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Effects of Imazethapyr on Soybean Root Growth and Soil Microbial Communities in Sloped Fields

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Abstract: The herbicide imazethapyr was previously recommended for controlling weeds in soybean fields. However, the effects of imazethapyr on soil microbial communities and their relationship with crop root growth in sloped soils remain unclear. In this study, a field experiment was conducted on a sloped field to explore the effects of imazethapyr on crop root growth, microbial communities, microbial co-occurrence networks, and the interactions between microbes and crop root growth. The field experiment included two factors: slope and imazethapyr. The slope factor included three different slope gradients: 5° (S1), 10° (S2), and 15° (S3). The imazethapyr factor included two treatments: with (I1) and without (I0) imazethapyr. Thus, six total combinations of slope and imazethapyr treatments were tested in this study: S1I1, S2I1, S3I1, S1I0, S2I0, and S3I0. The results show that, compared to the I0 treatments, the I1 treatments significantly increased the soybean root length, surface area, and volume by 11.7~26.5 m, 171.7~324.2 cm^2, and 1.8~3.1 cm^3, respectively, across all the slopes. The *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* bacterial phyla and Ascomycota and Basidiomycota fungal phyla were found to be the top phyla represented bacterial and fungal communities. These five phyla were scattered in co-occurrence networks of bacterial and fungal communities, suggesting these phyla play critical roles in enhancing the stability of co-occurrence networks. Compared to the I0 treatments, the I1 treatments increased nodes from *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* phyla by 6.4%, 9.1%, and 11.2%, respectively, in the bacterial co-occurrence network. Similarly, in the fungal co-occurrence network, the I1 treatments improved nodes from Ascomycota and Basidiomycota phyla by 1.8% and 5.8%, respectively. Compared to the I0 treatments, the I1 treatments increased positive relations by 8.3% and 3.2%, respectively, in the bacterial and fungal co-occurrence networks. Moreover, the I1 treatments increased the relative abundance of root-promoting biomarkers and suppressed root-limiting biomarkers. However, the application of imazethapyr reduced the diversity and richness of bacterial and fungal communities in general. Furthermore, the nodes and links of bacterial co-occurrence networks in the I0 treatments were 9.2% and 78.8% higher than these in the I1 treatments. Similarly, the I1 treatments also decreased 17.9% of fungal community links compared to the I0 treatments. Our data also show that compared to the I0 treatments, the I1 treatments decreased almost all gene families encoding nitrogen and carbon cycling pathways. In conclusion, the application of imazethapyr increased soybean root growth by increasing root-promoting biomarkers and improved the stability and cooperation of co-occurrence networks of bacterial and fungal communities. However, the application of imazethapyr had some negative impacts on microbial communities, such as reducing the diversity of bacterial and fungal communities and nitrogen and carbon cycling pathways.

Keywords: soybean root growth; microbial community; nitrogen and carbon cycling pathways; sustainable crop production

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Citation: Wang, Z.; Wang, X.; Wang, T. Effects of Imazethapyr on Soybean Root Growth and Soil Microbial Communities in Sloped Fields. *Sustainability* **2022**, *14*, 3518. https://doi.org/10.3390/su14063518

Academic Editor: Helvi Heinonen-Tanski

Received: 16 February 2022
Accepted: 14 March 2022
Published: 17 March 2022

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1. Introduction

The application of herbicides has become one of the key agronomy practices used to limit the growth of weeds and improve crop growth [1]. According to reports, the total

area featuring the application of pesticides exceeded 2.8×10^8 ha [2], and approximately 1.8 million tons of pesticides are applied every year in China [3,4]. Imazethapyr is the most common herbicide and is widely used throughout China, especially in soybean fields in Northeast China [2,5]. For example, Zhang et al. [6] reported that imazethapyr was applied to almost 80% of farmland planted with soybean to limit the growth of weeds. Many studies have been conducted to research the effects of imazethapyr on crop growth and the environment [7–9]. However, the effects of imazethapyr residues in the soil on microbial communities remain unclear.

Recently, many studies have demonstrated that the soil microbiome plays a vital role in crop growth [10–12]. On the one hand, crop roots secrete various chemicals, including sugars, amino acids, organic acids, and phenolic compounds, which recruit beneficial microbes [13–15]. On the other hand, these recruited microbes can increase the tolerance of crops to a range of biotic and abiotic stresses. These symbiotic relationships between root microorganisms and crops are very important for microorganisms and crop growth. Upon this background, studies have focused on the impacts of herbicides on soil microbial communities [9,16,17]. For example, Liu et al. [17] investigated the effects of imazethapyr on the rhizosphere microbes of *Arabidopsis thaliana* root exudates and found that imazethapyr helps *Arabidopsis thaliana* recruit some beneficial microbes to resist the imazethapyr stress by promoting secretion of amino acids, organic acids, and other attractive compounds. Qu et al. [1] found that after the use of S-metolachlor, the richness of the soil microbiome was markedly reduced, but plants recruited potential beneficial microbes to improve tolerance to S-metolachlor stress. However, in most of these studies, a model organism, such as *Arabidopsis thaliana*, was used as an experimental material. In addition, most of these studies were conducted in controlled environments. In real fields, crop growth and the soil microbiome are affected by many environmental factors, including weather properties, soil types, and crop types. Thus, the effects of herbicides on the soil microbiome observed in real fields might be different than those observed under incubation. For example, Dennis et al. [18] found that the application of the three herbicides, glyphosate, glufosinate, and paraquat, had no significant impacts on the microbial diversity and structure in soil. To our best knowledge, the effects of imazethapyr on soil microbial communities in real fields remain largely unclear.

