

## Article

# Effects of Nitrogen Application Rate on Rhizosphere Microbial Diversity in Oilseed Rape (*Brassica napus* L.)

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**Abstract:** Nitrogen is one of the essential nutrients for rape growth and development, of which the demand is large. In order to reveal the response of rhizosphere microbial diversity on oilseed rape to the nitrogen fertilizer, four nitrogen application rates of N (170 kgN/hm<sup>2</sup>), N50% (85 kgN/hm<sup>2</sup>), N70% (119 kgN/hm<sup>2</sup>) and N150% (255 kgN/hm<sup>2</sup>) were set. The diversity and community structure of soil bacteria and fungi in seedling, flowering and mature stages of oilseed rape were analyzed based on the high-throughput sequencing technology. The results of rhizosphere soil microbial analysis showed that the dominant bacteria phyla were *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes* and *Cyanobacteria*. The dominant fungi phyla were *Ascomycota*, *Olpidiomycota* and *Basidiomycota*. NMDS analysis showed that the community structure of soil bacteria and fungi changed significantly under nitrogen treatment. Cluster analysis showed that the bacteria at seedling and flowering stage had little effect under the condition of less nitrogen application, while the fungi had little effect on the rhizosphere soil microbial flora at flowering stage. At seedling stage, the diversity and richness of bacterial community in the rhizosphere of oilseed rape were lower under low nitrogen application (85 kgN/hm<sup>2</sup>). Bacteria and fungi in the rhizosphere soil of flowering oilseed rape maintained a higher community diversity under the condition of high nitrogen (255 kgN/hm<sup>2</sup>). The diversity of rhizosphere bacterial community was higher under conventional N application than under other N application.

**Keywords:** *Brassica napus* L.; nitrogen application; rhizosphere; soil microbial community



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## 1. Introduction

As an important oil crop, oilseed rape is widely planted in the world. It is not only an important source of edible oil, but also an important raw material of biomass energy. It was found that incorporating oilseed rape into the rotation system could significantly increase the yield of subsequent crops [1,2]. Oilseed rape planting is highly dependent on fertilizer, and proper application of nitrogen, phosphorus, and potassium fertilizer can significantly improve the yield components of winter oilseed rape to achieve yield increase, among which nitrogen has the most comprehensive and significant effect [3,4]. In the case of oilseed rape planting without nitrogen fertilizer, the yield of direct seeding winter oilseed rape decreased by 78.7% and that of transplanting winter oilseed rape decreased by 66.7%, and direct seeding winter oilseed rape was more sensitive to nitrogen deficiency [3]. Compared with normal fertilization, nitrogen, phosphorus and potassium deficiency in direct seeding of winter oilseed rape decreased seed yield by 61%, 38.3% and 14.4%, respectively [4]. Among the N, P, and K nutrients, the absolute yield reduction of winter oilseed rape caused by N deficiency was the highest. Nitrogen is mainly absorbed by inorganic salt ions and a small amount of amino acids, polypeptides, or proteins from the soil solution by the root hairs in the mature area of root tips [5,6] and transported

to the aboveground. Adequate nitrogen nutrition is very important to maintain plant photosynthesis and growth. Nitrogen is one of the essential nutrients for the growth and development of oilseed rape with a large demand [7], and winter oilseed rape has a high demand for nitrogen [8,9].

Oilseed rape is a crop with large nitrogen demand, and sufficient nitrogen supply can effectively promote photosynthesis of oilseed rape, increase dry matter accumulation and nitrogen absorption of plant, and increase the number of branches and pod so as to improve grain yield, which is an important guarantee for high and stable yield of oilseed rape [8,10,11]. Nitrogen uptake in oilseed rape is mainly at the pre-anthesis and flowering stage, and the amount of nitrogen uptake after anthesis is significantly reduced. During pod development, 73% of nitrogen comes from the transfer of leaf, stem and root system [12]. Studies have shown that the yield of oilseed rape is closely related to the rate of material accumulation from seedling stage to pre-flowering stage [13,14]. In the latter stage of oilseed rape, the leaf is defoliated, and the yield and harvest index of two oilseed rape varieties with different nitrogen efficiency types are significantly reduced [15]. It is concluded that nitrogen accumulation before anthesis and nitrogen transfer after anthesis are of great significance to the formation of oilseed rape yield. Li et al. [16] applied nitrogen fertilizer in different growth stages of oilseed rape, and found that the application of nitrogen fertilizer before sowing had the most obvious effect on oilseed rape yield increase. Fatty acid composition of oilseed rape is mainly determined by the characteristics of varieties, but also affected by nitrogen application to a certain extent [17,18].

Soil microorganisms are an important factor affecting soil ecological processes, closely related to soil nutrient cycling, decomposition of organic matter, and transformation of carbon, nitrogen and other elements [19–21], and play an important role in maintaining soil quality and ecosystem stability. Soil nitrogen mineralization is affected by soil temperature, moisture, soil properties (microorganisms, soil pH, aeration, etc.) [22–24]. Microorganisms as the most active components of soil ecosystem, play an important role in plant growth and development and community succession. Studies have shown that soil microorganisms can interact with the roots of crops, and changes in their quantity and species directly affect plant growth and soil quality, while plant species and planting methods also have a stable impact on the structure and function of soil microbial community [25–27]. At the same time, soil microbial community structure is an important indicator reflecting soil fertility and health status [28–30]. Therefore, soil microbial community determines and promotes the material circulation and energy flow of farmland soil ecosystem. The functional diversity of soil microorganisms, the range of functions that soil microbial communities can perform and the implementation process of these functions are closely related to soil functions, and are of great significance to soil ecological functions and natural element circulation.

Rhizosphere is the closest surface between plant and soil, and it is also the place where plant roots, soil, microorganisms and environment interact. The change of rhizosphere microbial community structure and functional diversity resulted in the decrease of beneficial microorganisms and the increase of fungal diseases, leading to crop yield reduction [31]. Crop growth period is also one of the factors affecting the abundance of key microbial functional genes in soil [32,33]. Nutrient requirements and different types of root exudates at different growth stages are the important reasons for the difference in microbial abundance. Because different crops release different types and quantities of secretions from their roots during the growth process, crop growth will have specific effects on soil microorganisms [34]. Studies have shown that the inoculation of *S.rhizophila*, *R.sphaeroides*, and *B.amyloliquefaciens* in oilseedrape rhizosphere soil can promote ammoniation, promote the conversion of organic nitrogen to inorganic nitrogen, and significantly increase the total nitrogen content of the plant [35]. The addition of rhizosphere growth-promoting bacteria can enhance the ability of plants to obtain nutrients by stimulating root growth, dissolving soil insoluble phosphorus and fixing nitrogen [36,37].

