

## Article

# Response of the Endophytic Microbial Composition in Amaranthus Roots to Different Fertilization Treatments

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**Abstract:** To explore how endophytic microbial compositions in amaranth roots are influenced by various fertilization methods and to determine whether these microbes are associated with amaranthin formation, we conducted an analysis of the endophytic microbial community structure. The roots of amaranth plants subjected to different fertilization treatments—conventional fertilization without potassium (NP), conventional fertilization without phosphorus (NK), conventional fertilization without nitrogen (PK), and balanced fertilization (NPK)—were examined. The results showed that the proportions of *Streptomyces*, *Actinospica*, and *Burkholderia-Caballeronia-Paraburkholderia* in the amaranth roots under the balanced fertilization (NPK) treatment were all greater than those in the amaranth roots under the nitrogen (PK), phosphorus (NK), and potassium (NP) deficiency fertilization treatments. In contrast, the proportions of *Phenyllobacterium*, *Acrocalymma*, *Neocosmospora*, *Fusarium*, *Acidovorax*, *Gibellulopsis*, *Cladosporium*, *Dactylonectria*, and *Gibberella* in the amaranth roots under the nutrient deficiency fertilization (NP, NK, and PK) treatments were higher than those in the amaranth roots under the balanced fertilization treatment. Additionally, a significantly positive correlation was found between *Streptomyces* and the amaranthin content. Furthermore, *Acrocalymma*, *Neocosmospora*, and *Fusarium* exhibited significantly negative correlations with the amaranthin content. The above results suggested that endophytes could easily colonize in amaranth roots as beneficial microorganisms under balanced fertilization conditions. In other words, the balanced fertilization (N, P and K fertilizers are 188.0, 53.0 and 50.0 kg·hm<sup>-2</sup>, respectively) could recruit more beneficial endogenous microorganisms in amaranth roots for improving their growth and quality.

**Keywords:** Amaranth (*Amaranthus tricolor* L.); balanced fertilization; endophytic bacteria; endophytic fungi; Amaranthin



**Citation:** Lin, X.; Yang, D.; Zhou, X.; Wei, X.; Xie, Y.; Yang, S. Response of the Endophytic Microbial Composition in Amaranthus Roots to Different Fertilization Treatments. *Agronomy* **2024**, *14*, 965. <https://doi.org/10.3390/agronomy14050965>

Academic Editor: Jerzy Wielbo

Received: 1 April 2024

Revised: 25 April 2024

Accepted: 1 May 2024

Published: 3 May 2024



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## 1. Introduction

Amaranth (*Amaranthus tricolor* L.) is an annual vegetable renowned for its abundance of carotene, vitamins, and minerals, encompassing a diverse array of plant pigments, such as chlorophyll, amaranthin, and carotenoids [1–4]. Currently, its cultivation is expanding across Asia, North America, and Europe [5]. As an important vegetable crop in agricultural production, amaranth occupies significant portions of the cultivation area and the consumer market. However, with the modernization of agriculture and the widespread use of fertilizers, the issue of excessive fertilization has become increasingly prominent [6,7]. Excessive fertilizer application may lead to problems such as soil and water pollution, as well as impaired plant growth, thereby affecting both the yield and quality of amaranth [8,9]. Therefore, studying an appropriate fertilization scheme to enhance the health and increase the yield of amaranth is of paramount importance.

Endophytic microorganisms are pivotal contributors to plant growth and development because of their sheer abundance and diversity [10,11]. Notably, endophytic bacteria exhibit a repertoire of functions, including phosphorus solubilization, nitrogen fixation,

iron carrier production, the synthesis of antibacterial agents, and the generation of plant growth hormones [12]. Their multifaceted roles include not only serving as facilitators of host plant growth but also actively contributing to nutrient accumulation and enhanced stress resistance, such as in disease, pest, or drought stress [13]. For example, endophytes can establish reciprocal interactions with host plants by exchanging nutrients and producing antibiotics and other substances to protect plants from stressful conditions [14]. The endophytic microbial composition of the plant root system is an important determinant of these symbiotic relationships, which are significantly influenced by soil type, field management (irrigation, fertilizer and pesticide application, etc.), and host genotype [15–18].

The impact of fertilization on endophytic microorganisms is intricate and profound, significantly altering the microbial composition within plant roots [19]. Research indicates that different fertilization methods can induce variations in the structure of endophytic microbial communities, thereby impacting plant growth, development, and stress resistance [20]. For instance, nitrogen, phosphorus, and potassium fertilizers can modify the pH and chemical properties of the soil environment, thus influencing the survival and activity of endophytic microorganisms [21]. Moreover, plant root nutrient secretion under various fertilization conditions can also affect the diversity and abundance of endophytic microorganisms [22]. For example, Pedraza et al. [23] observed that nitrogen fertilization markedly influenced the diversity of endophytic bacteria in plants, with greater microbial community diversity observed with moderate nitrogen fertilization. Similarly, Liu et al. [24] noted that moderate nitrogen fertilization in rice led to an increased diversity and abundance of endophytic bacteria and fungi compared to rice subjected to insufficient or excessive nitrogen inputs. Furthermore, excessive fertilization has been linked to a decreased diversity of endophytic bacteria in wheat roots, along with a reduction in the abundance of potential biocontrol bacteria, such as *Bacillus*, *Streptomyces*, and *Burkholderia*. Additionally, excessive fertilization suppressed antibiotic biosynthesis, exacerbating the occurrence of wheat crown rot [25]. Therefore, investigating the impact of balanced fertilization on the structure of plant endophytic microbial communities is of great significance.

Amaranthin, a pioneering natural colorant, finds application in various food systems, such as beverages, candy, cosmetics, and wine [26]. Acting as a colorant, amaranthin serves as a potent antioxidant, efficiently scavenging free radicals [27]. This dual function enhances its coloring properties and alleviates oxidative stress in plants. Antioxidants, crucial in plants, counteract harmful free radicals from physiological and environmental sources, aiding plants in combating stressors such as ultraviolet radiation, drought, and pollution, thereby enhancing overall plant health and resilience [28,29]. Previous studies have shown that endophytic microorganisms, as producers of plant hormones, can influence the synthesis of pigments (such as amaranthin) by producing hormones [30,31]. Meanwhile, betalain (amaranthin is a category of betalain) can also be produced by endophytic microorganisms, including certain fungal lineages and some nitrogen-fixing bacteria, such as *basidiomycetes* and *Gluconacetobacter diazotrophicus* [32].

Previous studies have focused mainly on the rhizosphere soil microbial composition under different fertilization treatments. Therefore, this study investigated the changes in endophytic bacterial and fungal community structures in the roots of amaranth under four different fertilization treatments. Specifically, the objectives of this study were to determine (1) the variations in the endophytic microbial community structure in amaranth roots under different fertilization treatments and (2) the correlation between endophytic microorganisms and amaranthin.

## 2. Materials and Methods

### 2.1. Field Site, Test Material, and Fertilizer Descriptions

The experiment was initiated on February 10, 2021, at the vegetable base of the Agricultural College, Guangxi University (108°17'15" E and 22°51'02" N). The soil type was red loam with 8.42 g·kg<sup>-1</sup> of organic matter and a pH of 5.71. The total nitrogen, phosphorus, and potassium concentrations were 0.51 g·kg<sup>-1</sup>, 0.67 g·kg<sup>-1</sup>, and 7.21 g·kg<sup>-1</sup>,

respectively. The available phosphorus, potassium, and alkali hydrolyzable nitrogen contents were  $0.59 \text{ mg}\cdot\text{kg}^{-1}$ ,  $51.01 \text{ mg}\cdot\text{kg}^{-1}$ , and  $13.17 \text{ mg}\cdot\text{kg}^{-1}$ , respectively.