Crop roots have a selective effect on the soil microbiome. In turn, the soil microbiome can enhance the absorption of soil nutrients and thereby improve the growth of crop roots [19,20]. Although many studies have been conducted to explore the impacts of imazethapyr and other herbicides on soil microbial communities [1,21], few have focused on the impacts of the soil microbiome on crop root growth. Therefore, the interaction between the soil microbiome and crop root growth is not well understood. Furthermore, previous studies that investigated the influence of imazethapyr or other herbicides on soil microbial communities have always focused on the bacterial community [9,17,21]. Indeed, fungi play a vital role in crop growth, especially mycorrhizal fungi [22]. Thus, the impacts of imazethapyr or other herbicides on fungal communities should be further studied. It is also worth noting that the co-occurrence relations between microbes have significant impacts on crop growth and soil function profiles [23,24]. The application of imazethapyr or other herbicides was found to change the diversity and structure of microbial communities [1,17], which could further change the co-occurrence relations among microbes. However, to our best knowledge, few studies have investigated the impacts of imazethapyr on the co-occurrence networks of soil bacterial and fungal communities.

Changes to soil microbial diversity, structure, and co-occurrence relations would almost certainly result in the alteration of the microbial function profiles involving nitrogen and carbon cycling pathways [9,25]. In this sense, the application of herbicides can affect microbial diversity, co-occurrence relations, and, consequently, soil functions. Although previous studies have investigated the impacts of imazethapyr and other herbicides on the environment [26], the influences of imazethapyr and other herbicides on carbon and nitrogen turnover processes remain largely unclear.

Ultimately, the purpose of this study was to investigate the effects of imazethapyr on crop root growth, microbial communities, microbial co-occurrence networks, and the interactions between microbes and crop root growth in a real field. The influences of imazethapyr on carbon and nitrogen cycling pathways were also further explored. As imazethapyr is mainly used in soybean fields [9], the present experiment was conducted in a soybean field. In addition, in the northeast of China, the total area of sloped fields is over 0.95 million ha. These sloped fields play a vital role in soybean production in the region. Additionally, to reduce soil erosion in sloped fields, almost all are planted with soybean. Thus, the present study was conducted in sloped soils with planted soybean. We hypothesized that the application of imazethapyr would enhance the interactions between microbes and soybean root growth in sloped farmlands, and, at the same time, the application of imazethapyr would reduce soil microbial diversity and carbon and nitrogen cycling pathways. The specific objectives were (1) to study the impacts of imazethapyr on soybean root growth; (2) to investigate the impacts of imazethapyr on soil bacterial and fungal community diversity, structures, and co-occurrence networks; (3) to explore the relationship between soil microbes and soybean root growth; and (4) to assess the effects of imazethapyr on carbon and nitrogen cycling pathways.

2. Materials and Methods

2.1. Site Characterization and Experiment Design

Field experiments were conducted at Shenyang Agricultural University experimental station (41°44′ N, 123°27′ E, 44.7 ma.s.l), Shenyang city, Liaoning Province, China, from 2018 to 2020. The soil in the experiments was mainly characterized as silt loam soil. The cation exchange capacity in the area is 8.27 cmol L^{-1}, and the average soil bulk density is 1.49 g cm^{-3}. The details of soil characteristics are listed in Table S1. There is a meteorological station located about fifty meters from the experimental field. The experimental site is characterized by a continental monsoon climate. The average annual air temperature is 8.0 °C, with a frost-free period of 155 to 180 days and average annual precipitation of 702.9 mm, and with 79% of the rain falling in the spring soybean growing season (May to September). The details of rainfall and air temperatures during the soybean growth period in 2018 to 2020 are presented in Figure S2.

The experiment included two factors: slope and imazethapyr. The slope factor included three different slope gradients: 5° (S1), 10° (S2), and 15° (S3). The imazethapyr factor included two treatments: with imazethapyr (I1) and without imazethapyr (I0). Thus, six combinations of the slope with and without imazethapyr were tested in this study: S1I1, S2I1, S3I1, S1I0, S2I0, and S3I0. Each treatment was performed using three replications. The total area of each plot was 8 m^2 (8 m × 1 m). The soybean was irrigated using an artificial rainfall simulation system under a large-scale open–closed-type rain-proof shelter that was always open except during rainy days (Figure S1). The irrigation amount of soybean during the growth period was determined according to local farmers, and the details of irrigation are listed in Table S2. The application rate of imazethapyr (active ingredient 5%, Shandong CYNDA (Chemical) CO., LTD, Jinan, China.) was 140 g ha^{-1}, as recommended by the manufacturers and local farmers.

The soybean variety was "LiaoDou 15", cultivated by the Liaoning Academy of Agricultural Sciences. The soybean was planted on 5th June, 25th May, and 6th June, and harvested on 21st September, 16th September, and 22nd September in 2018, 2019, and 2020, respectively. Each plot was planted with two rows of soybean crops, with plant spacing of 20 cm and row spacing of 30 cm. Before planting, the slopes were irrigated to ensure that the soil had the necessary moisture to germinate the plants. Before soybean emergence (7 days after sowing), an imazethapyr water agent was prepared and sprayed uniformly on the sloped soil with a sprayer. For chemical fertilizer, 27.0 kg ha^{-1} N, 30.1 kg ha^{-1} P, and 0 ha^{-1} K were applied as base fertilizers.

2.2. Sampling and Measurements

After three years of imazethapyr application, in the third year of the experiment (2020), soil samples at 0–20 cm were collected in the mature stage of soybean to measure soil bacterial and fungal communities. Specifically, in each plot, we collected nine samples from upslope to downslope and then mixed these to form one sample. Thus, each treatment had three replicates. In addition, soil samples were collected from the soybean rhizosphere. These soil samples were stored at −80 °C for DNA extraction.