In recent years, studies on functional diversity of microbial communities in oilseed rape rhizosphere soil have found that oilseed rape rhizosphere bacteria can improve the

ability of oilseed rape rhizosphere remediation, promote the degradation of the pollutant phenol, and improve the remediation efficiency of Cd, Pb and Zn contaminated soil [38–41]. However, there are a few studies on functional diversity of microbial communities in the rhizosphere soil of oilseed rape under nitrogen application during cultivation. The study on the diversity of rhizosphere soil microflora of oilseed rape will provide great importance for rational application of nitrogen fertilizer, improvement of rhizosphere microflora composition and promotion of growth and development of oilseed rape.

## 2. Materials and Methods

### 2.1. Materials

The tested oilseed rape was winter oilseed rape XY15 (*Brassica napus* L.), which was provided by Hunan Branch of National Oilseed Crops Improvement Center. The experiment was carried out in Yunyuan Teaching and Experimental Base of Hunan Agricultural University (26°10' N, 119°23' E) from October 2019 to June 2020. The experimental soil was field soil. In the experimental field, rice and oilseed rape were rotated in irrigated and upland fields all the year round. The soil pH was 5.65, total nitrogen 1.98 g/kg, alkali-hydrolyzed nitrogen 127.90 mg/kg, available phosphorus 43.10 mg/kg, available potassium 137.51 mg/kg and organic matter 23.83 g/kg. The fertilizers used in the study are carbamide (N 46%), superphosphate (P<sub>2</sub>O<sub>5</sub> 12%), and muriate (K<sub>2</sub>O 60%).

### 2.2. Test Design

Using potted planting, four treatments were set up, i.e., conventional nitrogen application (170 kgN/hm<sup>2</sup>; N), 50% routine nitrogen application (85 kgN/hm<sup>2</sup>; N50%), 70% conventional nitrogen application (119 kgN/hm<sup>2</sup>; N70%), and high nitrogen fertilization (255 kgN/hm<sup>2</sup>; N150%), 1.96 g, 0.98 g, 1.372 g, and 2.94 g carbamide were applied to each basin. One plant of oilseed rape was planted in each pot, and nine pots were planted in each treatment, a total of 36 pots. Except for the differences in the dosage of nitrogen fertilizer, the use amount of phosphate fertilizer and potassium fertilizer are constant—90 kg P<sub>2</sub>O<sub>5</sub>/hm<sup>2</sup> of phosphate and 120 kg K<sub>2</sub>O /hm<sup>2</sup> of potassium that are evenly applied into the pot as the form of base manure.

The top diameter of the pot is 28 cm, the bottom diameter is 20 cm, and the height is 18 cm. The pot is filled with 6 kg of screened air-dried soil. The bottom of the basin is sealed to prevent water and nutrients from leaching away. The oilseeds were sowed on 11 October, and one robust seedling was transplanted in each pot on 8 November. During oilseed rape growth, transparent baffle was used to block rain, according to the soil moisture, ensuring appropriate watering.

### 2.3. Sampling

The rhizosphere soil was sampled at the seedling stage (90 days after sowing), flowering stage (161 days after sowing) and pod maturity stage (202 days after sowing). Three pots of rhizosphere soil were collected for each stage and each treatment. Only rhizosphere soil was collected. When oilseed rape was pulled out, the soil close to the root surface was gently peeled off with aseptic scissors. The rhizosphere soil was immediately taken back to the laboratory and stored in a refrigerator at 4 °C for the determination of rhizosphere soil microorganisms.

### 2.4. DNA Extraction

Soil DNA was extracted using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manual. Purity and quality of the genomic DNA were checked on 1% agarose gels and a NanoDrop spectrophotometer (Thermo Fisher Scientific: Beijing, China).

### 2.5. PCR Amplification

The V3-4 hypervariable region of bacterial 16S rRNA gene were amplified with the primers 338F(5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') [42]. Fungal ITS region was amplified on Eppendorf Mastercycler Gradient Thermocycler, with the primers ITS1F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS2 (5-TGCGTTCTTCATCGATGC-3) [42]. For each soil sample, 8-digit barcode sequence was added to the 5' end of the forward and reverse primers (provided by Allwegene Company, Beijing, China). The PCR was carried out on a Mastercycler Gradient using 25  $\mu$ L reaction volumes, containing 12.5  $\mu$ L 2 $\times$ Taq PCR Master Mix, 3  $\mu$ L BSA (2ng/ $\mu$ L), 1  $\mu$ L forward primer (5  $\mu$ M), 1  $\mu$ L reverse primer (5  $\mu$ M), 2  $\mu$ L template DNA, and 5.5  $\mu$ L ddH<sub>2</sub>O. Cycling parameters were 95 °C for 5 min, followed by 28 cycles of 95 °C for 45 s, 55 °C for 50 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. The PCR products were purified using a Agencourt AMPure XP Kit.

### 2.6. High Throughput Sequencing

Deep sequencing was performed on Miseq platform at Allwegene Company (Beijing, China). After the run, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

### 2.7. Microbial Community Analysis

The raw data were first screened and sequences were removed from consideration if they were shorter than 230 bp, had a low quality score ( $\leq 20$ ), contained ambiguous bases or did not exactly match to primer sequences and barcode tags, and separated using the sample-specific barcode sequences. Qualified reads were clustered into operational taxonomic units (OTUs) at a similarity level of 97% [43] use Uparse algorithm of Vsearch (v2.7.1) software. For bacteria, the Ribosomal Database Project (RDP) Classifier tool was used to classify all sequences into different taxonomic groups against SILVA128 database [44]. For fungi, the BLAST tool was used to classify all sequences into different taxonomic groups against Unite database.