The Red Leaf 262 (Shenzhen Xiliang Seed Co., Ltd., Shenzhen, China) amaranth (*Amaranthus tricolor* L.) variety was used, and the seeds were purchased from the Nanning Vegetable Seed Market. Urea (46.4% N content), monoammonium phosphate (10% N content and 43%  $\text{P}_2\text{O}_5$  content), and potassium sulfate (51%  $\text{K}_2\text{O}$  content) were used as the N, P, and K fertilizers, respectively. They were all purchased at the Agricultural Material Market, Nanning City, from the Anhui Jinqiu Fertilizer Company (Fuyang City, Anhui Province, China).

## 2.2. Experimental Design

Four treatments were set up in this experiment, i.e., conventional fertilization without potassium (NP), conventional fertilization without phosphorus (NK), conventional fertilization without nitrogen (PK), and balanced fertilization (NPK), and three replications were carried out for each treatment. The experiment was carried out in pots with a 16 cm radius and a 20 cm height, and 15 kg of red loam soil was filled in each pot. In January 2021, indoor breeding was conducted as follows: Healthy, dry seeds were selected and soaked in  $50^\circ\text{C}$  warm water for 20 min with continuous stirring. Subsequently, the water was cooled to  $20\text{--}25^\circ\text{C}$ , and the seeds were soaked for 4–8 h. The seeds were then placed in a constant-temperature incubator at  $25^\circ\text{C}$  for germination. When the germination rate reached 80%, the seedlings were transferred to nutrient-rich soil, grown until they developed 2–3 leaves, and then transplanted into potting soil. The entire amaranth cultivation process was carried out in a sunlight greenhouse. The daytime temperature in the greenhouse ranged from  $25$  to  $30^\circ\text{C}$ , while the nighttime temperature ranged from  $16$  to  $18^\circ\text{C}$ . The humidity level was maintained between 60% and 80%. Watering was carried out once a week, and manual weeding was performed regularly. The main diseases and pests of amaranth include stem rot and damping-off disease. A 25% carbendazim solution was used at a dilution of 600 times, typically sprayed once every 7–10 days and 2–3 consecutive times. All fertilization treatments and weeding, irrigation, and pest control measures were consistent across the experimental groups. The optimal fertilization rates for amaranth in terms of N, P, and K are 188, 53, and  $50 \text{ kg}/\text{hm}^2$ , respectively [33], and all fertilizers were applied as basal fertilizers. The application rates are shown in Table 1.

**Table 1.** Amounts of N, P, and K applied in the different fertilization treatments.

Treatment	N Fertilizer (mg/kg)	P Fertilizer (mg/kg)	K Fertilizer (mg/kg)
NP	83.6	23.6	-
NK	83.6	-	22.2
PK	-	23.6	22.2
NPK	83.6	23.6	22.2

## 2.3. Sample Collection

Sampling was conducted when the amaranth entered maturity. Briefly, a pot with an amaranth plant was turned upside down to remove the whole plant, and then the soil adhering to the roots was carefully shaken off. The roots were rinsed with sterile water several times to obtain root samples, and then the root samples were placed in sterile plastic bags filled with ice and transported to the laboratory.

In the laboratory, the root samples were rinsed with sterile water to remove impurities and adherents; subsequently, they were washed with 75% ethanol and a 1%  $\text{NaClO}$  solution for 1 min and 3 min, respectively. Then, they were washed 3 times using sterile water for 0.5 min. To determine the success of amaranth root surface sterilization,  $100 \mu\text{L}$  of the last sterile aqueous wash was placed on Luria–Bertani (LB) agar plates (g/L) ( $\text{NaCl}$ -10, trypsin-5, yeast extract-5, and agar-20) and incubated for 7 days at  $25^\circ\text{C}$ . As there were no colonies on the plate, disinfection was recorded as complete [34].

#### 2.4. Analysis of the Amaranthin Content

First, 0.2 g of the leaves were weighed and ground uniformly with a small amount of methanol to a homogenous state. The precipitate was then centrifuged for 10 min (4 °C, 8000 r/min), the supernatant was discarded, and precipitates were obtained three times in succession. The precipitate was then extracted with 15 mL of pure water for 30 min and centrifuged for 15 min (4 °C, 8000 r/min). The supernatant was taken for a colorimetric analysis using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 538 nm. The content of amaranthin was calculated according to Pitelli's [35] method.

#### 2.5. Analysis of the Endophytic Microbial Compositions

The total DNA was extracted according to the instructions of an E.Z.N.A. DNA Kit (Omega, Norcross, GA, USA). The DNA concentration and purity were detected with a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA), and the extracted genomic DNA was detected via 1% agarose gel electrophoresis. The extracted root microbial DNA was used as a template, and the primers 799F (5'-AACMGGATTAGATACCCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3') were used for the PCR amplification of the 16S rRNA V6 region of the endophytic bacteria. The primers ITS1F (5'-CTTGGTCATTTAGA GGAAGTAA-3') and ITS2F (5'-GCTGCGTTCTTCATCATCGATFC-3') were used for the PCR amplification of the ITS rRNA region of the endophytic fungi. The PCR apparatus used was an ABI GeneAmp<sup>®</sup> 9700 (Thermo Scientific, Wilmington, DE, USA). PCR products were recovered via 2% agarose gel electrophoresis, purified with an Axy PrepDNA Gel Recovery Kit (AXYGEN) (Axygen, San Francisco, CA, USA), and eluted with Tris\_HCl. The PCR products were detected and quantified using a QuantiFluor<sup>™</sup>-ST blue fluorescence quantitative system (Promega, Madison, WI, USA). According to the Illumina MiSeq platform standard operating procedures, purified amplified fragments were constructed into libraries. Sequencing was performed using the MiSeq PE300 and MiSeq PE250 platforms from Illumina (Shanghai Meggie Biomedical Technology Co., Ltd., Shanghai, China).

#### 2.6. Statistical Analyses

The experimental data were analyzed using Excel 2019 and IBM SPSS 21. All the results are shown as the means with their standard deviation (mean ± SD). Mothur is an open-source software package used for processing, analyzing, and visualizing DNA sequence data. In this study, Mothur (version v.1.30.2, <https://mothur.org/wiki/calculators/>, accessed on 10 November 2023) was employed to calculate the diversity (Shannon index) and richness (ACE index) of the endophytic bacterial and fungal communities in amaranth roots. A principal coordinate analysis (PCoA) was conducted using the vegan 2.6.4 package [36] in R based on the Bray–Curtis distance. Then, partial least squares discriminant analysis (PLS-DA) was conducted using the mixOmics package in R (version 3.3.1) (<http://fiehnlab.ucdavis.edu/staff/kind/Statistics/Concepts/OPLS-PLSDA>, accessed on 10 November 2023). Endophytic microbial community composition and Venn diagrams were generated using the R language (version 3.3.1) tool for mapping and analysis. Linear discriminant analysis (LDA) is a technique used in multivariate statistical analyses and pattern recognition to measure the differences between data points within the same category. Linear discriminant analysis (LDA) was performed using LEfSe ([http://huttenhower.sph.harvard.edu/galaxy/root?tool\\_id=lefse\\_upload](http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload), accessed on 10 November 2023) on samples, according to different grouping conditions based on taxonomic composition, to identify the differences in endophytic bacteria and fungi in the root system of amaranth. The networkx (version 1.11) tool was used to determine the correlation between microorganisms and between amaranthin and endophytic bacteria and fungi. Online data analyses were carried out using the Majorbio Cloud Platform (<http://www.majorbio.com>, accessed on 29 September 2022) of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

### 3. Results

#### 3.1. Effect of Different Fertilization Treatments on the Amaranthin Content

As shown in Table 2, the amaranthin contents were not significantly different among the NK, PK and NPK treatments, except in the NP treatment. However, it was significantly greater in amaranth under the NPK treatment than those in amaranth under the conventional fertilization without potassium (NP). As shown in Table 2, the amaranthin contents were not significantly different among the NK, PK, and NPK treatments. However, it was significantly greater in amaranth under the NPK treatment than in amaranth under conventional fertilization without potassium (NP).

