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Microbial Changes in the Rhizosphere of Paper Mulberry (*Broussonetia papyrifera*) Mutant with a High Concentration of Crude Protein

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Abstract: Recently, many studies involving plant-microorganism relationships in the rhizosphere of multitudinous important economic crops revealed a clear signature of the host plant in shaping its rhizosphere microbial composition and structure. The nutrient preference of host plant was suggested to be one important factor determining the structure and assembly of the rhizosphere microbiome, but the proof for this hypothesis is still not enough. In this study, soil microbiomes in the rhizosphere of two Paper mulberry varieties with different nitrogen absorption and utilization efficiency were investigated using a short term pot experiment in controlled greenhouse, and the physicochemical properties were also determined. The results showed that, compared to the control plants, the mutated Paper mulberry variety with high N demand reduced the microbial growth significantly and changed the bacterial and the fungal composition in the rhizosphere soils, and alkaline nitrogen was identified to be the most significant factor affecting soil microbial community. Moreover, the effects of excessive consumption of soil nutrient during Paper mulberry cultivation on the microbiome was revealed, and it could be employed in field water and fertilizer management of Paper mulberry planting. This study further confirmed that the soil nutrient status resulting from the plant nutrient preference drives the development of a plant-specific microbiome.

Keywords: Paper Mulberry, Nutrient Preference, MiSeq Sequencing, Rhizosphere, Soil Microbial Community

1. Introduction

The rhizosphere of plants, as the zone of interaction between plants' roots and microbes, plays an important role in the plant-microbe-soil system, facilitating host plant growth and development by the supply of various necessary nutrients [1], and also advancing activity and biodiversity of the microbial community through supporting organic carbon such as plant root exudates, mucilage, and sloughed-off root cells [2]. In the rhizosphere, the plant and the microbial community interacted with each other. Host plants influence soil microorganisms surrounding their roots by root morphology, excretion of exudates, and other substances that can be both repellent and chemoattractant signals, and the presence of dead root cells [3-4]. Conversely, the soil microorganisms colonizing in the rhizosphere affect the host plants through regulation of plant growth hormone [5], activation of soil nutrients [6], and degradation of toxic and harmful substances by synthesis and secretion of secondary metabolites [7], and long-distance transport of water and nutrients [8]. The plant-associated microbial community in the rhizosphere also referred to as the second genome of the plant, is crucial for plant growth and health [9-10].

Among various factors influencing the soil microbial composition in the rhizosphere of the host plants, plant species have been recognized as the best predictor of the composition of fungal and bacterial communities [11]. However, this microbial community is also influenced by the physicochemical properties of the soil [12]. Recent advances in plant-microbe interactions have revealed a clear signature

of the host plant in shaping its rhizosphere microbiome, as evidenced by the specific microbial communities hosted by different plant species growing in the same soil [13-14]. However, whether these host-specific communities thriving in the rhizosphere are directly recruited by plant-derived carbons (root exudates) or via preferences for specific soil conditions, such as mineral nutrients and physical structure, is still unknown. The research by Ai et al. [14] indicated that a limited capability of root-derived products to influence the rhizosphere microbiota. Thus, considering the specificity of plant species with respect to the level and types of soil nutrients absorbed, plant nutrient preferences may also play a key role in recruiting the host-dependent rhizosphere microbiome.

Paper mulberry *(Broussonetia papyrifera*) is an ecologically, economically, and medicinally important plant with variety of features such as fast growth, ease of breeding, strong tillering ability, and pruning resistance [15]. Belonging to the nitrogen-fixing clade of Rosales and Moraceae, Paper mulberry might fix atmospheric nitrogen with formation of nodules by symbiotic microbes and transformed it into protein stored in leaf [16]. More recently, Paper mulberry has also been used as forage to address the shortage of feedstuff because of its digestible crude fiber and high protein contents [17-18]. To further improve forage quality of Paper mulberry, various efforts were taken in several areas to increase crude protein content, including breeding new varieties and improving planting technology. A new hybrid of Paper mulberry with a high crude protein concentration has been cultivated by Shen and Peng [19] and planted in almost all provinces of China as part of a national project to resolve the silage deficiency. Based on tissue culture of the hybrid material, we further obtained a new mutated variety of Paper mulberry (named as FT temporarily) with higher crude protein concentration taking advantage of accelerated heavy ion irradiation. Nitrogen was quickly absorbed, transported, and stored in different tissues by the FT plants, which led to serious nitrogen deficiency in the soil if the soil nitrogen pool can not be supplemented in time. How nitrogen preference of the FT plants affected soil physicochemical properties and the microbial community was still unknown.

Due to the great consequence of plant-microorganism relations in the rhizosphere soil for nutrient cycling and plant growth and health, it is essential to recognize factors affecting microbial parameters in the soil environment. And the role of plant nutrient preferences in recruiting the host-dependent rhizosphere microbiome was speculated. Therefore, to verify this hypothesis, the rhizosphere microbiomes of two varieties of Paper mulberry with different nitrogen absorption and utilization efficiency were determined using high-throughput MiSeq sequencing as well as the soil nutrient properties in a monocropping system with a short term pot experiment in control. The aims of this study were (1) to determine whether nitrogen nutrient preference is involved in assembling a host-specific rhizosphere microbiome and (2) to elucidate how Paper mulberry nitrogen nutrient preference shapes the rhizosphere microbiome.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The hybrid paper mulberry does not produce flowers, so the first-generation vegetative propagators were used for the following experimental materials. Regenerated plantlets (wild type (CK) and the mutant with a high concentration of crude protein (FT) cultivated by accelerated $12C^{6+}$ irradiation, ~5 cm in height) were prepared and transferred to 300-ml plastic pots (one plant per pot) into a mixture (1:1, by vol.) of peat soil: sand. The mixture had pH 4.32, EC 16.43 dS/m, organic carbon 38.02 g/kg and 340.2 mg/kg available N (alkaline $KMnO_4$ extractable), 223.48 mg/kg Olsen-P (NH_4F-HCl extractable) and 21.86 mg/kg available K (1 M NH_4OAc extractable).