### 2.8. Statistical Analyses

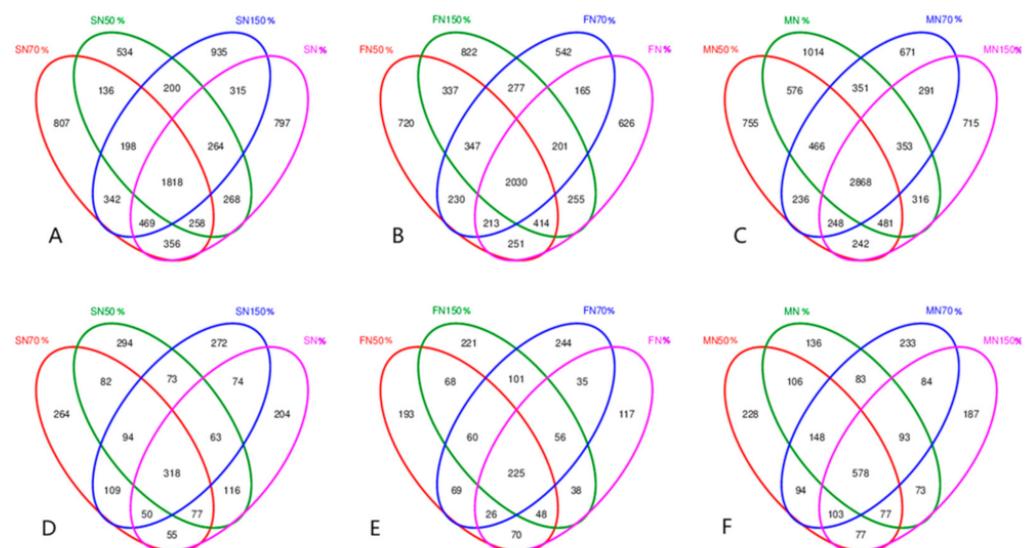
QIIME (v1.8.0) was used to generate rarefaction curves and to calculate the richness and diversity indices based on the OTU information. To compare the membership and structure of communities in different samples, heatmaps were generated with the top 20 OTUs using Mothur [45]. Based on the results of taxonomic annotation and relative abundance, R (v3.6.0) software was used for bar-plot diagram analysis. To examine the similarity between different samples, clustering analyses and PCA were analyzed by R (v3.6.0) based on the OTU information from each sample [46]. The evolution distances between microbial communities from each sample were calculated using Bray Curtis algorithms and represented as an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering tree describing the dissimilarity (1-similarity) between multiple samples [47]. A Newick-formatted tree file was generated through this analysis.

## 3. Results

### 3.1. OTUs Number Analysis of Bacteria and Fungi

It can be seen from Figure 1 that the total number of bacterial OTUs treated with conventional nitrogen (N), 50% conventional nitrogen (N50%), 70% conventional nitrogen (N70%) and high nitrogen fertilizer (N150%) in oilseed rape seedling stage were 4545, 3676, 4384 and 4541, respectively. The total number of fungal OTUs was 957, 1117, 1049 and 1025, respectively. The total number of bacterial OTUs in the four treatments at flowering stage was 4155, 4542, 4005, 4683, and the total number of fungus OTUs was 615, 759, 816, 817, respectively. The total number of bacterial OTUs in the four treatments at maturity stage were 6425, 5872, 5484, 5514, and the total number of fungal OTUs were 1294, 1411, 1416, 1272, respectively. The number of unique bacterial OTUs in the four treatments at

seedling stage accounted for 17.54%, 14.53%, 18.41%, and 20.59% of the total number of each treatment, and the number of fungus OTUs accounted for 21.32%, 26.32%, 25.17% and 26.54% of the total number of each treatment. The number of unique bacterial OTUs in the four treatments at flowering stage accounted for 15.07%, 15.85%, 13.53% and 17.55% of the total number of each treatment, and the number of fungus OTUs accounted for 19.02%, 25.43%, 29.90% and 27.05% of the total number of each treatment. The number of unique bacterial OTUs in the four treatments at maturity stage accounted for 15.78%, 12.86%, 12.24% and 12.97% of the total number of each treatment, and the number of fungus OTUs accounted for 10.51%, 16.16%, 16.45%, and 14.7% of the total number of each treatment. It can be seen that at the seedling stage, the number of bacterial OTUs treated by N50% is the least, while that of fungi is just the opposite. The number of OTUs of bacteria and fungi was the highest under N150% treatment at flowering stage. The number of OTUs of bacteria and fungi with conventional nitrogen application was the highest at the stage of siliques maturity.

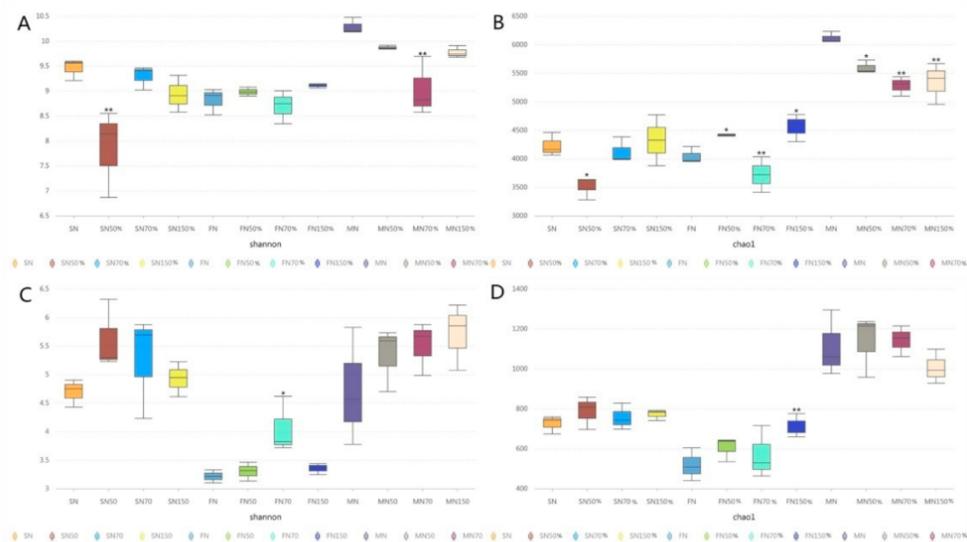


**Figure 1.** Venn diagram based on OTUs of bacteria and fungi in oilseed rape rhizosphere soil. (A): OTUs number of bacteria in Seedling Stage; (B): OTUs number of bacteria in flowering stage; (C): OTUs number of bacteria in maturity stage; (D): OTUs number of fungi in Seedling Stage; (E): OTUs number of fungi in flowering stage; (F): OTUs number of fungi in maturity stage.

### 3.2. Alpha Diversity Index Analysis

The Chao1 index measures the species richness, that is, how many species are there, and the Shannon index is the species diversity. Alpha diversity index of bacteria and fungi in oilseed rape rhizosphere soil is shown in Figure 2. Shannon index and Chao1 index under N50% treatment at seedling stage were significantly lower than those under other treatments, and the diversity and richness of bacteria were the lowest. Fungi Shannon index and Chao1 index under N50% treatment were higher than those under the other three treatments, but the differences were not significant. Nitrogen application at seedling stage had little effect on the diversity of the soil fungal community. At flowering stage, the Shannon index of bacteria under N150% treatment was the highest, while that under N treatment was the lowest, with no significant difference among all treatments. The Shannon index of fungal N70% treatment was the highest, significantly higher than that of the other three treatments. The Chao1 index of bacteria under N150% treatment was significantly higher than that under N and N70% treatment, and the Chao1 index of bacteria under N50% treatment was also significantly higher than that under N70% treatment. The Chao1 index of fungi under N150% treatment was the largest, while that under N treatment was the smallest, and the difference was significant. At maturity stage, the Shannon index and

Chao1 index of bacteria under N treatment were the highest, and the Chao1 index under N treatment was significantly higher than that under the other three treatments. There was no significant difference in Shannon index and Chao1 index among fungal treatments. It can be seen that different nitrogen application levels at seedling stage had little effect on the diversity of soil fungal community, and the diversity and richness of bacterial community in the rhizosphere of oilseed rape were lower under low nitrogen application conditions. Bacteria and fungi in the rhizosphere soil of flowering oilseed rape maintained a higher community diversity under the condition of high nitrogen. The rhizosphere bacterial community diversity under conventional N application was higher than that under other N application, but there was no significant difference in fungal community diversity among different treatments.