**Table 2.** Amaranthin content in amaranth plants under different fertilization regimens.

Treatment	Amaranthin Content (mg/g FW)
NP	0.48 ± 0.11 b
NK	0.70 ± 0.28 ab
PK	0.81 ± 0.05 ab
NPK	0.84 ± 0.12 a

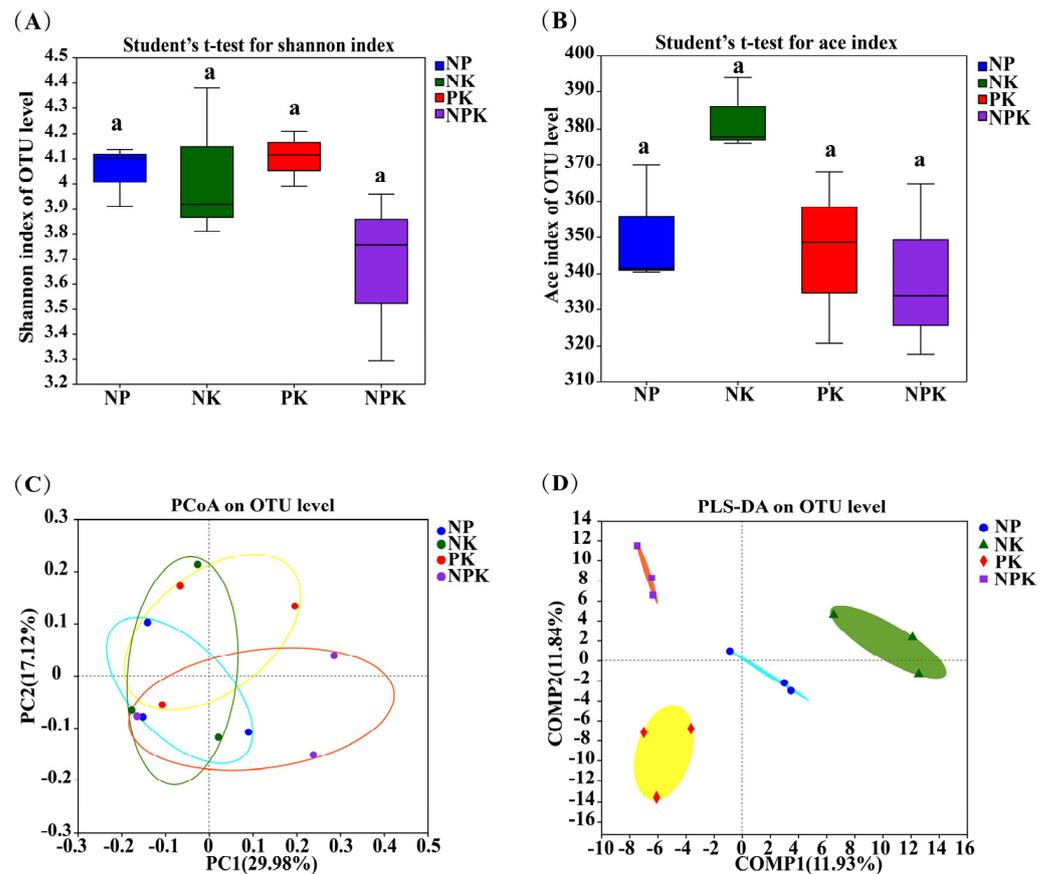
All the data are presented as the means ± standard deviations (SD). Different letters in the same column indicate significant differences among treatments at  $p < 0.05$ . NP: conventional fertilization without potassium; NK: conventional fertilization without phosphorus; PK: conventional fertilization without nitrogen; NPK: balanced fertilization. "mg/g FW" indicates milligrams of amaranthin per gram of fresh sample.

#### 3.2. Effect of Different Fertilization Treatments on the Composition of Endophytic Bacteria in the Amaranth Root System

The Shannon index, which was used to describe the endophytic bacterial diversity, and the ACE index, which was used to describe the endophytic bacterial richness in amaranth roots, were not significantly different between the different fertilization regimens (Figure 1A,B). These results indicate that the endophytic bacterial diversity and richness in amaranth roots were not significantly affected by the application of the different fertilization treatments. In addition, to assess the similarity of the endophytic bacterial communities, we also performed principal coordinate analysis (PCoA) and partial least squares discriminant analysis (PLS-DA) at the operational taxonomic unit (OTU) level (Figure 1C,D). As shown in Figure 1C, PCoA1 and PCoA2 contributed 29.98% and 17.12%, respectively, to the overall change in the endophytic bacterial structure. In addition, PLS-DA showed that the endophytic bacteria were clustered into four separate groups. The results show that the application of different fertilization treatments significantly altered the structure of the endophytic bacterial community in amaranth.

As shown in Figure 2B, at the genus level, the number of unique endophytic bacterial genera in the roots of amaranth under the NP, NK, PK, and NPK treatments was 1, 7, 6, and 7, respectively. At the OTU level, the number of unique endophytic bacterial genera in the roots of amaranth under the NP, NK, PK, and NPK treatments was 9, 28, 20, and 21, respectively (Figure 2C). The results indicate that the different fertilization treatments significantly altered the composition of the endophytic bacterial communities in the amaranth roots. Specifically, compared to the NK, PK, and NPK treatments, the number of unique endophytic bacteria enriched in amaranth roots was the lowest under the NP treatment.

At the phylum level, the compositions and proportions of the dominant endophytic bacteria (i.e., relative abundance > 1%) in the amaranth roots under the conventional fertilization without potassium (NP) treatment were *Proteobacteria* (69.46%), *Actinobacteriota* (27.85%), and other bacteria (2.30%). In contrast, the predominant taxa were *Proteobacteria* (73.04%), *Actinobacteriota* (23.49%), *Myxococcota* (1.14%), and others (2.32%) in the amaranth roots under the conventional fertilization without phosphorus (NK) treatment; *Proteobacteria* (66.07%), *Actinobacteriota* (29.67%), and others (3.54%) in the amaranth roots under the conventional fertilization without nitrogen (PK) treatment; and *Proteobacteria* (68.65%), *Actinobacteriota* (28.44%), and others (2.45%) in the amaranth roots under the balanced fertilization (NPK) treatment (Figure 2A).