Paper mulberry plants were grown in a random design in the climate-controlled chamber (16 h photoperiod at a light density of approximately 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation, 23-25°C and 60% relative humidity) at the Jiangxi academy of sciences for 45 days (4 plants per variety) and watered with tap water once a week.

2.2. Harvesting and Sampling

At harvest, rhizosphere soil was carefully collected from fine roots by gently scraping adhering soil using fine forceps. The soil samples were passed through 2 mm mesh to remove plant debris, thoroughly homogenized and divided into subsamples. Subsamples for molecular analysis were frozen stored at -80°C, and subsamples for analysis of soil physicochemical properties were air-dried.

Leaf, stem, and root were separated, washed, and gently dried with paper towels before being weighed. Total fresh weights of tissues were determined, and a weighed leaf sample (100-200 mg) was taken for determination of Chlorophyll concentration. Plant tissues were dried at ~45°C for 3-4 days before dry weight was determined.

2.3. Plant Growth and Soil Physicochemical Characteristics Analysis

The total leaf dry weight was determined from the total fresh weight and the fresh/dry weight ratio of the sample. Excised leaflets were collected in plastic bags and were weighed within one hour of sampling and extracted by 100% dimethyl sulfoxide for 24 h in the dark, to determine the content of Chla, Chlb, total chlorophylls as described previously [20]. Dried plant tissues were ground, and stem and leaf were mixed together as shoot samples. Crude protein concentration was determined by the Kjeldahl method.

Soil pH was determined using glass electrode pH meter (FE20–Five Easy Plus™, Switzerland) in a 1: 2.5 soil/water (w/v) suspension [21], and electrical conductivity (EC) was measured for the water extracts containing the 1:5 soil/water ratios (w/v). Soil organic carbon (SOC) was determined by the

$K_2Cr_2O_7$ oxidation oxidation-reduction titration method [22]. Soil inorganic nitrogen was assayed by using the alkaline hydrolysis method [23] and soil available phosphorus (P) by Mo-Sb colorimetry method [24]. Microbial Biomass C in Soils was determined by the Chloroform Fumigation-Extraction Method.

2.4. Soil DNA Extraction and Sequencing

Soil DNA was extracted from 0.5 g subsample of each soil sample using the Fast DNA®SPIN Kit for Soil (MP Biomedicals, Solon, Ohio, USA). The extracted total DNA, dissolved in 30 μl sterilized deionized water, was checked on a 1% agarose gel, and the DNA concentration and quality were estimated using a Nanodrop®ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). Amplicon libraries for bacteria were produced by using bacteria-specific primers 515F and 806R designed for the V4 region of the 16S rRNA gene according to Bergmann et al. [25]. Each pair of primers used to amplify a certain sample was barcoded with a unique error-correcting 10-12 bases barcode on both forward and reverse primers. All amplifications were performed in 2-fold 25 μl reactions, while each PCR reaction was carried out in 25 μl volume, including 1 μl of template DNA (20 ng/μl), 1 μl of each primer (10 pmol μl^{-1}), 12.5 μl of 2× PCR reaction mix (TAKARA Biotechnology Co. Ltd), and 0.5 μl of Taq DNA polymerase (2.5 U μl^{-1}; TAKARA Biotechnology Co. Ltd), the final volume was added up to 25 μl with sterilized deionized water. The protocol of amplification was as follows: 5 min initial denaturation at 95°C, followed by 35 cycles of denaturation for 45 s at 94°C, 45 s primer annealing at 58°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. Amplicon libraries for fungi were prepared using an identical approach, except that we chose a specific primer pair ITS1F and ITS2 [26], designed for the internal transcribed spacer (ITS) 1 region for Illumina sequencing. PCR products of each sample were pooled and purified by agarose gel electrophoresis with a QIAquick PCR Purification Kit (QIAEX Ⅱ Gel Extraction Kit, QIAGEN Sciences, Maryland), estimated by using a Nanodrop®ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) and mixed together with an equal amount of 150 ng for each sample. The pooled samples were sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd, China, and sequenced on an Illumina Miseq2000 platform (Illumina, San Diego, CA, U.S.A.).

2.5. Bioinformatics and Statistical Analysis

Data yielded from Illumina sequencing was analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (v.1.9.1), as described previously by Caporaso et al. [27]. All the raw sequence obtained was assigned to each sample based on their specific barcode sequence. Reads with an average quality value of < 30 and not having both universal primer sequences were all filtered off. For bacteria, following quality control, removal of chimeras and nontargeted sequences, the left sequences were aligned with the Silva-ARB database (version 119) [28], and clustered into operational taxonomic units (OTUs) at 97% identity by using the unsupervised Bayesian clustering algorithm CROP [29]. For fungi, data were analyzed with an identical procedure except that ITS1 region was extracted from each sequence by Fungal ITS Extractor 1.1 after removal of primers [30], and sequences were aligned against the UNITE ITS database (version 6.0) [31]. To estimate the final ranking, the abundance-based coverage estimator (ACE), the estimated asymptotic microbial taxon richness (Chao), and the observed richness (sobs) were calculated. After an identical number of reads (43 804 and 49 172 for bacteria and fungi respectively) subsampled from each sample, microbial diversity was compared between varieties based on the calculated Shannon index. Finally, sequences were assigned to taxonomic groups by using the RDP classifier (Rel. 11).