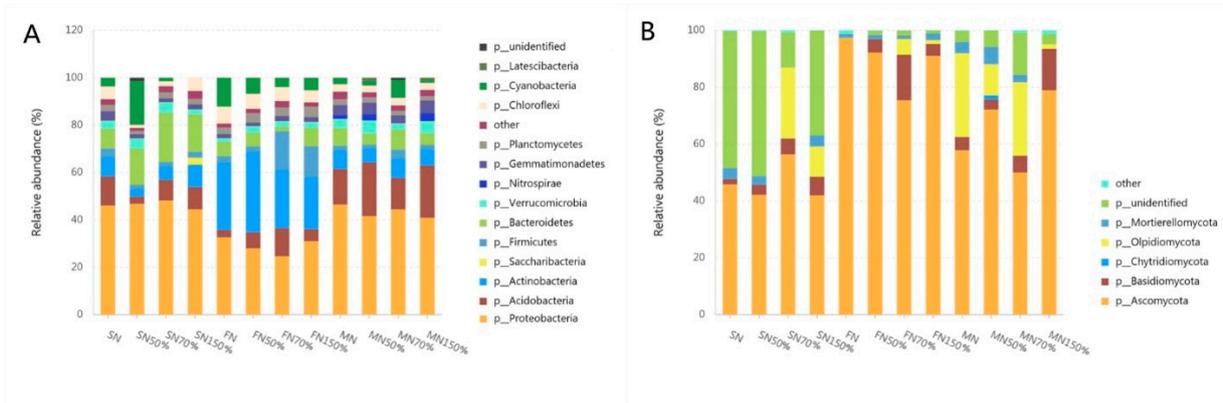
**Figure 2.** Alpha diversity index analysis of rhizosphere soil bacteria and fungi. (A,B) represent Scheme 1 index of bacteria; (C,D) represent Shannon index and Chao1 index; \* and \*\* indicate significant differences at the 0.05 and 0.01 levels, respectively.

### 3.3. Analysis of Microbial Community Structure

#### 3.3.1. Isolation and Analysis of the Total Samples by the Colonization Structure of the Hydrophylum

Classified groups with 1% relative abundance of bacteria and fungi were selected at the phylum level, and the other species were merged into others as shown in Figure 3. Unclassified represents species that have not received taxonomic notes. Relative abundance of bacteria 1% *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Saccharibacteria*, *Nitrospirae*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, *Latescibacteria*, and *Cyanobacteria* accounted for 13 species, accounting for 97.68% of the bacterial community. The dominant phyla were *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, etc., and their relative abundance was all above 5%. *Proteobacteria* had the highest average relative abundance (39.60%), followed by *Actinobacteria* (13.56%), *Acidobacteria* (11.07%), and *Bacteroidetes* (8.89%), respectively. *Proteobacteria* was insensitive under different nitrogen application levels, and the relative abundance of *Proteobacteria* was not significantly different at seedling and mature stages, but decreased only under low nitrogen application at flowering stage. The relative abundance of *Actinobacteria* and *Acidobacteria* was significantly affected by the nitrogen concentration at the seedling stage. The relative abundance of *Actinobacteria* and *Acidobacteria* was also lower with the lower nitrogen application rate. Compared with the normal nitrogen application rate, the relative abundance of *Actinobacteria* and *Acidobacteria* decreased by 5.06% and 9.18%, respectively. The relative abundance of *Actinobacteria* at flowering stage was significantly higher than that at seedling and mature stage, and the relative abundance of N150% was lower than

that at N condition, 6.33%, and 11.81%, respectively, than that at N50%. The relative abundance of *Acidobacteria* was significantly affected by the amount of nitrogen application at the flowering stage, and the relative abundance of N70% increased by 8.71% and 6.85%, respectively, compared with the amount of nitrogen application. The relative abundance of *Bacteroidetes* in seedling stage was higher than that in flowering and mature stage, and there were differences between different nitrogen application levels in seedling stage and flowering stage. The relative abundance of *Cyanobacteria* under the treatment of 50% at seedling stage was significantly higher than that of other nitrogen application levels, the highest at the treatment of N at flowering stage and the highest at the maturity stage.



**Figure 3.** Analysis of difference in colony structure of each sample at phylum level. (A): Bacteria; (B): Fungi.

The species in the fungal community were *Ascomycota*, *Basidiomycota*, *Mortierellomycota*, *Chytridiomycota*, *Olpidiomycota*, the dominant phyla with relative abundance greater than 5% were *Ascomycota*, *Olpidiomycota* and *Basidiomycota*. There was no significant difference in the relative abundance of *Ascomycota* at the seedling stage, but the N treatment had the highest relative abundance at the flowering stage, reaching 97.42%. The relative abundance of *Ascomycota* at maturity stage was the lowest under N70% treatment, which was significantly different from other N treatments. The relative abundance of *Olpidiomycota* under N and N50% treatments was less than 1% at seedling and flowering stage, and only the relative abundance of *Olpidiomycota* under N70% and N150% treatments was detected, and the relative abundance of *Olpidiomycota* under N70% treatment was significantly higher than that under N150%. At maturity stage, the relative abundance of N treatment was the highest (29.49%), followed by N70% (25.88%), N150% (1.63%) and N50% treatment (11%). The relative abundance of *Basidiomycota* at seedling stage was the lowest (1.82%) under N treatment, and the highest (6.53%) under N150% treatment. *Basidiomycota* at flowering stage was the highest (15.98%) under N70% treatment, which was significantly higher than the other three treatments. *Basidiomycota* had the highest relative abundance (14.55%) at maturity stage under high nitrogen treatment, which was significantly higher than the other three treatments.

Under different nitrogen application backgrounds, the significance test and analysis of the differences between dominant phylum treatments in the rhizosphere samples of oilseed rape at seedling, flowering, and mature stages showed (Table 1) that the structure of soil microbial community in the rhizosphere of oilseed rape significantly changed, and there were differences in bacteria and fungi, as well as some differences in the changes under different nitrogen application levels.