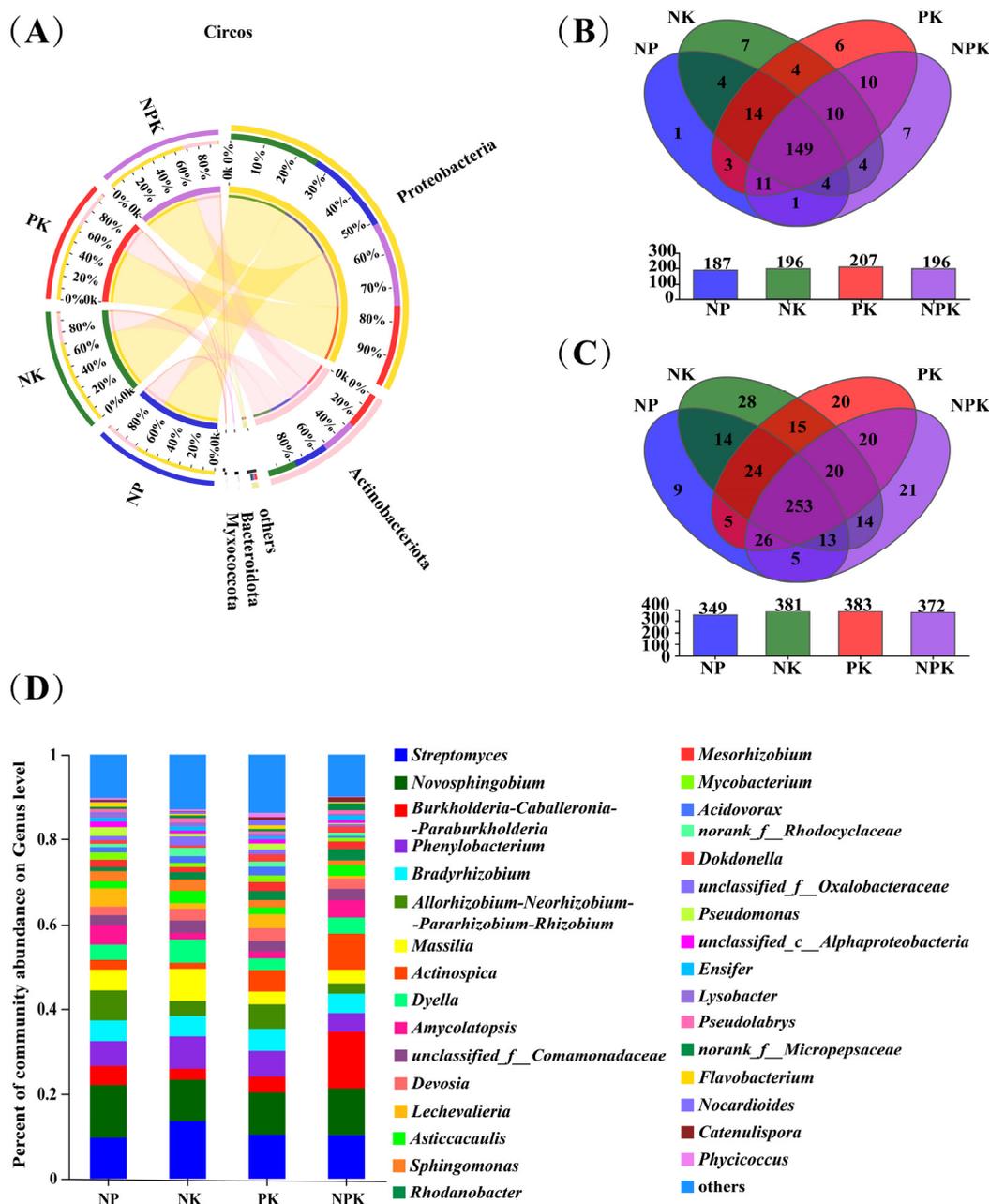


**Figure 1.** The Shannon (A) and ACE (B) indices of endophytic bacteria in amaranth roots under different fertilization treatments; PCoA (C) and PLS-DA (D) of endophytic bacteria in amaranth roots under different fertilization treatments at the OTU level. Same letters on bars within a figure indicate no significant differences in mean ranks among treatments at  $p > 0.05$ .

At the genus level, 22, 23, 25, and 20 dominant endophytic bacterial genera (i.e., relative abundance  $> 1\%$ ) were detected in the amaranth roots under the NP, NK, PK, and NPK treatments, respectively (Figure 2D).

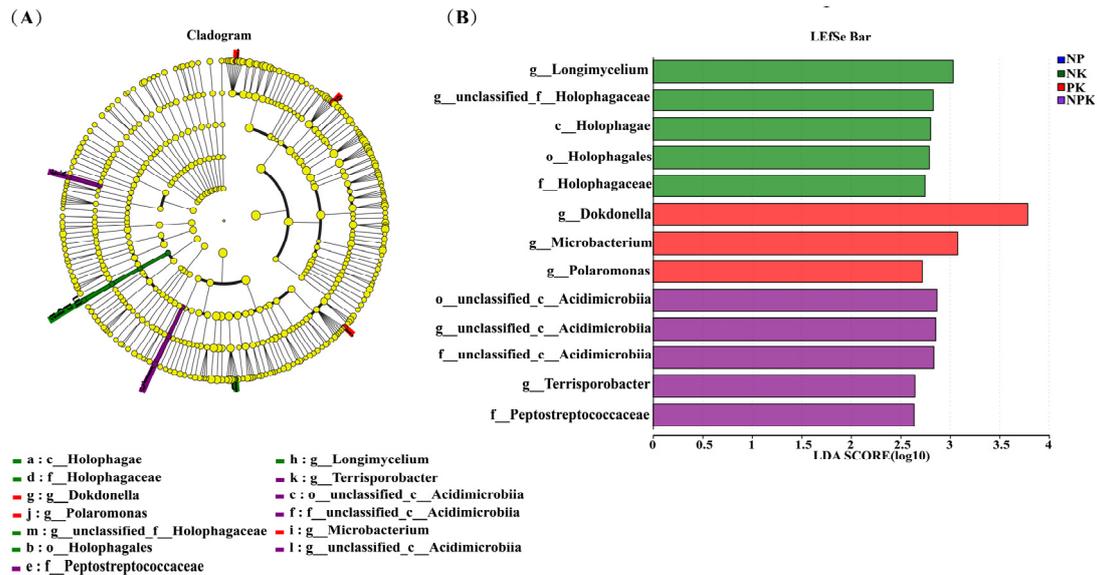
In comparison with those in the NP, NK, and PK treatments, the proportions of *Burkholderia-Caballeronia-ParaburthoLderia* and *Actinospica* increased, but the proportions of *PhenyLobacterium*, *Bradyrhizobium*, *Sphingomonas*, and *Allorhizobium-Neoruzobium-Pararhixobium-Rhixobium* decreased in the NPK treatment. It is worth noting that *Streptomyces* accounted for over 10% in the NK, PK, and NPK treatments, but only 6% in the NP treatment. In addition, *Acidovorax*, *Lechevalieria*, *Mycobacterium*, and *unclassified\_f\_Oxalobacteraceae* were the unique dominant bacterial genera in the NP, NK, and PK treatments, respectively, but they were not present in the NPK treatment. In contrast, *norank\_f\_Micropepsaceae* and *Catenulispora* were the unique dominant bacterial genera in the NPK treatment. Moreover, *Lysobacter* and *unclassified\_c\_Alphaproteobacteria*, *Ensifer*, and *Pseudolabry*, and *Nocardioides*, and *Phycoccus* were the unique dominant bacterial genera in the NP, NK, and PK treatments, respectively. The above results show that the application of different fertilization treatments significantly changed the community structure of endophytic bacteria in the amaranth roots.

The LEfSe analysis revealed the species with differences between groups (Figure 3A,B). At the genus level, the results showed that *Longimycelium* and *unclassified\_f\_Holophagaceae* were significantly enriched in the NK treatment ( $p < 0.05$ ). *Dokdonella*, *Microbacterium*, and *Polaromonas* were significantly enriched in the PK treatment ( $p < 0.05$ ). *Unclassified\_c\_Acidimierobia*, and *Terrisporobacter* were significantly enriched in the NPK treatment ( $p < 0.05$ ). However, a significant enrichment of endophytic bacteria at the phylum or genus level was not found in the roots of the amaranth plants under the NP treatment.