All microbial community-related analyses were based on the relative abundance of OTUs per sample unless otherwise stated, and the taxonomic community dissimilarity was calculated by using the Bray-Curtis index among varieties. Principal Co-ordinates Analysis (PCoA) of microbial communities was carried out by using the APE package in R [32]. Taxonomic community composition was compared between the mutant and CK by using ANOSIM [33]. Differences in observed parameters between the mutant and CK were tested by one-way ANOVA with the software package SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The phylogenetic trees were constructed by using the Maximum Likelihood method based on the Kimura 2-parameter model, with the nucleotide sequences of the 30 most abundant species.

To investigate molecular-level functional traits, a PICRUSt (https://picrust.github.com) was performed for the bacterial community [34]. 16S rRNA gene abundance levels were normalized against the known gene copy number and function predictions were performed based on the OTU table with PICRUSt. Functional predictions were categorized into enzyme and statistical analysis was performed using the SPSS 18.0 software package (SPSS 18.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Plant Growth and Soil Physicochemical Fluctuation

The results showed that FT exhibited fast growth, and had a higher dry matter and nutrient accumulation. In terms of biomass, leaf dry weight increased 24.07% in FT, stem increased 74.68% and root increased 74.62% (Table 1). Similar to biomass, chlorophyll a, chlorophyll b and total chlorophyll concentration were higher in FT than CK (the control plant) ($P < 0.05$). As the main nutritional component of animal feed, crude protein becomes the most concerned objective of feed crop breeders. Crude protein concentration in FT was found to be higher than that in the control, increasing by 12.68% in shoot and 25.53% in the root.

The growth of paper mulberry greatly consumed soil

nutrients and reduced soil pH and electrical conductivity. Both for FT and CK, the soil physicochemical characteristics were much lower than that before paper mulberry cultivation. However, except for pH and Olsen-P, the soil physicochemical characteristics for FT were lower than those for CK (Table 2, $P < 0.05$). Notably, soil microbial C was lower for FT than that for CK (Figure 1), with a similar pattern of the soil physicochemical characteristics.

Table 1. *Plant growth, Chlorophyll concentration, and matter accumulation between the two varieties of Paper mulberry.*

	Biomass (g)			Chlorophyll (mg/g)			Crude protein (mg/g)	
	Leaf	Stem	Root	a	b	a+b	Shoot	Root
CK	2.41	0.79	1.30	1.17	0.24	1.41	160.44	124.44
FT	2.99	1.38	2.27	1.28	0.34	1.63	180.78	156.21
Sig	*	*	**	*	***	**	*	**

Notes: Sig., significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 2. *The physicochemical properties of the mixtures after paper mulberry cultivation.*

	pH	Electrical conductivity （mS/cm)	Organic carbon (g/kg)	Alkaline nitrogen (mg/kg)	Olsen-P (mg/kg)
CK	3.85±0.02	15.51±0.10	37.57±1.42	105.03±2.50	168.20±13.34
FT	3.76±0.04	14.57±0.11	32.36±1.16	76.30±8.47	179.89±9.17
Sig.	ns	**	*	*	ns

Notes: Sig., significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2. Sequencing Results and Microbial Diversity Indices

After quality control, removal of chimeras and nontargeted sequences, 351 986 bacterial sequences (ranging from 43 804~69 289 sequences per sample), and 380 943 fungal sequences (ranging from 49 172~73 122 sequences per sample) were used for further analysis. A total of 1 996 bacterial operational taxonomic units (OTUs) and 1 924 fungal OTUs were identified in rhizosphere soils of Paper mulberry at a cut-off of 97% sequence similarity. The fungal OTUs detected in rhizosphere soil were more abundant in CK than FT ($P = 0.022$), but the difference was not significant for bacteria ($P = 0.225$). Rarefaction waves showed that the number of OTUs increased with the number of sequences obtained in each of the soil samples, and the curves already reached a plateau at 97% similarity level (Figure 2), suggesting that the sequencing was deep enough to cover the microbial community in the rhizosphere soil of Paper mulberry.

For bacteria, Chao1 at the dissimilarity of 0.03 was higher in the rhizosphere soil of CK than FT, but the Shannon index and ACE showed no significant difference. However, the Shannon index, ACE and Chao1 were all higher in the rhizosphere soil of CK than FT for the fungal community (Table 3). Overall, the fungal community alpha diversity was significantly lower in the rhizosphere soils of FT, but the difference for bacteria was not significant. Bacterial community dissimilarity was significantly lower in the rhizosphere soils of FT (Figure 3C, $P = 0.042$), and the fungal community dissimilarity showed the same trend (Figure 4C, $P = 0.029$), which indicated that both bacterial and fungal community in rhizosphere soils of FT were more similar in composition.

