteria and Bacteroidetes abundance [12,23]. The reason for these differing results might be related to the different environments to which these bacteria have adapted. Acidobacteria with genes that catalyze the metabolism of N could effectively reduce nitrate and nitrite, and participate in nitrogen nutrient circuits. It also contains biosynthetic gene clusters that encode a wide range of polyketides and promote the production of secondary metabolites. In addition, the Acidobacteria were also involved in carbon fixation and metabolism of methane and sulfur [74,75]. This phylum favored an oligotrophic lifestyle and acidic-neutral conditions (pH 3.0–6.5) [10,23,76]. However, the Proteobacteria could decompose organic matter, dissolve phosphate, fix nitrogen, and secrete extracellular polymers in alkaline–sandy soil [12,77]. The application of N fertilizer increased the relative abundance of Proteobacteria, Bacteroidetes, and Firmicutes [11,78]. In the present study, the treatments of T2 and T4 were both nitrogen-limited (T2: low N, high P-K; T4: T2 + MF) and occurred in a neutral acidic environment (pH 6.0–6.5). This indicated that high P-K or high P-K plus MF would be beneficial in increasing the relative abundance of Acidobacteria and Proteobacteria. These microorganisms could improve the carbon fixation, nitrogen fixation, and phosphorus dissolution of the soil.

Gemmatimonadetes play a denitrifying role in the nitrogen cycle and could mitigate the harmful effects of nitrate accumulation on plants [79]. In contrast, Nitrospira could carry out the soil nitrification reactions of converting nitrite (NO_2^-) to nitrate (NO_3^-), which plays an essential role in N metabolism, especially in neutral or acid soils [80–82]. NPK fertilization markedly increased the relative abundance of Gemmatimonadetes [46], while the application of N fertilizer alone decreased their relative abundance [79]. Moreover, N addition also declined the relative abundances of both Nitrospirae and soil microbial diversity [46,83]. Previous studies have shown that Gemmatimonadetes might be specifically adapted to arid environments and had less coherent ecological responses to N fertilisation, which might explain the inconsistent results of previous studies [84,85]. In the present work, the T2 treatment (low N) increased the relative abundance of Nitrospirota and Gemmatimonadota; this was consistent with the findings of Cederlund et al. [79] and Wang et al. [83], who reported that the abundances of these two phyla were not significantly affected by the T4 treatment (low N, high P-K with MF fertilizer). These results indicated that applying chemical fertilizer alone would affect the nitrifying bacteria's utilization of nitrogen and the balance of nitrate nitrogen and ammonium nitrogen in the soil. A combined chemical and microbial fertilizer treatment might utilize other

digestive bacteria, such as Acidobacteria and Proteobacteria, to regulate the balance of NO_3^- and NH_4^+ in the soil, thereby improving its NPK absorption capacity in the soil. This is consistent with our results for the T4 treatment, which could increase the nitrate nitrogen and ammonium nitrogen in the soil of Ginkgo seedlings.

Using the KEGG pathway, we annotated the metabolic functions of the top 27 soil bacteria. Carbohydrate, amino acid, energy, etc., metabolism was the main metabolic pathway under different fertilizer treatments. Furthermore, FAPROTAX functional predictions indicated increased activity in several functional groups, including nitrogen metabolism, photosynthesis, and chemoheterotrophy, which include nitrate ammonification, denitrification, aerobic chemoheterotrophy, oxygenic photoautotrophy, and photoheterotrophy. These functional groups are closely related to C and N cycling [12]. Nitrate denitrification, nitrite respiration, and xylanolysis also increased with the T2 treatment (low N, high P-K). This may be related to the simultaneous increase in the relative abundance of Nitrospirota and Gemmatimonadota under the T2 treatment. This indicates that the short-term application of chemical fertilizers alone may promote growth and the synthesis of secondary metabolites. However, long-term application of chemical fertilizer alone (even with an appropriate NPK proportion) might disturb the nitrogen balance in the soil and increase nitrate denitrification. This needs to be verified through further experiments. This also demonstrated that N limitation drives microbial communities to prioritize alternative energy acquisition strategies, such as phototrophy and anaerobic nitrogen respiration, in order to compensate for reduced nitrification efficiency [64,71,80].

Notably, the combined application of low N, high P-K plus microbial fertilizer (T4) also enhanced nitrogen respiration, methanol_oxidation, methylotrophy, ureolysis, and aliphatic non-methane hydrocarbon degradation. It also improved the metabolism of manganese, thiosulfate, and iron, aromatic hydrocarbon degradation, except for increasing the functions of nitrogen metabolism, photosynthesis, and chemoheterotrophy. These findings were consistent with the idea that inoculation with functional microorganisms enhanced nitrogen fixation, nitrification, and aerobic ammonia oxidation, thereby improving soil quality [12]. Additionally, microbial fertilisers played a dual role in enriching keystone taxa of nitrogen fixation and sulfur oxidation, priming soil organic matter turnover through enzymatic diversification. T5 treatment increased the high levels of relative abundance of Planctomycota and Myxococcota in the rhizosphere soil, which might improve soil fertility through decomposing plant debris.

5 Conclusions

Our work results indicate that a low N, high P-K in fertilizer (in the short term), or plus MF was beneficial in improving the yield and quality (high levels of flavonoids) of Ginkgo leaf-harvesting forests, especially in T2 and T4 treatments. These treatments (T2–T4) also improved the physical and chemical properties of the soil by elevating nitrate-N, AP, and AK, urease, and phosphatase levels in the rhizosphere soil (RS). The effect of the T4 treatment was the best.

The Acidobacteriota, Proteobacteria, Verrucomicrobiota, Nitrospirota, and Gemmatimonadota were found to be highly abundant in the Ginkgo RS. T2 decreased the diversity of RS bacteria while increasing the abundance of these bacterial phyla in the RS and enhancing microbial functions. Applying chemical fertilizer plus MF (T4) had a minimal effect on the bacterial diversity. However, the T4 treatment increased the abundance of the *Acidobacteria bacterium* WWH8 and the *delta-proteobacterium*, enhanced nitrogen metabolism, photosynthesis, ureolysis, methylotrophy, etc.

In conclusion, our results showed that optimizing P-K and reducing N level, or plus MF, created a rhizosphere environment conducive to nutrient balance and microbial-mediated nutrient cycling. This

approach improved ammonium-N and P-K availability, reconfigured enzyme activities to favor sustainable nutrient use, and promoted the growth of trunk-cutting seedlings, as well as the synthesis of flavonoids and terpene lactones. This increased the yield and quality of *G. biloba*.

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Supplementary Materials: The supplementary material is available online at https://www.techscience.com/doi/10.32604/phyton.2026.085745/s1. Figure S1: The Venn diagram, the coincidence OTU number; Figure S2: The PCoA graph and beta diversity analysis and multi-group Shannon curves; Figure S3: The heatmaps of genus correlation network in the top 43 genus (Phylum).

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