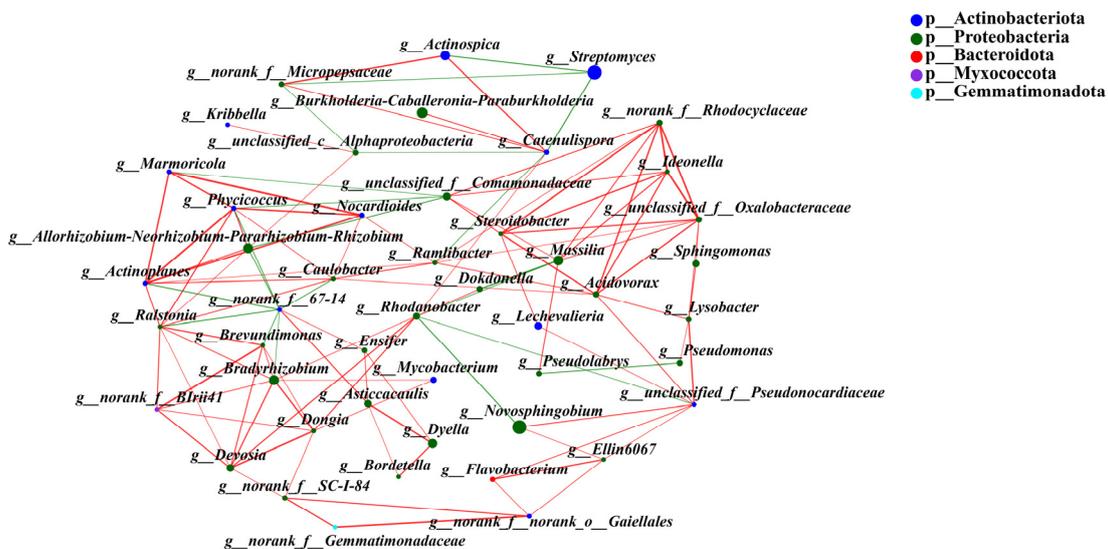


**Figure 2.** (A) Compositions and proportions of endophytic bacteria in amaranth roots under different fertilization treatments at the phylum level. (B) Venn diagram of endophytic bacteria in amaranth roots at the genus level. (C) Venn diagram of endophytic bacteria in amaranth roots at the OTU level (Blue: NP; Green: NK; Red: PK; Purple: NPK). The numbers in the Venn diagram represent the number of endophytic bacteria in amaranth roots in different fertilisation treatments. (D) Compositions and proportions of endophytic bacteria in amaranth roots under different fertilization treatments at the genus level.

At the genus level, the top 50 endophytic bacteria obtained from a network analysis showed 47 nodes and 116 edges. Among them, 95 and 21 edges were significantly positively and negatively correlated, respectively. Furthermore, *Ralstonia*, *norank\_f\_\_67-14*, *Phycococcus*, *unclassified\_f\_\_Oxalobacteraceae*, *Rhodanobacter*, *Acidovorax*, *Actinoplanes*, and *Bradyrhizobium* were the most strongly correlated with the other endophytic bacteria (degree  $\geq 8$ ) (Figure 4).



**Figure 3.** Cladogram (A) and LefSe bar (B) of the root endophytic bacterial communities under the different fertilization treatments (Blue: NP; Green: NK; Red: PK; Purple: NPK). Pathologically, nodes indicate microbial taxa that are significantly enriched in the corresponding group and have a significant effect on the differences between groups (p, phylum; C, class; O, order; f, family; and g, genus). ( $p < 0.05$ , LDA scores  $\geq 2$ ).

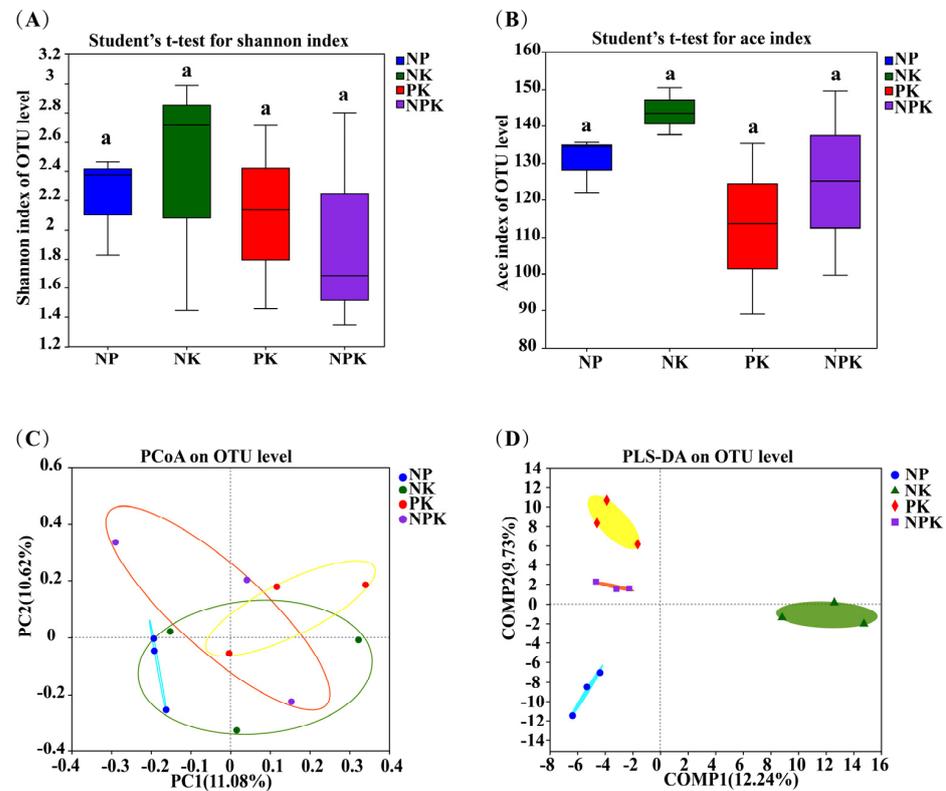


**Figure 4.** Co-occurrence network analysis of endophytic bacterial communities under different fertilization treatments. Different levels are indicated by different prefixes (p, phylum; g, genus). The size of the nodes in the graph indicates the size of the species abundance, and different colors indicate different species; the colors of the connecting lines indicate positive and negative correlations, with red indicating positive correlation and green indicating negative correlation ( $p < 0.05$ ); the thickness of the lines indicates the size of the correlation coefficient; the coarser the line is, the greater the correlation between the species; and the greater the number of lines is, the closer the connection between the species and other species.

### 3.3. Effects of Different Fertilization Treatments on Endophytic Fungi in Amaranth Roots

As shown in Figure 5A,B, the indices of endophytic fungal diversity (Shannon) and richness (ACE) were not significantly different among the NP, NK, PK, and NPK treatments. Taken together, these findings suggest that the endophytic fungal diversity and richness in amaranth roots were not significantly altered by the application of the different fertilization

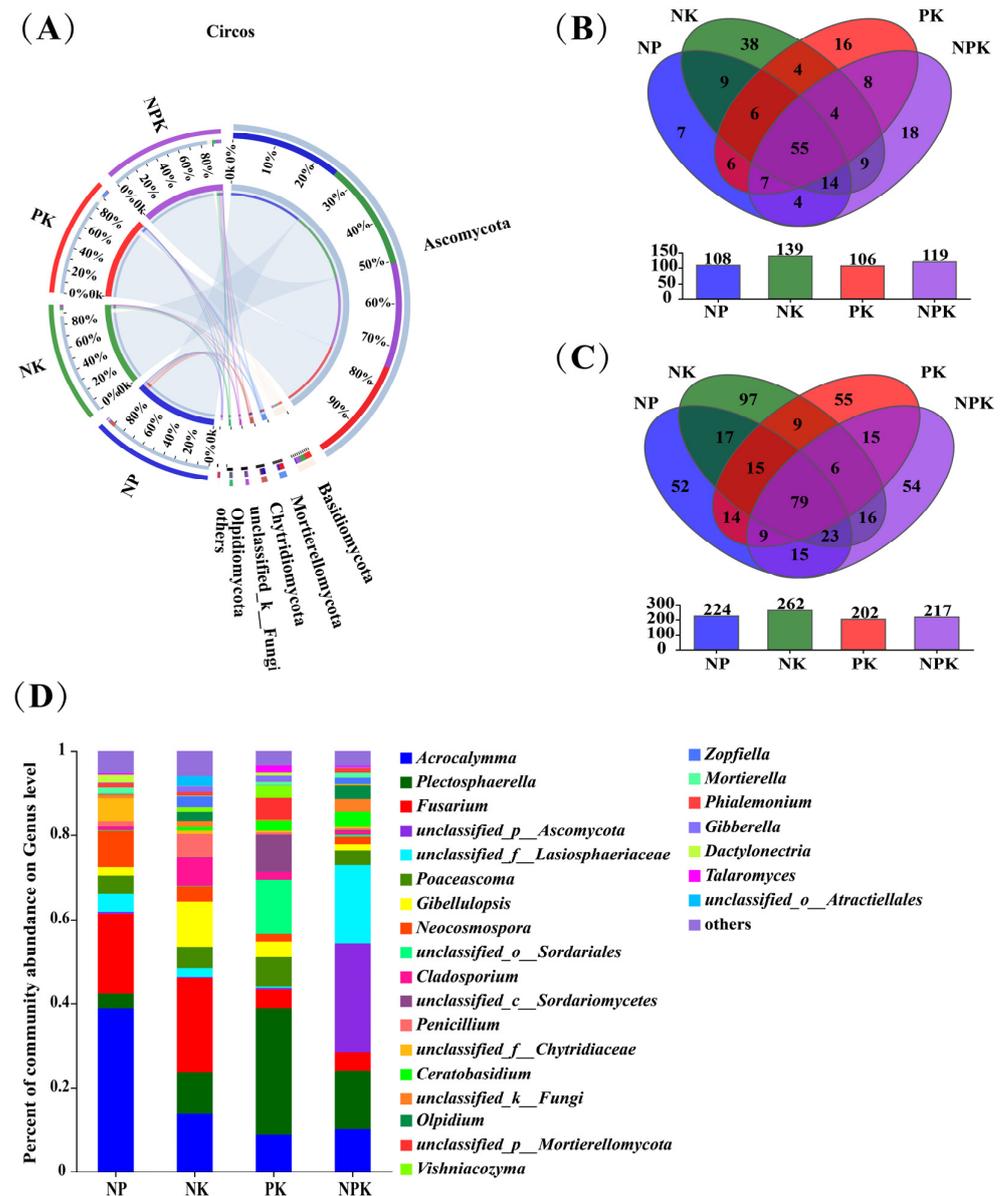
treatments. In addition, the PCoA revealed that PCoA1 and PCo2 contributed 29.98% and 17.12%, respectively, to the overall changes in the endophytic fungal structure. Meanwhile, according to the PLS–DA results, the endophytic bacteria were clearly clustered into four classes, which were distributed in the negative and positive directions of Comp2. Thus, the results show that the application of different fertilizers significantly altered the structure of the endophytic fungal community in amaranth.