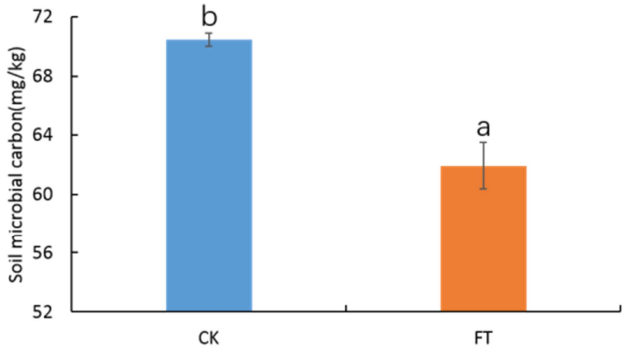

Figure 1. *Soil microbial carbon concentration after paper mulberry cultivation.*

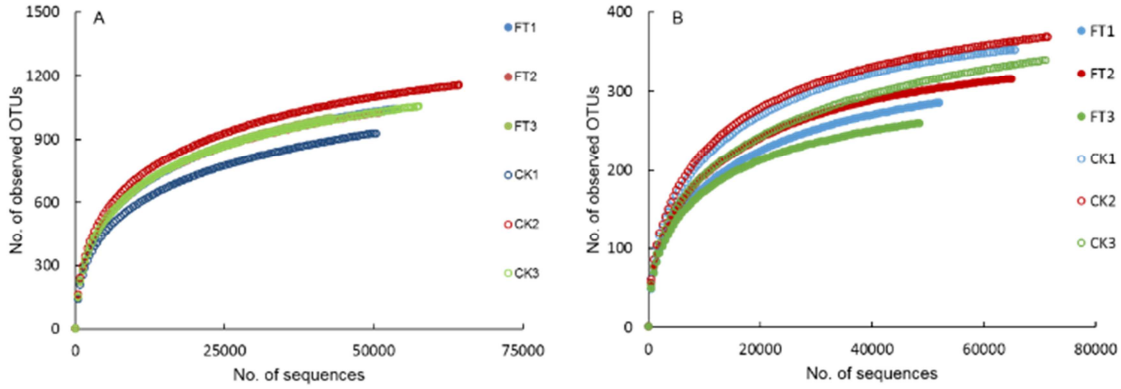

Figure 2. *Rarefaction curves of bacteria (A) and fungi (B) associated with the rhizospheric soil of Paper mulberry depicting the effects of 3% dissimilarity on the number of OTUs identified. Hollow circles, the control; Solid circles, mutant with high protein concentration.*

Table 3. *Microbial diversity indexes in the rhizosphere soils of Paper mulberry.*

		OTUs	Shannon	Ace	Chao1
Bacteria	CK	343.33±13.57	4.16±0.10	376.60±10.54	379.87±11.18*
	FT	322.00±6.08	4.23±0.01	351.72±3.45	346.58±5.59
Fungi	CK	354.00±8.39*	3.05±0.11*	395.99±6.82*	385.59±6.13*
	FT	287.33±16.18	2.76±0.13	322.29±17.94	325.47±19.74

Notes: *, $P < 0.05$.

3.3. Microbial Community in the Rhizosphere of the Paper Mulberry

PCoA based on the relative abundance of OTUs separated microbial community in the rhizosphere soils of FT from CK at the first axis (Figure 3A and Figure 4A). The hierarchical clustering tree on the OTU level further strengthened the difference of microbial community between FT and the control plants (Figure 3B and Figure 4B). By classifying all OTUs into taxonomic groups, we identified 20 bacterial phyla and 7 fungal phyla associated with rhizosphere soils (Table 4). The dominant bacterial phyla in both FT and CK were Actinobacteria, Proteobacteria, Chloroflexi, Patescibacteria, Bacteroidetes, Acidobacteria, and Planctomycetes. By the T-test, Gemmatimonadetes and Patescibacteria were found to be less abundant (P = 0.02, 0.006, respectively), but Acidobacteria more abundant in FT, compared to the control plants (P = 0.026). On the other side, the dominant fungal phyla were Ascomycota, Basidiomycota, and Mortierellomycota. Basidiomycota and Rozellomycota were more abundant in FT than the control plants (P = 0.046, 0.011, respectively).

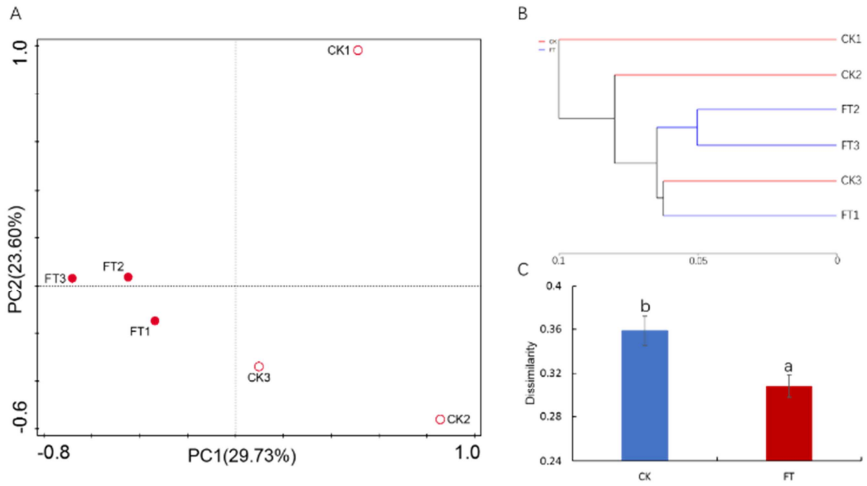

Figure 3. *The structure of microbial community for bacteria associated with rhizospheric soil of Paper mulberry. A, Principal coordinate analyse (PCoA) on OTU level; B, Hierarchical clustering on OTU level; C, Comparison on average taxonomic dissimilarity (Bray-Curtis) of bacterial communities.*