Soybean root samples in the 0–40 cm soil layer were taken during the maturing stage. Root samples within an area of 40 cm × 40 cm around the root were dug out with a spade from soil layers at a depth of 0–40 cm. All root samples were carefully washed and then scanned by a scanner (Epson Perfection V700, Nagano, Japan). The root length, surface area, and volume of the soybean were calculated using the WinRHIZOPro software 2013e (Regent Instruments, 2013, Quebec City, QC, Canada).

2.3. DNA Extraction and PCR Amplification

Genomic DNA of the microbial communities was extracted from 0.5 g soil samples using an E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The extracted DNA was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using an ABI GeneAmp® 9700 PCR thermocycler (ABI, Los Angeles, CA, USA) [27]. The internal transcribed spacer (ITS) regions of the fungal genes were amplified by PCR using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) [27].

The raw 16S rRNA and ITS gene sequencing reads were then demultiplexed, quality-filtered via fastp version 0.20.0 [19], and merged using FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, truncated reads shorter than 50 bp were discarded, and reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequences. The maximum mismatch ratio of the overlap region was 0. Reads that could not be assembled were discarded; (iii) samples were distinguished according to their barcodes and primers, and the sequence direction was adjusted with exact barcode matching and 2 nucleotide mismatches in primer matching. Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using UPARSE version 7.1 [28,29], and chimeric sequences were then identified and removed. The taxonomy of each OTU representative sequence was analyzed via RDP Classifier version 2.2 [30] against the 16S rRNA database (e.g., Silva v138) and ITS database using a confidence threshold of 0.7.

2.4. Function Group Analysis

The relative abundance of gene families in the bacterial community was determined using the PICRUSt2 software [31]. The gene families involved in nitrogen and carbon cycling were then selected according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [32,33]. In this study, gene families involved in the nitrogen cycling pathways, including nitrification, denitrification, assimilation, nitrogen fixation, ammonification, dissimilatory nitrate reduction, and assimilatory nitrate reduction, were compared among treatments. For carbon cycling pathways, the main carbon cycling pathways (i.e., anaerobic carbon fixation, Calvin cycle carbon fixation, fermentation, aerobic respiration, methanogenesis, methane oxidation, and anaerobic CO oxidation) were compared among treatments. The microbes involved in these carbon cycling pathways were determined according to Llorens-Mares et al. [34]. The functions of the Calvin cycle, methane oxidation, and methanogenesis were predicted using the FAPROTAX database [35].

2.5. Data Analysis and Statistics

The differences between experimental variables and their interactions were analyzed using two-way analysis of variance (ANOVA) with the R software. The least significant difference (LSD) method was used to determine the significant differences between values. The contributions of slope and imazethapyr to soybean roots were calculated using R software. Principal coordinate analysis based on the Bray–Curtis distance and Adonis analysis was applied to evaluate the influence of treatments on the soil microbial community structure. The co-occurrence networks of bacterial and fungal communities were constructed via the molecular ecological network analysis (MENA) method [23]. Only the microbes observed at a genus level in more than six treatments were used to construct the co-occurrence network. Only significant Pearson correlation coefficients greater than 0.8 and 0.75 in the bacterial and fungal communities were retained in the co-occurrence network. The networks were constructed using the Gephi software (version 0.9.2). The interactions between biomarkers and soybean roots were calculated via the Spearman method using Origin software (version 2021, OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Root Growth

The root traits of soybean are illustrated in Figure 1. As shown in Figure 1A–C, compared to the I0 treatments (S1I0, S2I0, and S3I0), the I1 treatments (S1I1, S2I1, and S3I1) significantly increased the root length by 11.8, 26.5, and 11.7 m on 5°, 10°, and 15° slopes, respectively. For root surface area, the I1 treatment significantly increased the root surface area by 173.3, 324.2, and 171.7 cm^2, respectively, compared to the I0 treatment on the three slopes. In addition, the root volumes in the I1 treatments (S1I1, S2I1, and S3I1) were 2.0, 3.1, and 1.8 cm^3 greater, respectively, than those in the I0 treatments on the three slopes. These results indicated that the application of imazethapyr significantly increased soybean growth in the sloped soils. For the effects of the slope gradient on soybean root growth, the 10° slope had the highest values for root traits. Compared to the 5° and 15° slopes, the 10° slope increased root length, surface area, and volume by 78.0%, 51.3%, and 28.7% and 22.8%, 24.8%, and 26.5%, respectively. Figure 1D–F shows that the slope gradient and imazethapyr explained 94.1%, 96%, and 96.6% of the variance in root length, surface area, and volume, respectively, indicating that slope gradient and imazethapyr had large influences on soybean root growth. Figure 1D–F also shows that imazethapyr explained 53.2%, 71.8%, and 84.2% of the variance in root length, surface area, and volume, respectively, indicating that imazethapyr had a greater influence than slope gradients on soybean root growth.