**Table 1.** Analysis of the significance of differences between dominant phyla treatments in the rhizosphere samples of oilseed rape at seedling, flowering and mature stages.

Treatment	Sampling	Dominant Phylum of Bacteria				Dominant Phylum of Fungi			
		Proteobacteria	Actinobacteria	Acidobacteria	Bacteroidetes	Cyanobacteria	Ascomycota	Olpidiomycota	Basidiomycota
N	seedling stage	46.05% <sup>a</sup>	8.10% <sup>ab</sup>	12.23% <sup>a</sup>	8.29% <sup>b</sup>	3.73% <sup>b</sup>	45.82% <sup>a</sup>	0.33% <sup>b</sup>	1.82% <sup>b</sup>
N50%		46.75% <sup>a</sup>	3.04% <sup>c</sup>	3.05% <sup>b</sup>	15.32% <sup>a</sup>	18.52% <sup>a</sup>	42.15% <sup>a</sup>	0.21% <sup>b</sup>	3.50% <sup>ab</sup>
N70%		48.20% <sup>a</sup>	5.55% <sup>bc</sup>	8.66% <sup>a</sup>	20.74% <sup>a</sup>	1.50% <sup>b</sup>	56.40% <sup>a</sup>	24.95% <sup>a</sup>	5.53% <sup>a</sup>
N150%		44.47% <sup>a</sup>	9.54% <sup>a</sup>	9.28% <sup>a</sup>	15.81% <sup>a</sup>	0.93% <sup>b</sup>	41.95% <sup>a</sup>	10.68% <sup>ab</sup>	6.53% <sup>a</sup>
N	flowering stage	32.58% <sup>a</sup>	28.30% <sup>ab</sup>	3.15% <sup>c</sup>	5.99% <sup>a</sup>	12.21% <sup>a</sup>	97.42% <sup>a</sup>	0.09% <sup>b</sup>	0.70% <sup>b</sup>
N50%		27.99% <sup>ab</sup>	33.78% <sup>a</sup>	6.76% <sup>b</sup>	5.71% <sup>a</sup>	6.80% <sup>b</sup>	92.16% <sup>b</sup>	0.10% <sup>b</sup>	4.68% <sup>b</sup>
N70%		24.63% <sup>b</sup>	24.51% <sup>bc</sup>	11.86% <sup>a</sup>	1.88% <sup>b</sup>	3.98% <sup>b</sup>	75.43% <sup>c</sup>	5.52% <sup>a</sup>	15.98% <sup>a</sup>
N150%		31.02% <sup>a</sup>	21.97% <sup>c</sup>	5.01% <sup>bc</sup>	7.65% <sup>a</sup>	5.32% <sup>b</sup>	91.02% <sup>b</sup>	1.36% <sup>b</sup>	4.19% <sup>b</sup>
N	maturity stage	46.47% <sup>a</sup>	7.67% <sup>a</sup>	14.95% <sup>ab</sup>	7.36% <sup>a</sup>	2.78% <sup>ab</sup>	57.84% <sup>ab</sup>	29.49% <sup>a</sup>	4.61% <sup>b</sup>
N50%		41.59% <sup>a</sup>	5.57% <sup>b</sup>	22.62% <sup>a</sup>	4.57% <sup>a</sup>	1.92% <sup>b</sup>	72.07% <sup>a</sup>	11.00% <sup>ab</sup>	3.54% <sup>b</sup>
N70%		44.50% <sup>a</sup>	8.01% <sup>a</sup>	13.19% <sup>b</sup>	8.49% <sup>a</sup>	7.50% <sup>a</sup>	49.94% <sup>b</sup>	25.88% <sup>a</sup>	5.92% <sup>b</sup>
N150%		40.92% <sup>a</sup>	6.67% <sup>ab</sup>	22.04% <sup>a</sup>	4.82% <sup>a</sup>	1.16% <sup>b</sup>	78.91% <sup>a</sup>	1.63% <sup>b</sup>	14.55% <sup>a</sup>

Values are means ( $n = 3$ ). Different lowercase letters indicate significant differences among treatments ( $p < 0.05$ ).

### 3.3.2. The Whole Sample Belongs to the Breakdown Structure Analysis of Hydrophylla

Classifications with 1% relative abundance of bacteria and fungi were selected at the genus level, and other species were merged into others as shown in Figure 4. Unclassified represents species that have not received taxonomic notes. There were 45 bacterial genera and 34 fungal genera in species with 1% relative abundance of bacteria. The bacteria are *Pedobacter*, *Sphingomonas*, *Pseudomonas*, *Flavobacterium*, *Oryzihumus*, *Dyella*, *Bryum\_argenteum\_var\_argenteum*, *Massilia*, *Pseudarthrobacter*, and *Bacillus* ten species, were dominant strains, and showed different dominant genera in different periods. At seedling stage, the dominant strains of *Sphingomonas* and *Pseudomonas* maintained a high abundance under N and N150%, and the relative abundance of *Flavobacterium* was the highest under low N treatment. *Pseudarthrobacter* abundance at flowering stage was the highest under N50% treatment and decreased with the increase of N application rate, which was 1.22 and 2.98 times that of N and N150% treatment, respectively. The relative abundance of *Bacillus* was the highest under N70% treatment, which was 1.34 times of that under N150% treatment. Under the condition of N70% at mature stage, the number of dominant bacteria genera was abundant, including 4 species, including *Dyella*, *Massilia*, *Sphingomonas*, and *Pseudarthrobacter*. The dominant fungi in different treatment stages were *Cladosporium*, *Alternaria*, *Emericellopsis*, *Olpidium*, and *Fusarium*. *Alternaria* and *Cladosporium* were the dominant genera at flowering stage. The relative abundance of *Alternaria* increased significantly with the increase of nitrogen application rate, and increased by 29.94%, 27.79% and 31.35%, respectively, compared with that of N50%, N and N70%, while that of *Cladosporium* was the opposite. It was 22.07%, 8.16 and 3.78% lower than that under N50%, N and N70% treatment, respectively. *Olpidium* under N70% treatment maintained a higher relative abundance than other treatments at each stage, *Fusarium* only had a higher relative abundance under N treatment at seedling stage, and *Emericellopsis* had a higher relative abundance under N treatment at both flowering and maturity stage. Different nitrogen application levels significantly affected the rhizosphere soil bacterial and fungal community composition at different growth stages, especially the dominant bacterial genera were significantly different under low nitrogen and high nitrogen conditions, and the difference of fungi was mainly reflected in the flowering stage.