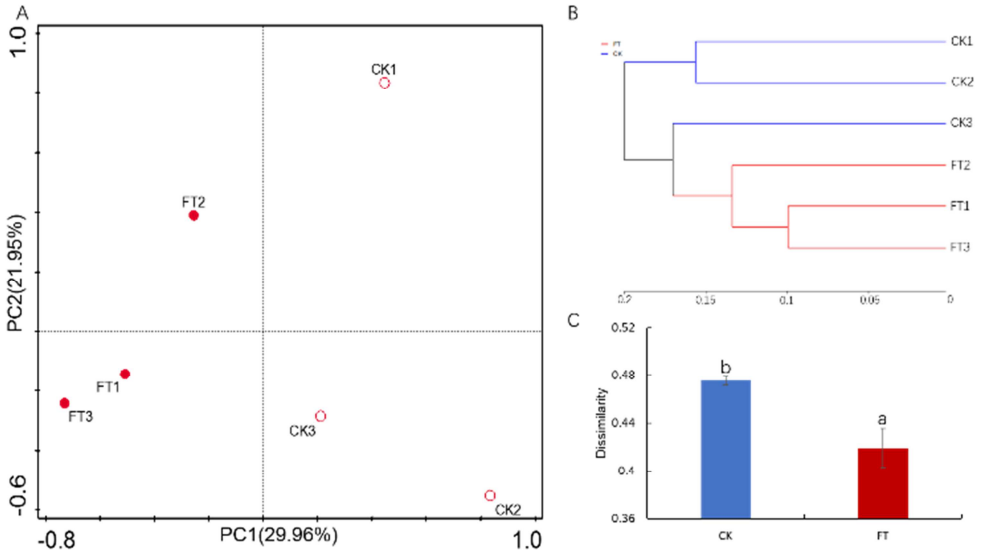

Figure 4. *The structure of microbial community for fungi associated with rhizospheric soil of Paper mulberry. A, Principal coordinate analyse (PCoA) on OTU level; B, Hierarchical clustering on OTU level; C, Comparison on average taxonomic dissimilarity (Bray-Curtis) of bacterial communities.*

Table 4. *Microbial compositions of FT and the control plant at the phylum level. Datas show the average percentage of phylum proportion in the rhizosphere soil of paper mulberry.*

	Phylum	Rhizosphere soil (%)		
		FT	CK	Sig.
Bacteria	Thaumarchaeota	0.12460	0.12774	NS
	FBP	0.06191	0.05564	NS
	WPS-2	0.13871	0.09874	NS
	BRC1	0	0.00392	NS
	Firmicutes	1.02504	0.62458	NS
	Entotheonellaeota	0	0.00157	NS
	Cyanobacteria	0.47177	0.36597	NS
	Rokubacteria	0	0.00313	NS
	Actinobacteria	46.03425	34.26903	NS
	Verrucomicrobia	0.14184	0.11755	NS
	Dependentiae	0.15987	0.16771	NS
	Gemmatimonadetes	0.65436 ↓	1.02817	*
	Bacteroidetes	1.63473	4.22162	NS
	Acidobacteria	2.52576 ↑	1.80792	*
	Planctomycetes	1.44744	2.22248	NS
	Chloroflexi	9.60699	6.76306	NS
	Proteobacteria	31.90392	40.68101	NS
	Nitrospirae	0.02429	0.02429	NS
	Armatimonadetes	0.02508	0.02038	NS
	Patescibacteria	3.47087 ↓	6.99894	**
	unclassified Bacteria	0.54857	0.39654	NS
Fungi	Ascomycota	76.39375	79.91581	NS
	Basidiomycota	12.82545↑	7.43539	*
	Chytridiomycota	0.03588	0.73008	NS
	Entorrhizomycota	0.00138	0	NS
	GS19	0.00138	0	NS
	Mortierellomycota	0.24980	0.24290	NS
	Rozellomycota	0.00759↑	0.00069	*
	unclassified Fungi	10.48477	11.67512	NS

Note: FT, the mutant plants; CK, the control plants; Sig., significance; **, $P < 0.01$; *, $P < 0.05$; NS, $P > 0.05$.

The 30 most abundant genus of the bacterial and the fungal community was selected to construct the phylogenetic tree respectively, and the abundance of the selected genus was compared between FT and CK. The results showed that the 30 most abundant bacterial genus belonged to just 5 phyla (Bacteroidetes, Actinobacteria, Proteobacteria, Patescibacteria, and Chloroflexi), and Streptomyces was found to be more abundant (P = 0.002), but norank_Chitinophagaceae less abundant in FT compared to the control plants (P = 0.02) (Figure 5). On the other side, the 30 most abundant fungal genus belonged to 4 phyla (Ascomycota, Mortierellomycota, Basidiomycota, and Chytridiomycota), and *Trichoderma*, *Fusarium*, *Cladophialophora*, and *Knufia* were all found to be less abundant in FT (P = 0.021, 0.003, 0.049, and P <0.001, respectively, Figure 6).

To further study the effects of environmental factors on the microbial community, Pearson correlation analysis was performed between soil physicochemical characteristics and microbial composition at the genus level. Alkaline nitrogen was found to be the most significant factor affecting soil microbial community, followed by electrical conductivity, organic carbon, and pH, but Olsen-P showed no significant effect. Alkaline nitrogen was found to negatively correlate with Streptomyces (P = 0.015), positive with Trichoderma, Fusarium, Cladophialophora, and Knufia (P = 0.015, 0.025, 0.036 and 0.015, respectively, Table 5). Streptomyces exhibited to negatively correlate with most of the soil physicochemical characteristics, and Cladophialophora showed the opposite trend.