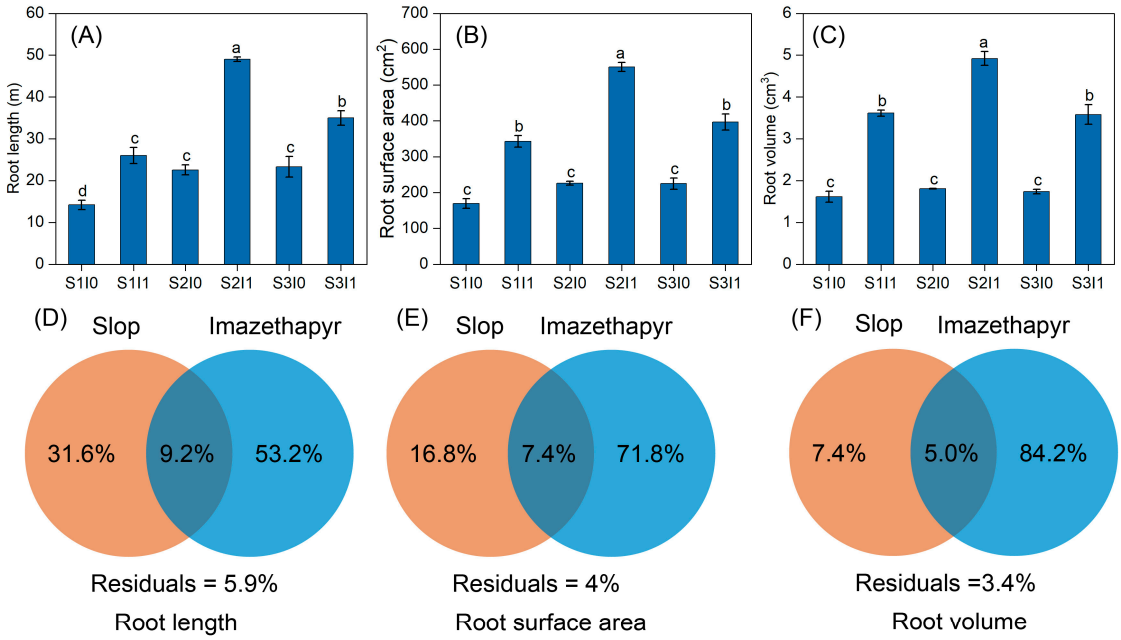


Figure 1. The effect of slope gradient and imazethapyr on soybean root growth. Note: I0 and I1 represent respectively the treatment with or without imazethapyr; S1 to S3 represent three slope gradients, respectively. Different letters in (**A**–**C**) indicate significant differences at $p < 0.05$. The values in the (**D**–**F**) indicate the percentage of the variance of soybean root traits explained by slope gradient (red) and imazethapyr (blue). The ANOVA result was listed in Table S3.

3.2. Soil Microbial Community Diversity and Composition

Considering that the influence of imazethapyr on soybean roots was significantly greater than that of the slope, we further analyzed the microbial mechanisms behind the influence of imazethapyr on soybean roots. Table 1 shows that the application of imazethapyr decreased the Shannon and Sobs indexes of the bacterial and fungal communities, except for the Sobs index of the fungal community on a 5° slope. This result indicated that the application of imazethapyr generally decreased the alpha diversity of bacterial and fungal communities. For beta diversity, Figure 2 shows that the application of imazethapyr had significant impacts on the structures of soil bacterial and fungal communities. As shown in Figure 2, the communities with and without imazethapyr treatment were clearly separated. The results of the Adonis analysis showed that the R^2 values of the bacterial communities on the three slopes were 0.54, 0.47, and 0.4, respectively, with *p*-values of 0.03. Similarly, the R^2 values of the fungal communities on the three slopes were 0.4, 0.28, and 0.46, respectively, with *p*-values lower than 0.05. Overall, imazethapyr decreased the diversity of bacterial and fungal communities and changed the bacterial and fungal structures.

Table 1. The diversity and richness of bacterial and fungal communities as indicated by Shannon and Sobs indices, respectively.

Treatment	Bacterial Community		Fungal Community	
	Shannon	Sobs	Shannon	Sobs
S1I0	3.47 ± 0.4^{ab}	611 ± 59^{a}	3.43 ± 0.4^{ab}	362.25 ± 59^{b}
S1I1	3.46 ± 0.3^{ab}	592.75 ± 38.5^{a}	3.44 ± 0.3^{ab}	551 ± 38.5^{a}
S2I0	3.85 ± 0.1^{ab}	563.25 ± 15.9^{a}	3.69 ± 0.1^{ab}	611 ± 15.9^{a}
S2I1	3.7 ± 0.1^{ab}	551 ± 52.9^{a}	3.84 ± 0.1^{ab}	520.5 ± 52.9^{a}
S3I0	4.23 ± 0.1^{a}	520.5 ± 31^{a}	4.23 ± 0.1^{a}	592.75 ± 31^{a}
S3I1	3.14 ± 0.2^{b}	362.25 ± 37.5^{b}	3.12 ± 0.2^{b}	563.25 ± 37.5^{a}

Note: I0 and I1 represent treatment with or without imazethapyr; S1 to S3 represent three slope gradients. Different letters in the same column indicate significant differences at $p < 0.05$.

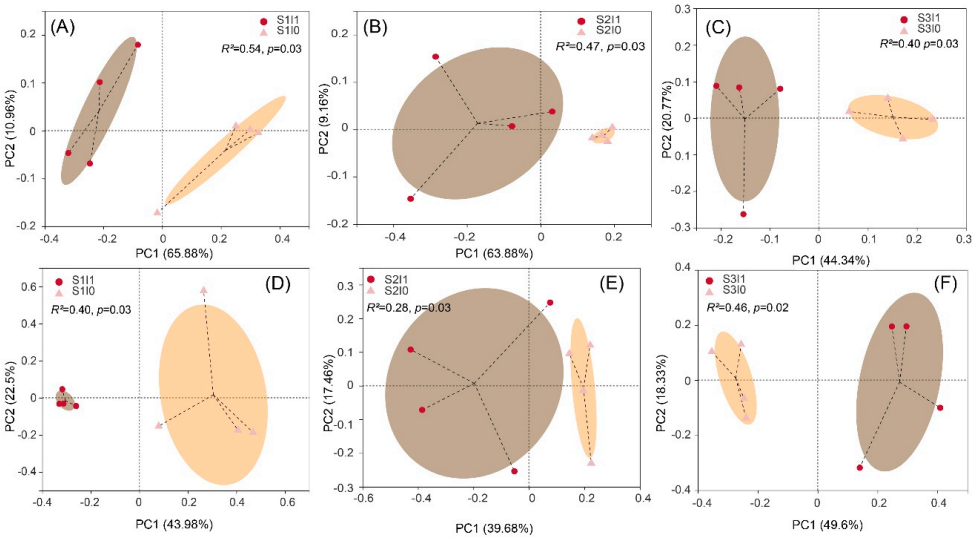


Figure 2. The effects of imazethapyr on bacterial and fungal community structures on different slope gradients. Note: I0 and I1 represent respectively the treatment with or without imazethapyr; S1 to S3 represent three slope gradients, respectively; (**A**–**C**) the effects of imazethapyr on bacterial community structures on the S1, S2, and S3 sloped fields, respectively; (**D**–**F**) the effects of imazethapyr on the fungal community structures on the S1, S2, and S3 sloped fields, respectively.