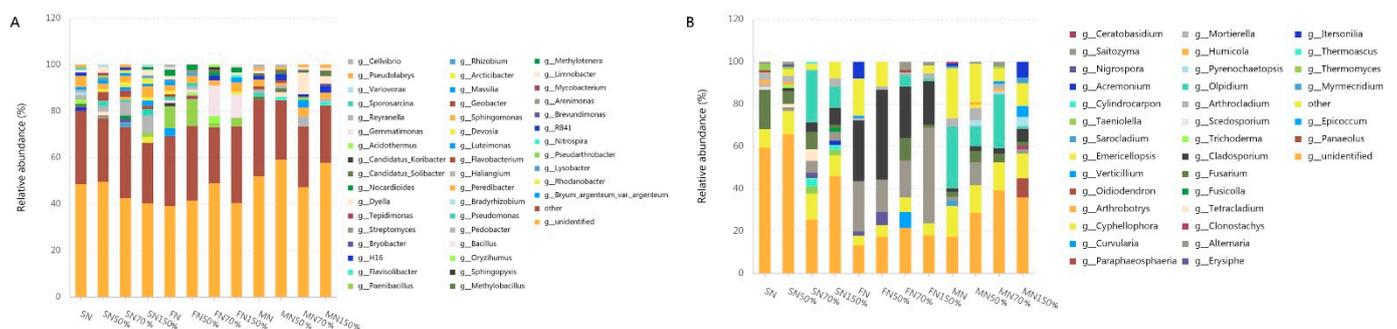
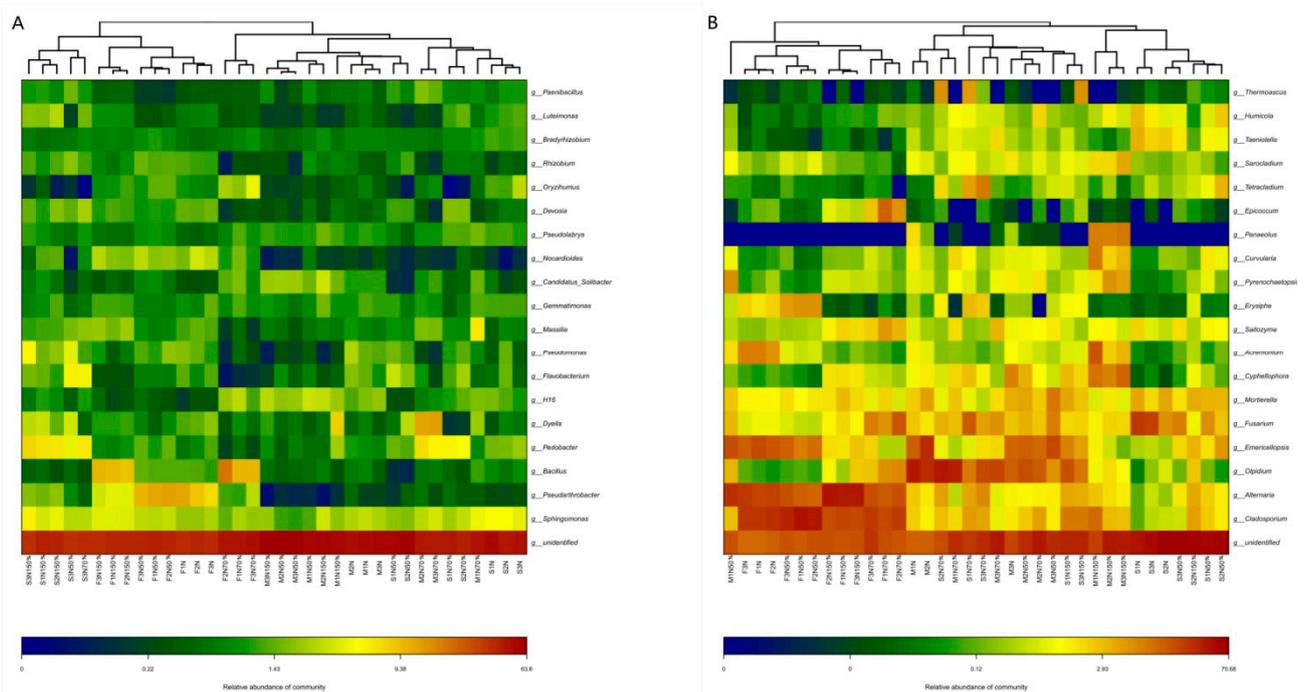


Figure 4. Microflora structure from all samples at the genus level. (A) Bacteria; (B) Fungi.

The results of heatmap and cluster tree analysis (Top20) showed that the dominant bacteria genera of the samples treated with higher nitrogen application rate were grouped into a similar group, and the dominant bacteria genera of the samples treated with higher abundance at seedling and flowering stage were grouped into a group. The dominant bacteria genera of the rhizosphere soil at seedling and flowering stage were more abundant under N150% treatment (Figure 5A). The results of heatmap and cluster tree analysis (Top20) showed that the fungal communities of all samples were basically the same and similar according to the growth period, and they clustered into a group. The dominant bacteria were mainly concentrated at the flowering stage, while the non-dominant bacteria were more abundant in the samples treated with higher nitrogen fertilizer at the mature stage (Figure 5B).



**Figure 5.** Histogram and heatmap from twenty samples at the level of genus. (A) Bacteria; (B) Fungi.

### 3.4. Beta Diversity Analysis between Samples

When the stress of non-metric multidimensional calibration method (NMDS) is less than 0.2, it indicates that the analysis has a certain reliability. The closer the sample is in the coordinate graph, the higher the similarity is. NMDS analysis of bacteria and fungi was obtained based on the Bray-Curtis distance algorithm, and the stress values of the analysis results were all less than 0.2, indicating that the NMDS analysis results were reasonable (Figure 6). PCA analysis showed that the bacterial and fungal community composition of the treated samples were significantly different in different quadrants (Figure 7). Bacterial PC1 and PC2 accounted for 23.99% and 14.49% of the results, and fungal PC1 and PC2 accounted for 25.64% and 15.06% of the results, respectively, which were contributed by growth period factor and nitrogen application rate factor, respectively. There was a positive correlation between N, N50%, N70%, N150% and the first and second principal components of bacteria and fungi at the maturity stage. There was a negative correlation between the four treatments and the first principal component and a positive correlation between the four treatments and the second principal component at the flowering stage. At the seedling stage, N, N50% and N70% of bacteria were positively correlated with the first principal component and negatively correlated with the second principal component, while N150% was negatively correlated with the first and second principal components. At the seedling stage, N, N50% and N150% were positively correlated with the first principal component and negatively correlated with the second principal component, while N70% was positively correlated with the first and second principal components. At flowering stage, N50% and N were negatively correlated with the first and second principal components, while N70% and N150% were negatively correlated with the first principal component and positively correlated with the second principal component. The results indicated that the amount of nitrogen fertilizer had a great influence on the rhizosphere soil microflora types in different growth stages of oilseedrape. Hierarchical cluster analysis of bacteria and fungi showed that bacteria and fungi clustered into two groups according to the growth stage, and each group clustered under different nitrogen application rates (Figure S1). Bacteria at seedling stage and flowering stage had less influence under less nitrogen application, while fungi showed less influence on rhizosphere soil microbial community types at flowering stage.