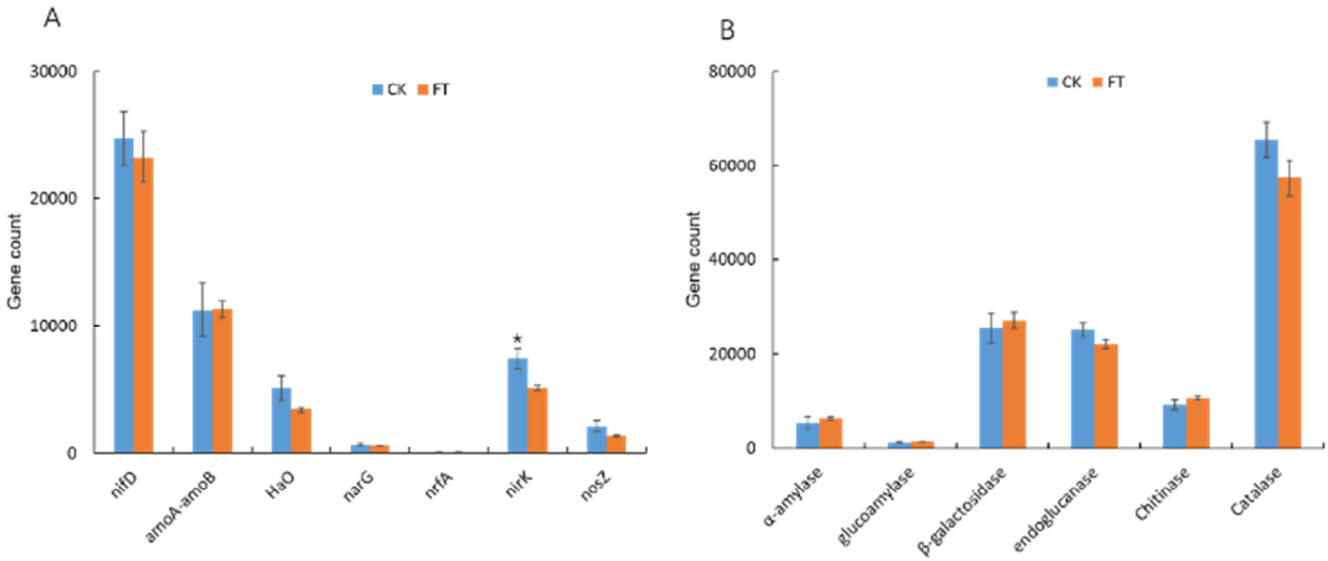

Figure 5. *Phylogenetic tree on the 30 most abundant genus for bacterial community. *, P < 0.05; **, P < 0.01.*

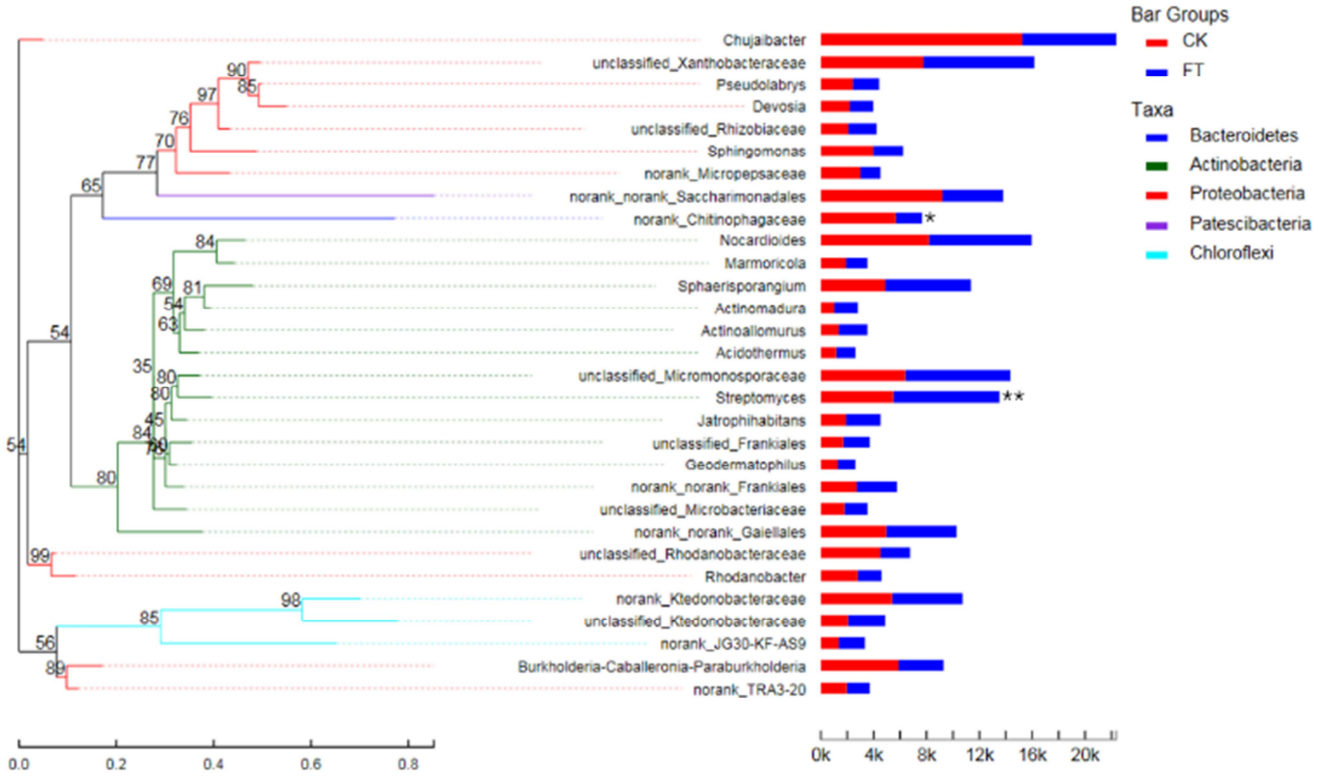

Figure 6. *Phylogenetic tree on the 30 most abundant genus for fungal community. *, P < 0.05; **, P < 0.01; ***, P ＜ 0.001.*