To further explore which microbes are changed using imazethapyr, we analyzed the effects of imazethapyr on the composition of soil bacterial and fungal communities. For the bacterial community, Figure 3 shows that at the phylum level, the relative abundance of *Actinobacteriota* was 35.7%, making it the dominant phylum. In addition, the *Actinobacteriota* counts were similar under the I0 and I1 treatments at 35.5% and 35.8%, respectively. *Proteobacteria* was the second most represented phylum, with a relative abundance was 24.6%. In addition, compared to the I0 treatment, I1 treatment increased the relative abundance of *Proteobacteria* by 41.7%, 35.8%, and 28.1% on the 5°, 10°, and 15° slopes, respectively. In addition, the I1 treatment improved the relative abundance of *Bacteroidota*, *Cyanobacteria*, and *Patescibacteria* by 47.6~174.1%, 242.1~1331.4%, and 49.6~298.0%, respectively, compared to the I0 treatment. For the fungal community at the phylum level, *Ascomycota, Basidiomycota*, and *Mortierellomycota* were the top three dominant phyla, with a relative abundance of 76.9%, 12.7%, and 5.8%, respectively. Moreover, the I1 treatment increased the relative abundance of *Ascomycota* by 73.2%, 15.7%, and 14.6% on the 5°, 10°, and 15° slopes, respectively, compared to the I0 treatment. In contrast, compared to the I0 treatment, the I1 treatment decreased *Basidiomycota* and *Mortierellomycota* by 28.0~74.6% and 77.9~93.9%, respectively.

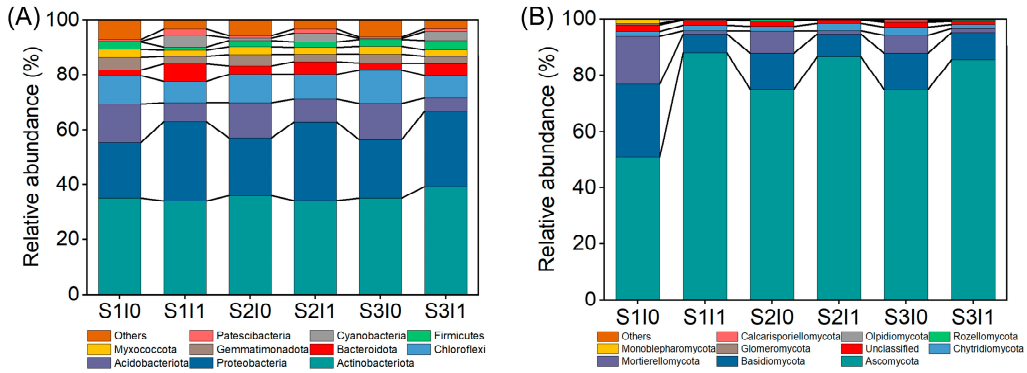


Figure 3. The composition of bacterial (**A**) and fungal (**B**) communities at the phylum level. Note: I0 and I1 represent respectively treatment with or without imazethapyr; S1 to S3 represent three slope gradients, respectively.

3.3. Co-Occurrence Networks

To further explore the impact of imazethapyr on the internal interactions between soil bacterial and fungal communities, we constructed co-occurrence networks of bacterial and fungal communities. As shown in Figure 4, in the bacterial co-occurrence networks of the I0 and I1 treatments, *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* were the three phyla with the highest numbers of nodes. In addition, these three phyla were scattered in the networks, indicating that these phyla played a vital role in connecting with each other in the networks. Compared to I0 treatment, I1 treatment increased *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* by 6.4%, 9.1%, and 11.2%, respectively. This result indicates that the use of imazethapyr increased the connectivity of the network. However, Figure 4C,D also shows that the hubs and connectors in the I0 network were higher than those in the I1 network. In addition, the nodes and links in the I0 network were 9.2% and 78.8% higher than those in the I1 network, indicating that the use of imazethapyr decreased the scale of the co-occurrence network of the bacteria. Although the scale of the co-occurrence network decreased with the use of imazethapyr, positive connections increased by 8.3%. This result indicated that imazethapyr increased the cooperation between microbes. Overall, for the bacterial co-occurrence network, the I1 treatment decreased the scale of the network but also improved the connectivity and positive relationships in the network.