**Figure 5.** The Shannon (A) and ACE (B) indices of endophytic fungi in amaranth roots under different fertilization treatments. PCoA (C) and PLS–DA (D) of endophytic fungi in amaranth roots under different fertilization treatments at the OTU level. Same letters on bars within a figure indicate no significant differences in mean ranks among treatments at  $p > 0.05$ .

Similarly, at the genus level, the total numbers of unique endophytic fungi in the roots of the amaranth plants under the NP, NK, PK, and NPK treatments were 7, 38, 16, and 18, respectively (Figure 6B). At the OTU level, the total numbers of unique endophytic fungi in the roots of the amaranth plants under the NP, NK, PK, and NPK treatments were 52, 97, 54, and 54, respectively (Figure 6C). These results suggested that different fertilization treatments could significantly alter the composition of the endophytic fungal communities in amaranth roots. Specifically, compared to the NK, PK, and NPK treatments, the number of unique endophytic fungi enriched in amaranth roots was the lowest under the NP treatment.

At the phylum level, the dominant endophytic fungi (i.e., relative abundance  $> 1\%$ ) in the amaranth roots were *Ascomycota* (91.41%), *Chytridiomycota* (5.27%), *Mortierellomycota* (1.36%), and *Basidiomycota* (1%) under the NP treatment. The predominant bacteria in the amaranth roots under the NK treatment were *Ascomycota* (90.11%), *Basidiomycota* (5.30%), *Olpidiomycota* (2.07%), and *unclassified\_k\_Fungi* (1.43%). In addition, *Ascomycota* (86.99%), *Basidiomycota* (6.08%), and *Mortierellomycota* (5.90%) were found in the amaranth roots under the PK treatment. In contrast, *Ascomycota* (87.77%), *Basidiomycota* (4.17%), *Olpidiomycota* (3.28%), *unclassified\_k\_Fungi* (2.96%), and *Mortierellaomycota* (1.32%) were found in the amaranth roots under the NPK treatment (Figure 6A).



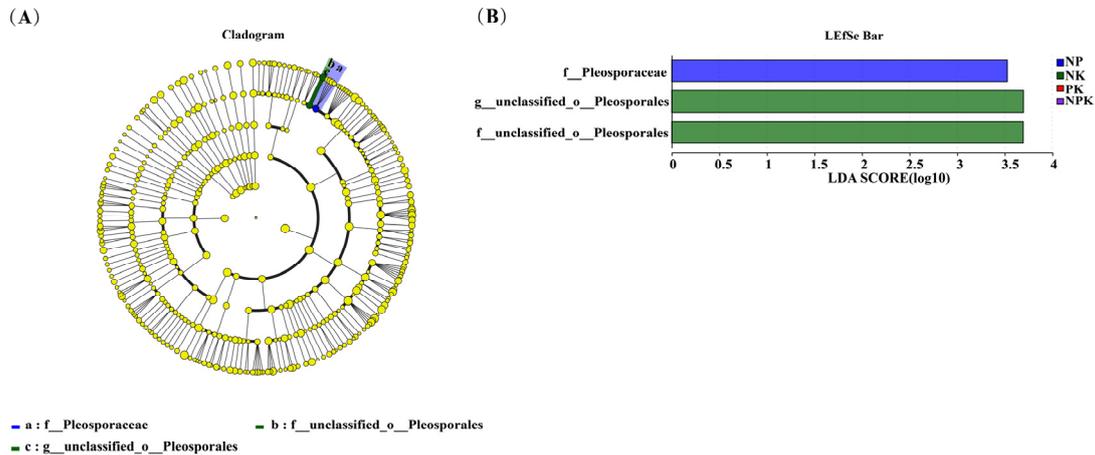
**Figure 6.** (A) Compositions and proportions of endophytic fungi in amaranth roots under different fertilization treatments at the phylum level. (B) Venn diagram of endophytic fungi in amaranth roots at the genus level. (C) Venn analyses of endophytic fungi in amaranth roots at the OTU level (Blue: NP; Green: NK; Red: PK; Purple: NPK). The numbers in the Venn diagram represent the number of endophytic fungi in amaranth roots in different fertilisation treatments. (D) Compositions and proportions of endophytic fungi in amaranth roots under different fertilization treatments at the genus level.

At the genus level, a total of 25 dominant endophytic fungal genera were detected in the amaranth roots under the different fertilization treatments. Among them, 12, 16, 14, and 14 dominant endophytic fungal genera were found under the NP, NK, PK, and NPK treatments, respectively (Figure 6D).

Compared with those under the NP, NK, and PK treatments, the proportions of *Poaceascoma*, and *Gibellulopsis* in the amaranth roots decreased under the NPK treatment. However, *Unclassified\_p\_Ascomycota* was particularly enriched in the amaranth roots under the NPK treatment. *Dactylonectria* was an endophytic fungi unique to the NP treatment, and *Gibberella*, and *Cladosporium* were endophytic fungi unique to the NK and PK treatments. *Neocosmospora* and *Acrocalymma* were endophytic fungi unique to the NP, NK, and PK