3.4. Rhizosphere Bacterial Metabolic Profile Prediction

Using 16S rRNA gene profiling information, PICRUSt [34] predicted the abundance of C and N functional genes and found some differences between the rhizosphere soil of FT and the control plants. Analysis of the most abundant N metabolism pathway genes and carbon degrading functional genes revealed that there was a significant decrease in the abundance of *nirK* involved in denitrification in the rhizosphere of FT, compared to the control plants ($P = 0.047$, Figure 7).

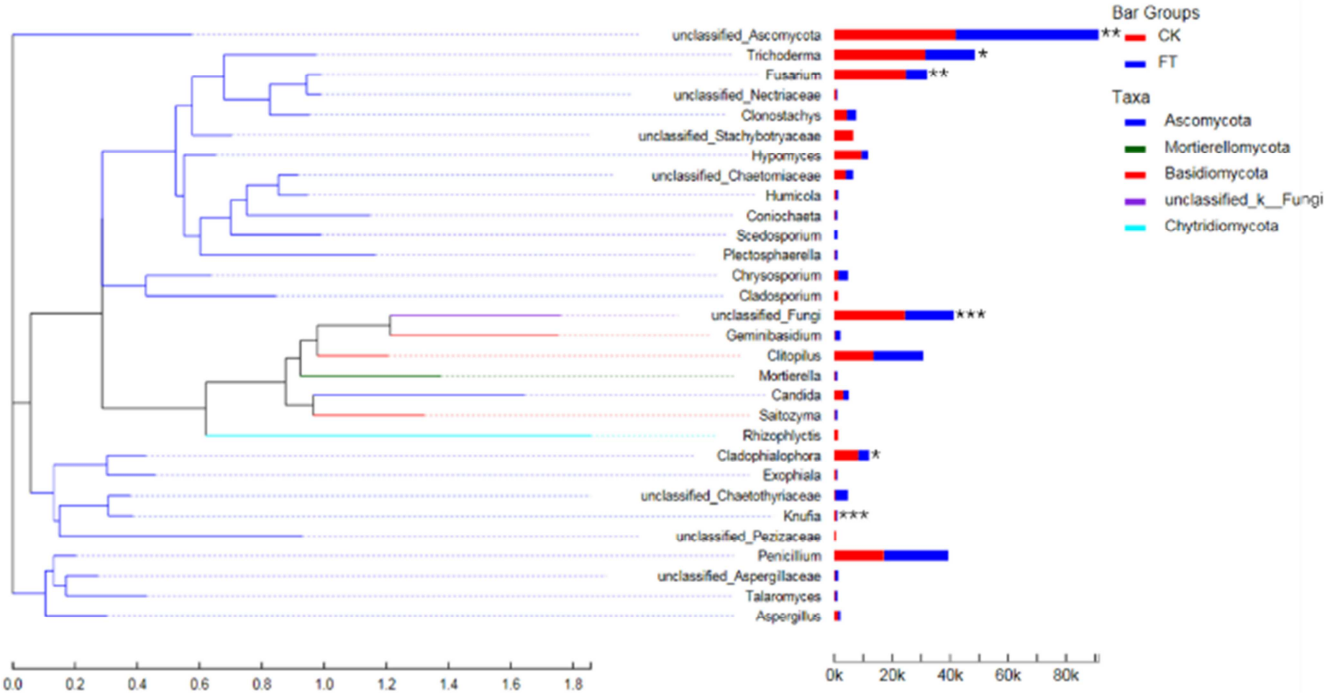

Figure 7. *Gene counts of the most abundant PICRUSt predicted N cycling genes (A) and C degrading genes (in order of complexity from labile to recalcitrant) (B).*

Table 5. *Correlation coefficients for relationships between soil characteristics and relative abundances of the genus in the rhizosphere of FT and the control plants.*

	Genus	Electrical conductivity	Organic Carbon	pH	Alkaline nitrogen	Olsen-P
Bacteria	*Streptomyces*	-0.878*	-0.814*	-0.599[a]	-0.897*	0.175[a]
	Norank Chitinophagaceae	0.725[a]	0.526[a]	0.574[a]	0.787[a]	-0.582[a]
	Unclassified Ascomycota	-0.280[a]	-0.017[a]	-0.133[a]	-0.239[a]	0.475[a]
Fungi	*Trichoderma*	0.743[a]	0.655[a]	0.696[a]	0.893*	-0.589[a]
	Unclassified Fungi	0.608[a]	0.410[a]	0.365[a]	0.583[a]	-0.375[a]
	Fusarium	0.899*	0.793[a]	0.776[a]	0.868*	-0.487[a]
	Cladophialophora	0.852*	0.884*	0.921*	0.841*	-0.460[a]
	Knufia	0.925**	0.837*	0.719[a]	0.899*	-0.323[a]

Notes: a, not significant; *, $P = 0.05$ significance; **, $P = 0.01$ significance.