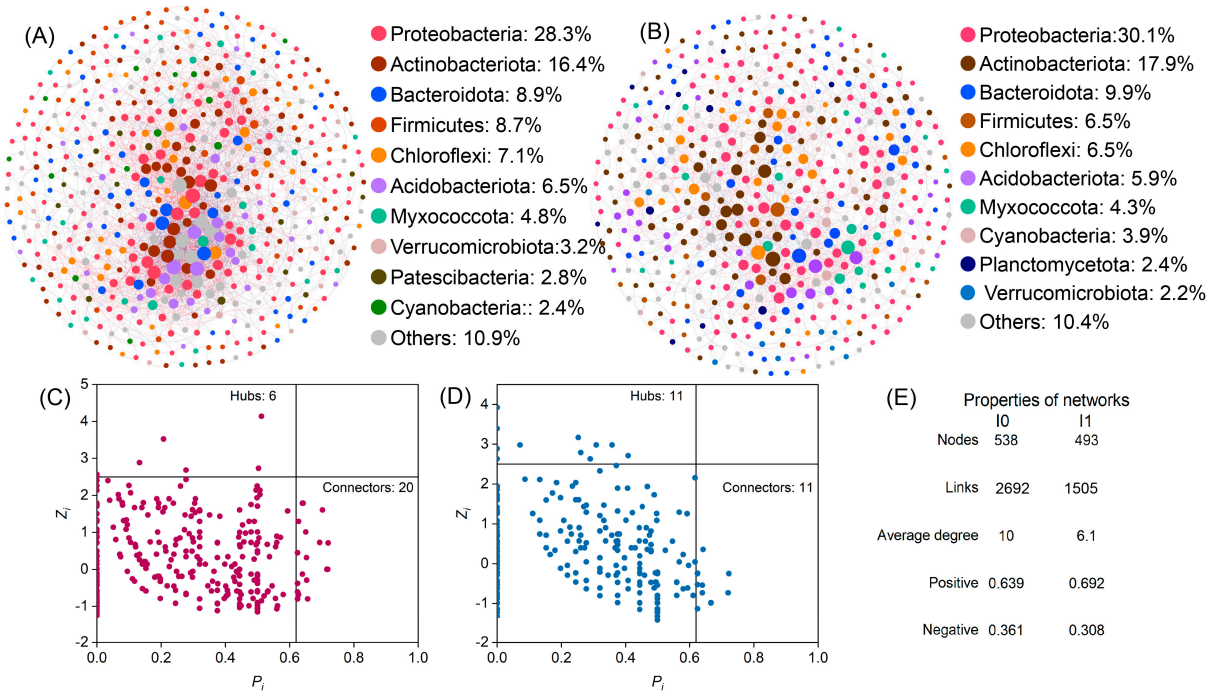


Figure 4. Cooccurrence networks of the bacterial community with and without imazethapyr. Note: (**A**,**B**) co-occurrence networks of the bacterial community with and without imazethapyr, respectively; (**C**,**D**) hubs and connectors in the co-occurrence networks of the bacterial community, respectively; (**E**) properties of the co-occurrence networks. I0 and I1 represent respectively treatment with or without imazethapyr; the nodes in the networks are colored by phylum; red and blue lines represent a positive correlation and a negative correlation, respectively; the genera with Z_i values over 2.5 were considered module hubs that play key roles in connecting modules; the genera with P_i values over 0.62 were considered connectors that play key roles in connecting the inner modules of nodes.

For the fungal co-occurrence network, most nodes were from the *Ascomycota* (68.6%) and *Basidiomycota* (22.8%) phyla in the fungal co-occurrence network (Figure 5). These two phyla were scattered evenly in the co-occurrence network, indicating that they play key roles in connecting the network. Moreover, compared to the I0 network, the I1 network increased *Ascomycota* and *Basidiomycota* by 1.8% and 5.8%, respectively. However, Figure 5E

shows that the links in the I1 network decreased by 17.9% compared to the I0 network, indicating that imazethapyr decreased the scale of the fungal co-occurrence network. In contrast, Figure 5E also shows that the positive relations in the I1 network were 3.2% higher than those in the I0 network, indicating that imazethapyr increased the cooperation relationship in the fungal co-occurrence network. Overall, similar to the bacterial co-occurrence network, the use of imazethapyr decreased the scale of the fungal co-occurrence network but increased the connectivity of the network and strengthened the positive relationships in the network.