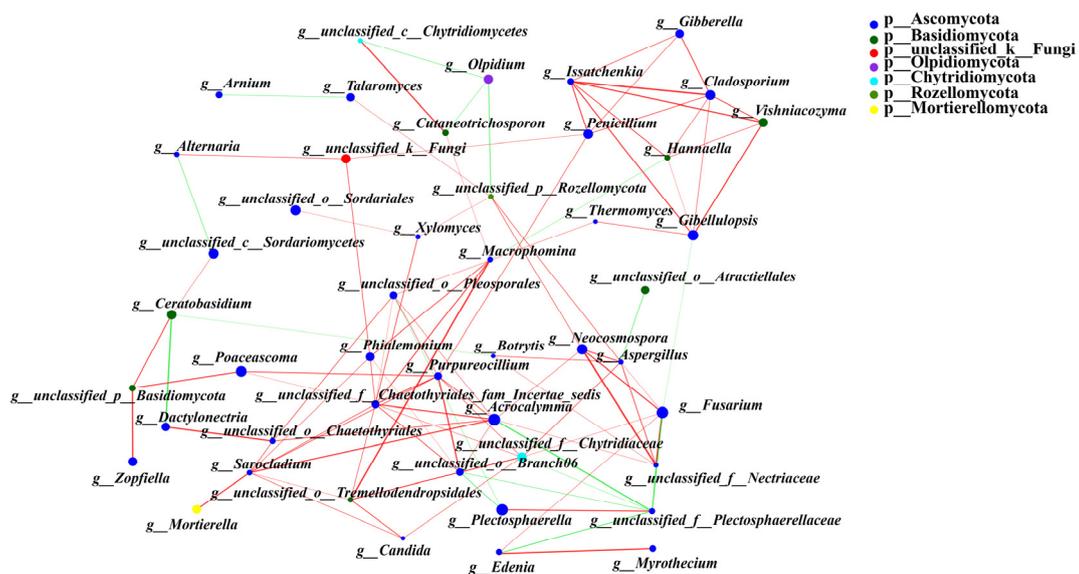
treatments. Meanwhile, the proportion of *Fusarium* increased in the NP treatment. All these results suggest that the application of different fertilization treatments can significantly alter the community structure of endophytic fungi in amaranth roots.

At the genus level, the results of the LEfSe analyses showed that only *unclassified\_0\_Pleosporales* were significantly enriched in the NPK treatment ( $p < 0.05$ ). However, a significant enrichment of endophytic fungi was not found in response to different fertilization regimens consisting of diverse NP, NK, and PK treatments (Figure 7).



**Figure 7.** Cladogram (A) and LEfSe bar (B) of the root endophytic fungal communities under the different fertilization treatments (Blue: NP; Green: NK; Red: PK; Purple: NPK). Pathologically, nodes indicate microbial taxa that are significantly enriched in the corresponding group and have a significant effect on the differences between groups (p, phylum; C, class; 0, order; f, family; and g, genus). ( $p < 0.05$ , LDA score  $\geq 2$ ).

Similarly, the results of the network analysis also revealed 48 nodes and 101 edges (Figure 8). Among them, 83 significantly positive and 18 significantly negative correlations were found. *Unclassified\_f\_Chaetothyriales\_fam\_Incertae\_sedis*, *Acrocalymma*, *Purpureocillium*, *Sarocladium*, *unclassified\_f\_Chytridiaceae*, and *unclassified\_o\_Branch06* were the most strongly correlated with the other endophytic fungi (degree  $\geq 8$ ).

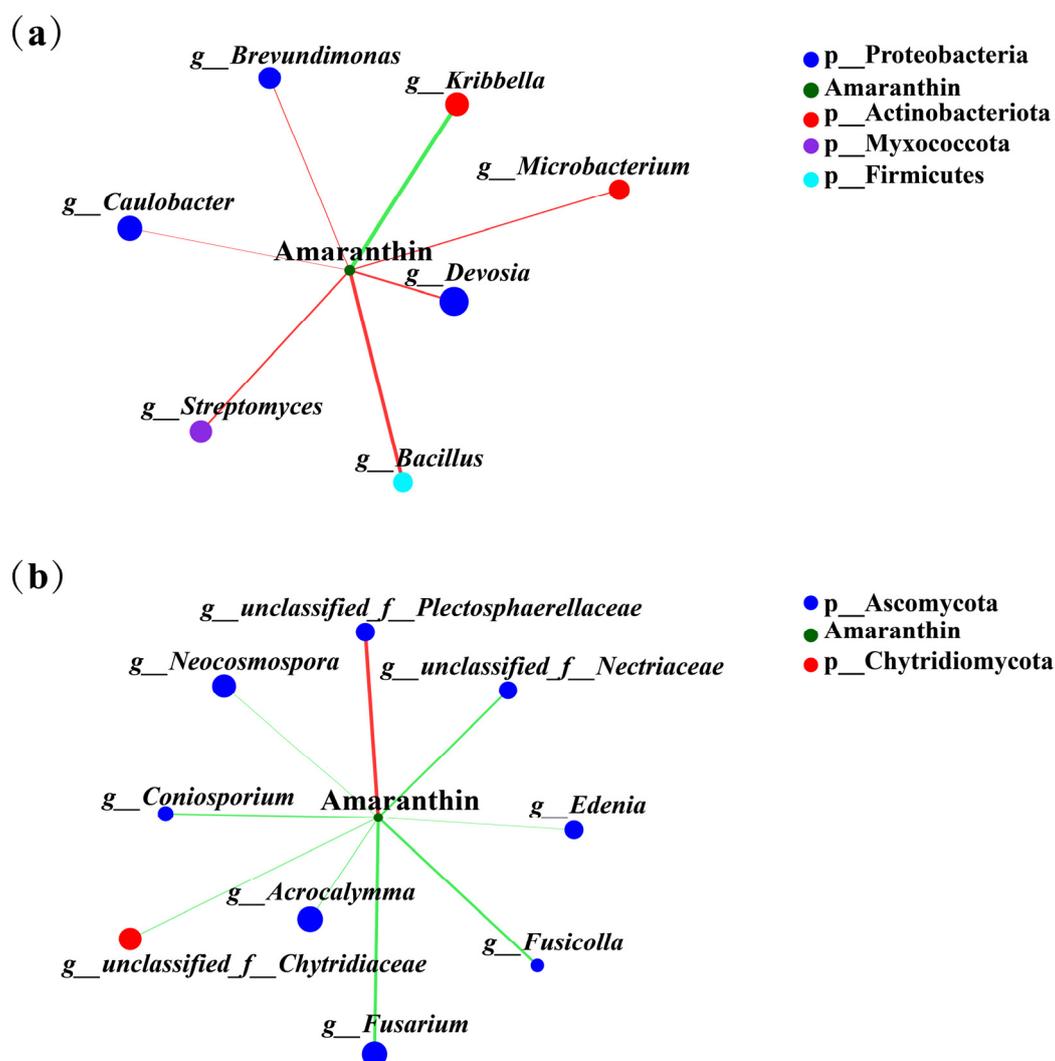


**Figure 8.** Co-occurrence network analysis of endophytic fungal communities under different fertilization treatments. Different levels are indicated by different prefixes (p, phylum; g, genus). The size of the

nodes in the graph indicates the size of the species abundance, and different colors indicate different species; the colors of the connecting lines indicate positive and negative correlations, with red indicating positive correlation and green indicating negative correlation ( $p < 0.05$ ); the thickness of the lines indicates the size of the correlation coefficient; the coarser the line is, the greater the correlation between the species; and the greater the number of lines is, the closer the connection between the species and other species.

### 3.4. Correlation Analysis of Amaranthin with Endophytic Bacteria and Fungi

To investigate the relationship between the amaranthin and endophytic microorganisms in the roots of amaranth, Spearman's correlation coefficient was used. The results showed that amaranthin was significantly positively correlated with *Devosia*, *Bacillus*, *Streptomyces*, *Caulobacter*, and *Brevundimonas*, and that it was significantly negatively correlated with *Kribbella* (Figure 9a). Additionally, a significant positive correlation between amaranthin and endophytic fungi, such as *unclassified\_f\_Plectosphaerellaceae*, was detected. In contrast, significant negative correlations were also detected between amaranthin and endophytic fungi, such as *unclassified\_f\_Nectriaceae*, *Edenia*, *Fusicolla*, *Fusarium*, *Acrocalymma*, *unclassified\_f\_Chytridiaceae*, *Coniosporium*, and *Neocosmospora* (Figure 9b).



**Figure 9.** Correlation analysis of amaranthin with endophytic bacteria (a) and fungi (b). Different levels are indicated by different prefixes (p, phylum; g, genus). The size of the nodes in the graph indicates the size of the species abundance, and different colors indicate different species; the colors of the connecting lines indicate positive and negative correlations, with red indicating positive correlation

and green indicating negative correlation ( $p < 0.05$ ); the thickness of the lines indicates the size of the correlation coefficient; the coarser the line is, the greater the correlation between the species; and the greater the number of lines is, the closer the connection between the species and other species.