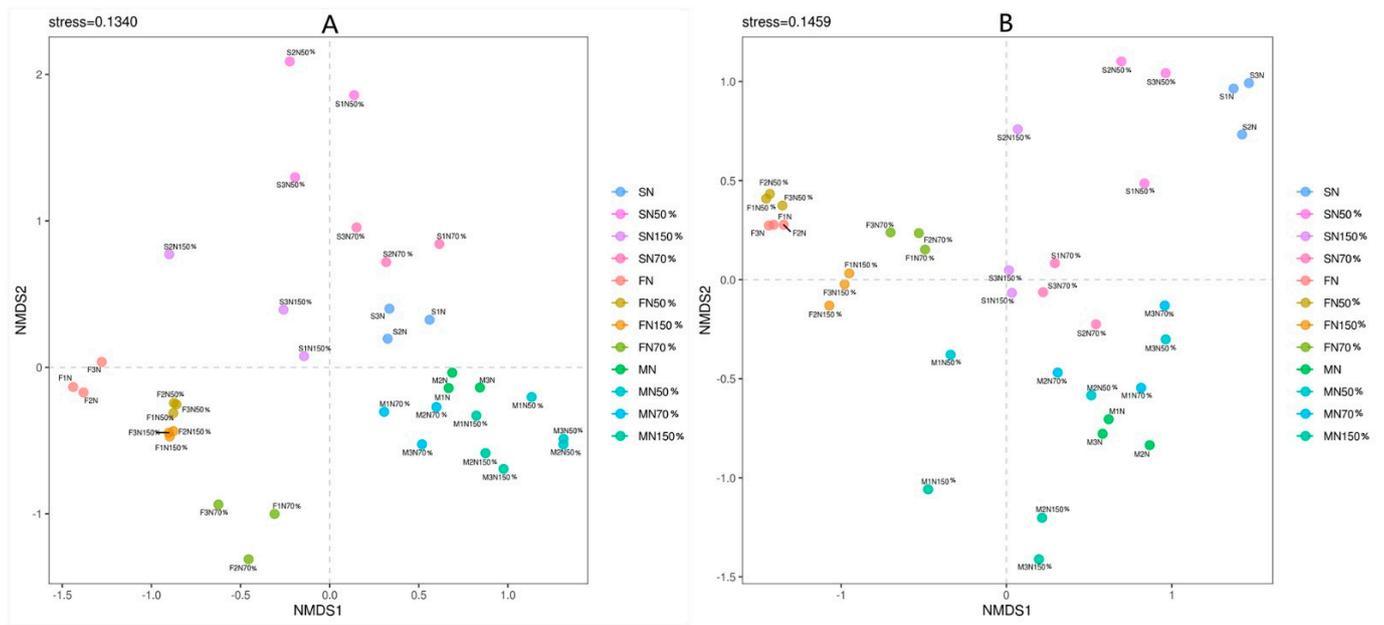


Figure 6. NMDS analysis of all samples. (A) Bacteria; (B) Fungi.

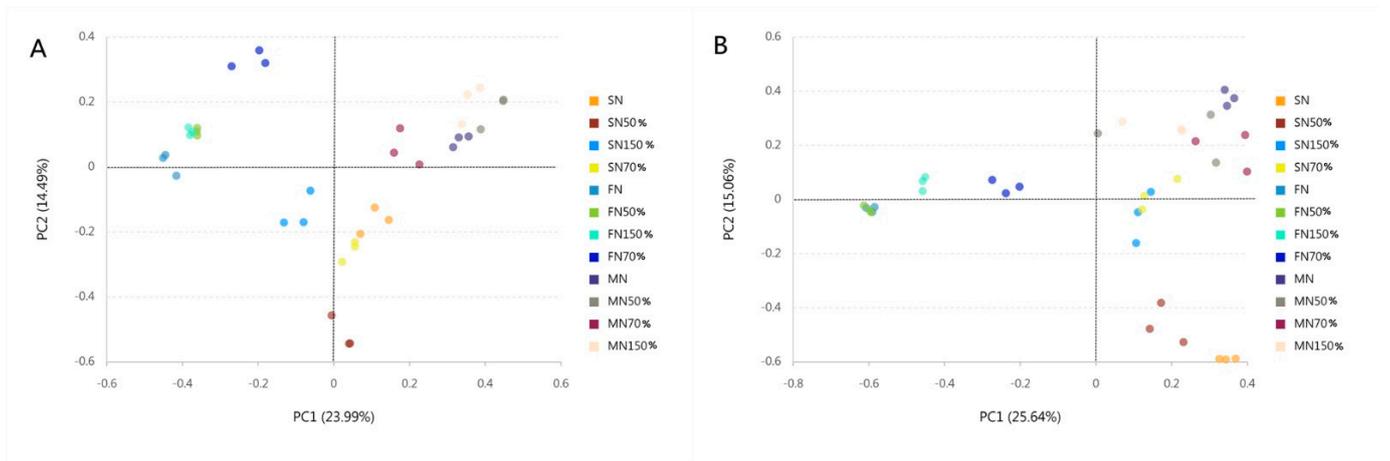


Figure 7. Principal component analysis (PCA) of all samples. (A): Principal component analysis of bacterial; (B): principal component analysis of bacterial fungi.

#### 4. Discussion

##### 4.1. The Growth and Development Stages of Oilseedrape Determine the Composition of Rhizosphere Microbial Community

Plants can control their rhizosphere microbial composition [48], select specific microbial functions according to growth and development needs [49], and support, restrict, or terminate microbial growth and activities [50]. Such interaction exists in both directions, and microorganisms can also adapt to the rhizosphere environment through locomotion, chemotaxis, quorum sensing and remodeling of bacterial community [50]. Rhizosphere microbial communities influence plant nutrition and health [51,52], and that, on the contrary, these communities may be influenced by plant growth stages [53]. For example, *Proteobacteria* had a high relative abundance at different nitrogen application levels at both seedling and maturity stages. However, at flowering stage, the abundance of *Proteobacteria* was significantly lower than that at seedling and maturity stages regardless of high nitrogen application or low nitrogen application. *Actinobacteria* showed the opposite trend. The relative abundance of *Actinobacteria* at flowering stage was significantly higher than that at seedling and maturity stage. The relative abundance of *Ascomycota* was 97.42% at the

flowering stage. Based on the analysis of oilseed rape rhizosphere community under different nitrogen application in three stages, our results were consistent with previous studies, in which oilseed rape growth stage was determined to be an important determinant of rhizosphere microbial composition [53,54].