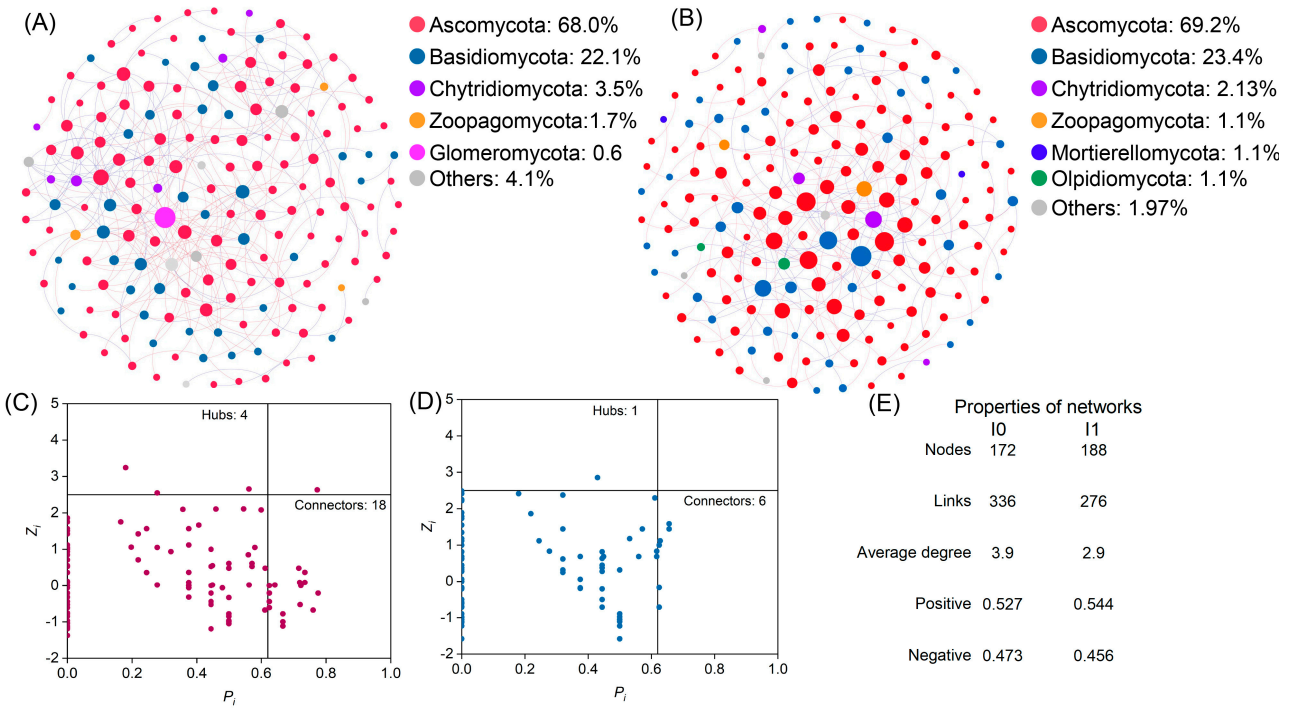


Figure 5. The co-occurrence networks of the fungal community with and without imazethapyr. Note: (**A**,**B**) co-occurrence networks of the fungal community with and without imazethapyr, respectively; (**C**,**D**) hubs and connectors in the co-occurrence networks of the fungal community, respectively; (**E**) properties of the co-occurrence networks. I0 and I1 represent treatment with or without imazethapyr, respectively; the nodes in the networks are colored by phylum; red and blue lines represent a positive correlation and a negative correlation, respectively; the genera with Z_i values over 2.5 were considered module hubs that play key roles in connecting modules; the genera with P_i values over 0.62 were considered connectors that play key roles in connecting nodes' inner modules.

3.4. The Interaction between Biomarkers and Soybean Roots

To further explore the impact of soil microbes on soybean roots, a total of 77 hubs and connectors in bacterial (Figure 4C,D) and fungal communities (Figure 5C,D) were considered as biomarkers, and the interactions between biomarkers and soybean root traits were calculated. As shown in Figure 6A, 30 genera had a significant relationship with soybean root length, surface area, and volume. Among these 30 genera, the relative abundance of genera that had significant positive relations with root length, surface area, or volume was significantly higher in the I1 treatment than that in the I0 treatment (Figure 6B). In contrast, the relative abundance of genera that had a negative influence on root length, surface area, or volume was higher in the I0 treatment than that in the I1 treatment. In this study, the genera that were positively or negatively related to soybean were considered root-promoting biomarkers or root-limiting biomarkers. This result indicated that the I1 treatment increased the relative abundance of root-promoting biomarkers. In addition, Figure 6 also shows that most root-promoting biomarkers were from the *Proteobacteria* and *Bacteroidota* phyla. Overall, compared to the I0 treatments, the I1 treatments improved the

relative abundance of root-promoting biomarkers and reduced the relative abundance of root-limiting biomarkers.

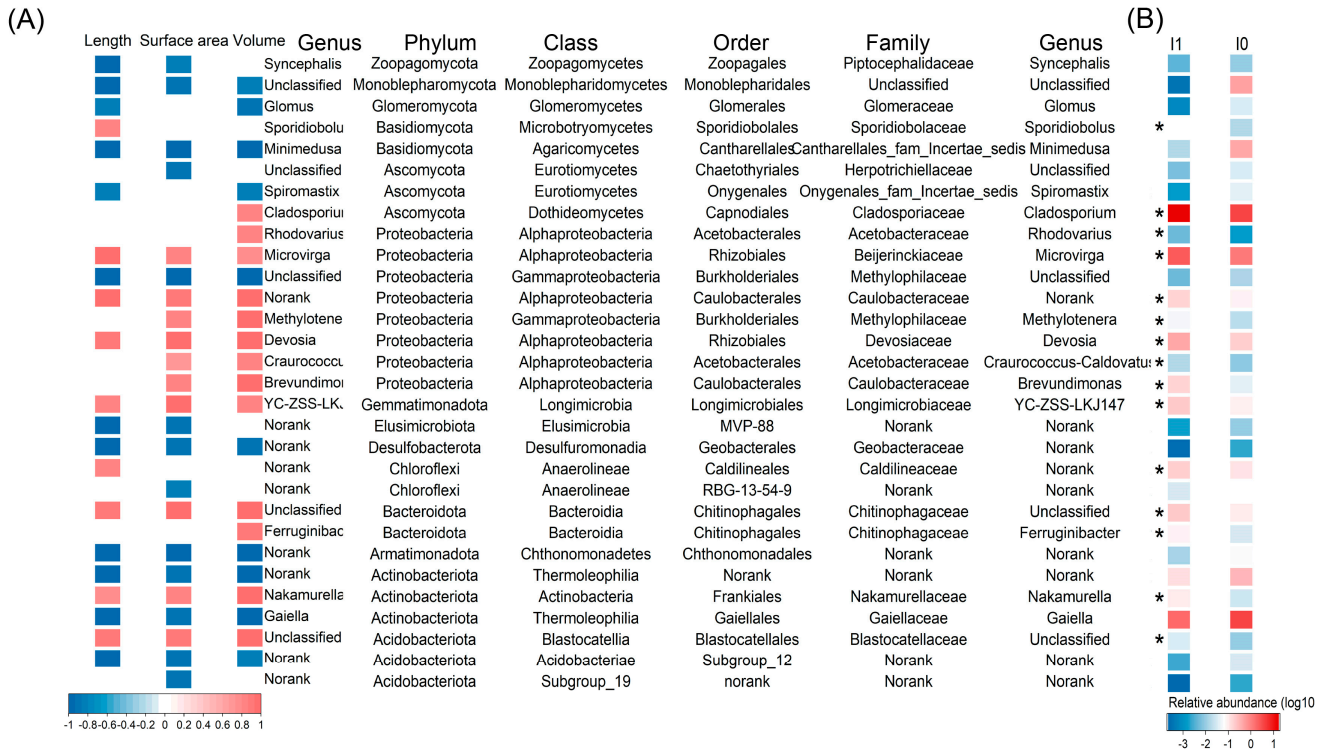


Figure 6. The relationships between soybean root traits and biomarkers (**A**) and the effect of imazethapyr on biomarkers (**B**). Note: I0 and I1 represent respectively treatment with or without imazethapyr. One asterisk means that the effects were significant at $p < 0.05$; the values in (**A**) represent the relative coefficient calculated by the Spearman method; the values in (**B**) represent the relative abundance of biomarkers (log10–converted) in I0 and I1 treatments.