#### 4. Discussion

##### 4.1. Effects of Different Fertilization Treatments on the Amaranthin Content

Previous studies reported that the synthesis of plant pigments, such as anthocyanins, could be significantly affected by fertilization, and could be reduced under low-nitrogen conditions [37]. However, when balanced fertilizer application is applied to strawberry plants, the anthocyanin content can increase significantly [38]. Neshev et al. [39] reported that different fertilization treatments had no effect on the carotenoid content in potato leaves, but that NP fertilization significantly increased the chlorophyll content in potato leaves. Our study also revealed that the amaranthin content was affected by different fertilization regimens; among these factors, the highest amaranthin content was found in the leaves of plants under the balanced fertilization (NPK) treatment. In contrast, the lowest amaranthin content was detected in the leaves under the conventional fertilization without potassium (NP) treatment. All above results suggested that nitrogen, phosphorus and potassium were the main factors affecting amaranthin synthesis. This is consistent with previous studies [40].

##### 4.2. Effects of Different Fertilization Treatments on the Endophytic Bacterial Community Structures in Amaranth Roots

*Proteobacteria*, a bioindicator of plant disease, has been reported to be the most common endophytic bacterial phylum in plant organisms [41–43]. Moreover, *Actinobacteriota*, which are producers of natural antibiotics, also widely inhabit plants [44,45]. In this experiment, *Proteobacteria*, and *Actinobacteria* were the main endophytic phyla in amaranth roots, but they exhibited significant changes under different fertilization treatments. Specifically, *Proteobacteria* were enriched under NK treatment, while *Actinobacteria* were enriched under PK treatment. All above results suggest that unbalanced fertilization may lead to amaranth producing antibiotics to protect themselves.

*Streptomyces* play an important role in controlling plant diseases, and many pathogens, such as ash mold, *Fusarium oxysporum*, *anthrax of capsicum*, and *Aspergillus flavus*, are significantly inhibited by their metabolic substances [46,47]. In addition, previous studies have shown that *Streptomyces* are associated with the synthesis of pigments, and gene sequencing has revealed the presence of a large number of cytochrome P450 genes in *Streptomyces* [48,49]. Cytochrome P450 enzymes are monooxygenases that catalyze various metabolic and biosynthetic chemical reactions [50,51]. Numerous studies have identified cytochrome P450 (CYP76AD1) as a key gene in the amaranthin metabolic pathway that plays an important role in amaranthin biosynthesis [52]. Moreover, a significant positive correlation between *Streptomyces* and amaranthin was found in our study. The abundance of *Streptomyces* was greater than 10% in the NK, PK, and NPK treatments and decreased only in the conventional fertilization without potassium (NP). This result suggested that *Streptomyces* was more affected by potassium than by other elements, such as nitrogen and phosphorus. It also suggested that potassium might influence the synthesis of amaranthin relating to the proportion of *Streptomyces* in amaranth roots.

*Actinospica* and *Burkholderia-Caballeronia-ParaburthoLderia* are beneficial bacteria for producing antibiotics and degrading pollutants [53,54]. *Phenylobacterium* and *Acidovorax* are harmful bacterial genera that can be found in the environment and cause cucumber disease [55,56]. Our results showed increased abundances of *Actinospica* and *Burkholderia-Caballeronia-ParaburthoLderia* in the NPK treatment, with a decreased abundance of *Phenylobacterium*. Additionally, *Acidovorax* was enriched in amaranth roots under NP, NK, and PK treatments, but absent under NPK treatment. These results indicate that more beneficial endophytic bacteria could be enriched by balanced fertilization. In contrast, unbalanced

fertilization may lead to an increase in the number of harmful endophytic bacteria in amaranth roots.

#### 4.3. Effects of Different Fertilization Treatments on the Endophytic Fungal Community Structures in Amaranth Roots

*Olpidiomycota* can break down cellulose and chitin, providing more nutrients for plant growth and promoting ecosystem matter [57]. *Chytridiomycota*, ubiquitous fungi, are specialized parasites of many higher plant roots that act as mediators of the transmission of some soilborne viruses [58,59]. We found that in the NPK treatment, the abundance of *Olpidiomycota* increased, while it decreased in the unbalanced fertilization treatments (NP, NK, and PK). Furthermore, in the NPK treatment, the proportion of *Chytridiomycota* was the lowest, whereas it was the highest in the unbalanced fertilization treatments, especially in the NP treatment.

*Acrocalymma* is a type of fungus that causes crown rot [60]. However, *Neocosmospora* can induce black rot at the base of the plant stem and roots [61]. Many studies have shown that *Fusarium* species are soilborne pathogens that cause plant root rot and can secrete toxins [62,63]. In this study, we found that *Fusarium*, *Acrocalymma*, and *Neocosmospora* were significantly negatively correlated with amaranthin. *Fusarium* was the most abundant genus in the NP treatment. *Neocosmospora* and *Acrocalymma* were endophytic fungi unique to the NP, NK, and PK treatments. Previous studies have confirmed that *Fusarium* can produce gibberellins [64]. Moreover, the exogenous application of Gibberellin(GA<sub>3</sub>) inhibited the accumulation of amaranthin in amaranth plants [65]. These results suggest that endophytic fungi related to amaranthin formation were significantly inhibited by conventional fertilization without potassium.

*Gibellulopsis* is also a pathogenic fungus that induces the yellowing and wilting of sugar beet leaves [66]. *Cladosporium*, a mycorrhizal fungus with conidia, can easily lead to leaf mold and spot disease [67]. *Dactylonectria* not only produces toxins but also causes root necrosis [68,69]. *Gibberella*, a parasitic fungus that affects a variety of grasses, can lead to malignant seedling disease in rice, and culm rot or cob rot in maize [70]. Our study revealed that the abundance of *Gibellulopsis* decreased in the roots of the amaranth plants under the NPK treatment compared to those under the NP, NK, and PK treatments. In addition, *Dactylonectria* was the unique dominant fungal genus in the roots of the amaranth plants under the NP treatment, and *Gibberella* and *Cladosporium* were the unique dominant fungal genera in the roots of the amaranth plants under the NK and PK treatments.

All these results indicated that unbalanced fertilizations might be more suitable for the growth of pathogenic fungi in a soil environment. Consequently, it triggered more pathogenic fungi colonizing in the amaranth roots, in association with a higher incidence of diseases. Conversely, balanced fertilization might enhance the resistance of plants to adverse environmental conditions, reducing the damage from environmental stress for plants.

## 5. Conclusions

The above results suggest that endophytes can easily colonize amaranth roots as beneficial microorganisms under balanced fertilization conditions. However, endophytes can easily colonize amaranth roots as harmful microorganisms under unbalanced fertilization conditions. Additionally, we also observed a significant positive correlation between *Streptomyces* and amaranthin, as well as significant negative correlations among *Acrocalymma*, *Neocosmospora*, *Fusarium*, and amaranthin. All above results suggested that different fertilizations could significantly alter the endophytic microbial community structures in amaranth roots. Balanced fertilization (N, P and K fertilizers are 188.0, 53.0, and 50.0 kg·hm<sup>-2</sup>, respectively) could recruit more beneficial endogenous microorganisms in amaranth roots for improving their growth and quality.

**Author Contributions:** X.L.: conceptualization, methodology, software, formal analysis, data curation, writing—original draft, writing—review and editing, and visualization. D.Y.: software, data curation. X.Z.: software, data curation. X.W.: software, formal analysis. Y.X.: software, formal analysis, data

curation. S.Y.: conceptualization, methodology, resources, writing—review and editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by the Key Research and Development Program of Guangxi (Guike AB23026076).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The original reads were stored in the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA963072).

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

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