4. Discussion

Surprisingly, after a short term of 45 days' cultivation, the soil in the rhizosphere of Paper mulberry differed significantly in physicochemical properties ($P < 0.05$) compared with those of the initial soil sample, and also the soil properties showed some difference between the two varieties with different nitrogen absorption and utilization efficiency. Correspondingly, soil microbial biomass and microbial composition also exhibited a significant difference between these two varieties. Via the Pearson correlation analysis, we further constructed the relationships between the microbial composition at the genus level and the soil physicochemical properties, and found that alkaline nitrogen was the most significant factor affecting soil microbial community.

Soil nutrients were significantly changed after Paper mulberry cultivation, and the FT plants exhibited stronger nitrogen preference compared to the control plants. Soil physicochemical indexes reduced significantly compared to the initial soil sample, including organic carbon, alkaline nitrogen, and Olsen-P, due to the consumption of Paper mulberry cultivation. Moreover, despite originating from the same soil, the nutrient status of the soil in the rhizosphere of the FT and the control plants differed significantly from each other after 45 days' cultivation (Table 2), especially for alkaline nitrogen and organic carbon. Nitrogen plays a key constituent of nucleic, protein, chlorophyll, alkaloids, vitamins, and hormones [35], is recognized as an essential macronutrient for plants. N acquisition is closely related to photosynthesis and, therefore, to the yield of crops [36]. In this study, the FT plants acquired a large amount of nitrogen from the soil, and converted to biomacromolecule (e.g. lipoproteins and glycoprotein) [37], exhibited a higher concentration of chlorophyll in leaves, and higher biomass (Table 1). Enhanced nitrogen acquisition from soil strengthened plant photosynthetic capacity and improved dry matter accumulation.

Using high-throughput MiSeq sequencing, the rhizosphere microbiomes of the two Paper mulberry varieties with different nitrogen absorption and utilization efficiency were determined. The α-diversity indexes for each sample shown in Table 3 indicated that the FT had a significantly lower fungal diversity (as shown by the lower ACE indices, Shannon index, and Chao1) than the control, however, just Chao1 showed a significant difference for the bacterial community. The principal coordinate analysis (PCoA), the Hierarchical clustering, and taxonomic dissimilarity (Figure 3 and Figure 4) revealed results consistent with those in Table 3, which suggested that the FT and the control plants had significantly different impacts on the composition of the rhizosphere microbiome, and the different impacts appeared more obvious with fungi. Environmental variables (e. g. C/N ratio) that regulate the soil microbiome may firstly affect the fungal composition and have consequences for microbial interactions, which favors soil nutrient cycling [38]. This may explain the different responses of bacteria and fungi to the change of soil physicochemical properties after the short term of the Paper mulberry cultivation (45 days). In the rhizosphere soils, the microbial community turned out to be more similar in the FT plants (Figure 3C and Figure 4C), which was an indication of biotic homogenization [39].

Soil microbial carbon showed significantly lower in the rhizosphere of the FT, compared to the control plants, suggested that soil nitrogen deficiency inhibited soil microbial growth, which was in accordance with the finding that microbiome alleviated nitrogen deficiency by self-feeding under the condition of extreme nitrogen deficiency [40]. The bacterial and fungal community composition at the phylum (Table 4) and the genus levels (Figure 5 and Figure 6) were furtherly determined in the rhizosphere soils of Paper mulberry, and the abundances in the FT and the control plants were compared. The microbial groups differed significantly in the rhizosphere soil of Paper mulberry with different nitrogen absorption and utilization efficiency mainly focused on a few phylum and genus, indicated that the effects of differentiated nitrogen consumption on the soil microbial community showed a strong selectivity, and this is a mode by which host plant nutrient preference shaped special microbiome in the rhizosphere. At the genus level, *Streptomyces* and norank_Chitinophagaceae from the bacterial community, and *Trichoderma*, *Fusarium*, *Cladophialophora*, and *Knufia* from the fungal community showed a significant difference between the FT and the control plants, however, the mechanism by which plant roots select specific microbes to assemble in the rhizosphere has not been revealed. Paper mulberry nitrogen preference reduced the microbial growth and changed the bacterial and the fungal composition in the rhizosphere soils.

Ai et al. [14] suggested that the structure and assembly of the rhizosphere microbiome also depended on other cues, more than the root exudates which were considered key determinants in several studies [13, 41]. However, Ai et al. [14] did not provide further direct evidence in support of this viewpoint. Based on a five-season continuous pot experiment with two plant species, tomato and cucumber, Cai et al. [42] preliminary confirmed that the plant nutrient preference played a key driver in the development of a plant-specific microbiome. The results from the Pearson correlation analysis showed that most of the significant correlations between soil physicochemical properties and microbial genus involved alkaline nitrogen that differed significantly between the two Paper mulberry varieties (Table 5). Also, via function prediction of the bacterial community, *nirK* involving in nitrogen conversion was found to be lower abundant in the rhizosphere soil of the FT, compared to the control plants (Figure 6). Hence, our study provided further evidence for the role of the nutrient preferences in shaping the rhizosphere microbiome.

5. Conclusion

In conclusion, the results obtained from the present study suggested that (i) soil microbial growth and microbial composition significantly differed between the two Paper mulberry varieties with different nitrogen absorption and utilization efficiency; (ii) alkaline nitrogen was identified to be the most significant factor affecting soil microbial community in the rhizosphere soil. Moreover, the effects of excessive consumption of soil nutrient during Paper mulberry cultivation on the microbiome was revealed, and it could be employed in field water and fertilizer management of Paper mulberry planting.

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