#### 4.2. The Rhizosphere Microbiome Was Selectively Enriched by Different Nitrogen Application Rates

Rhizosphere microorganisms are the regulators of nutrient transformation and transport between soil and root system. The diversity of soil microbial community reflects the overall dynamic change of the community. Studies have shown that the dominant phyla of soil bacteria include *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, *Firmicutes*, *Verrucomicrobia*, and *Gemmatimonadetes*. The dominant phyla of fungi include *Ascomycota*, *Basidiomycota*, *Basidiomycota*, and *Chytridiomycota* [55]. This study found that *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Verrucomicrobia*, *Ascomycota*, *Basidiomycota*, *Mortierellomycota*, and *Chytridiomycota* were basically consistent with the above studies. However, the rhizosphere of oilseedrape also showed some unique dominant phyla, examples include *Bacteroidetes*, *Saccharibacteria*, *Nitrospirae*, *Planctomycetes*, *Latescibacteria*, *Cyanobacteria*, and *Olpidiomycota*. *Bacteroidetes* and *Planctomycetes* have been reported as the dominant phyla of oilseed rape rhizosphere [56].

Nitrogen application changed the relative abundance of each microbial community. For example, the relative abundance of *Actinobacteria* and *Acidobacteria* at seedling stage under N50% treatment decreased by 5.06% and 9.18% compared with that under N treatment, respectively. The relative abundance of *Acidobacteria* was also significantly affected by the amount of nitrogen application at the flowering stage. Studies have shown that *Acidobacteria* is positively correlated with soil nitrogen content [57], and nitrogen application increases soil nitrogen content, thus increasing the relative abundance of *Acidobacteria*. Fierer et al. [58] believed that when nitrogen in the soil increased, the abundance of some eutrophic bacteria, such as *Proteobacteria*, would increase. In this study, the relative abundance of *Proteobacteria* was lower at seedling and mature stages than that of low nitrogen fertilization, which may be related to the vulnerability and sensitivity of the farmland soil ecosystem, but the specific mechanism needs to be further studied. The relative abundance of *Ascomycota* ranged from 41.95% to 97.42%, and *Ascomycota* occupied the dominant position of soil flora, which was most obvious at flowering stage. This may be related to its strong environmental adaptability. *Cyanobacteria* is a detected dominant phylum of oilseed rape rhizosphere soil microorganisms. *Cyanobacteria* can fix nitrogen [59], increase the availability of phosphorus and release auxin that promotes plant growth [60]. The relative abundance of *Cyanobacteria* in oilseed rape mature stage was the highest under N70% treatment, followed by N treatment. *Cyanobacteria* may be involved in promoting nitrogen supply in fertilizer and nitrogen accumulation in later growth stage of oilseed rape. Furthermore, *Cyanobacteria* can produce H<sub>2</sub> as a by-product of nitrogen fixation [61]. *Cyanobacteria* participates in the metabolism of *Chloroflexi* through a symbiotic way [35]. Therefore, *Chloroflexi* may be related to the nutrient supply capacity of soil and the promotion of plant growth, which is worthy of further study.

#### 4.3. Correlation between Nitrogen Uptake and Utilization Characteristics and Microbial Community in Growth Stage of Oilseed Rape

As an active element, nitrogen in plants is in a dynamic process of constant absorption, assimilation, transport, and distribution during the growth and development stage of crops [62]. Our previous studies found that with the increase of fertilizer application rate, oilseed rape root system preferred to take up nitrogen from fertilizer and could obtain higher nitrogen accumulation. This may be due to the adaptation of roots to the rhizosphere environment after application of nitrogen fertilizer. Host-microbiome interactions had been verified to prove that plant's stage of growth affects the formation of rhizosphere microbes [51,53]. For example, at the seedling stage, the number of bacterial OTUs was the lowest, while the number of fungus OTUs was the highest in N50% treatment. The

number of OTUs of bacteria and fungi was the highest under N150% treatment at flowering stage. The number of OTUs of bacteria and fungi treated by N was the highest at the stage of pod maturity. The results indicated that the increase of nitrogen application rate could maintain the number of microbial community in the later growth stage of oilseed rape. The UPGMA cluster tree also showed that the community composition of soil bacteria and fungi changed significantly with different nitrogen application levels. The results of Shannon index and Chao1 index showed that the bacterial diversity and abundance in N treatment at maturity stage were high, and there was no significant difference in fungal richness at maturity stage, but the abundance in N150% treatment was high.

## 5. Conclusions

The composition of dominant microflora in the rhizosphere of oilseed rape was not affected by the amount of nitrogen application and growth stage, but its relative abundance was related to the amount of nitrogen application, which was different in different growth stages. The bacterial community was more active than the fungal community at the late growth stage, and the community diversity was higher under the condition of high nitrogen fertilization. In the process of oilseed rape cultivation, the application of basal fertilizer should be considered to increase the application rate of nitrogen fertilizer, so as to meet the demand of nitrogen in the later growth period of oilseed rape and maintain a rich rhizosphere microbial community. In addition, we also found *Bacteroidetes*, *Saccharibacteria*, *Nitrospirae*, *Planctomycetes*, *Latescibacteria*, *Cyanobacteria*, and *Olpidiomycota*, and other endemic dominant bacteria phyla of oilseed rape rhizosphere. Whether these dominant fungi affect the nutrient uptake and utilization of oilseed rape roots remains to be further studied. In this study, the distribution difference of microbial community in the rhizosphere soil of oilseed rape was discussed under the background of different nitrogen application rates. The study on the diversity of microbial community in the rhizosphere soil of oilseed rape will provide important guiding significance for rational application of nitrogen fertilizer, improvement of microbial community composition in the rhizosphere of oilseed rape and promotion of growth and development of oilseed rape.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11081539/s1>, Figure S1: Hcluster of all samples. (A): Hierarchical clustering of bacterial; (B): Hierarchical clustering of fungi.

**Author Contributions:** Y.Z., C.G. and M.G. designed the experiment. M.X. and Y.Z. conducted the experiment. C.G. and M.G. helped and provided useful suggestions during the experiment. M.X. and Y.Z. processed and analyzed data and wrote the first draft. C.G. and M.G. revised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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