3.5. Nitrogen and Carbon Cycling Pathways

Figure 7 shows the effects of imazethapyr on the soil nitrogen and carbon cycling pathways on three slopes. For soil nitrogen cycling pathways, on the 5° slope, the I1 treatment increased the expression of gene families encoding enzymes for the conversion of NH_4^+ to NH_2OH (*amoABC*) and of NH_2OH to NO_2^- (*hao*) by 55.3% and 61.3%, respectively. However, the other gene families decreased by 13.8%~106.3% in the I1 treatment. On the 10° slope, compared to the I0 treatment, the I1 treatment decreased all gene families involved in nitrogen cycling pathways by 7.1~128.7%. Similar to the 5° slope, on the 15° slope, only gene families encoding enzymes involved in the conversion of NH_4^+ to NH_2OH and (*amoABC*) and of NH_2OH to NO_2^- (*hao*) had increased expression, while the expression of other gene families decreased under the I1 treatment compared to the I0 treatment. Overall, compared to the I0 treatment, the I1 treatment decreased almost all gene families encoding nitrogen cycling pathways, including nitrification, denitrification, nitrogen fixation, assimilation, ammonification, dissimilatory nitrogen reduction, and assimilatory nitrogen reduction pathways (Figure 7A–C). Similar to nitrogen cycling pathways, most carbon cycling pathways were limited under the I1 treatment, except for fermentation and anaerobia CO oxidation (Figure 7D–F). This result indicated that the use of imazethapyr suppressed the most soil nitrogen and carbon cycling pathways.

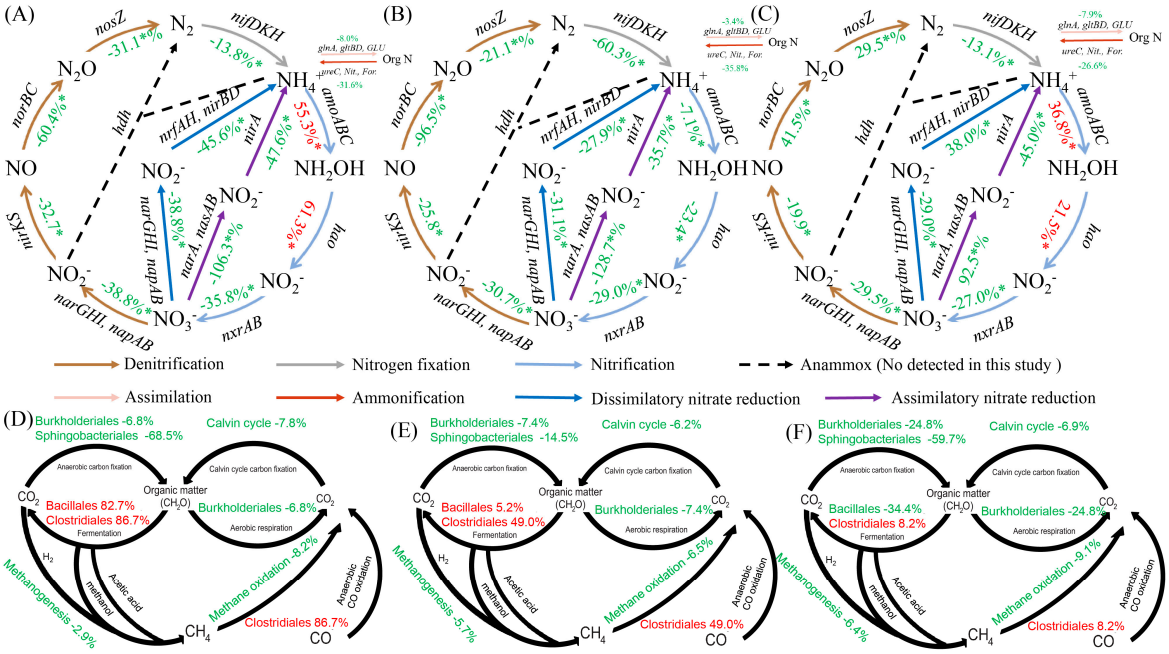


Figure 7. The effect of imazethapyr on nitrogen and carbon cycling pathways. Note: (**A**–**C**) main nitrogen cycling pathways; (**D**–**F**) main carbon pathways; (**A**,**D**) nitrogen and carbon cycling pathways in the 5° sloped field; (**C**,**D**) nitrogen and carbon cycling pathways in the 10° sloped field; (**C**,**F**) nitrogen and carbon cycling pathways in the 15° sloped field. A black asterisk means that the effects were significant at a $p < 0.05$ level. The italics in (**A**–**C**) are the names of gene families. The values were calculated as follows: (value in the treatment with imazethapyr/value in the treatment without imazethapyr − 1) × 100.

4. Discussion

In this paper, we investigated the effects of slope gradient and imazethapyr on soybean root growth and the alpha and beta diversity of bacterial and fungal communities. We also explored the effects of imazethapyr on the co-occurrence networks and biomarkers of the bacterial and fungal communities and how these biomarkers influence soybean root growth. We found that the application of imazethapyr can increase soybean root growth and significantly change the microbial diversity and structures associated with soybean root growth in sloped soils. We further found that the application of imazethapyr suppressed the nitrogen and carbon cycling pathways.

Many studies previously explored the effects of herbicide on crop growth [36–38]. However, most of these studies were conducted in normal fields. The effects of imazethapyr on soybean root growth in sloped soils remain scarce in the literature. In this study, compared to treatment without imazethapyr, the application of imazethapyr significantly increased the soybean root length, surface area, and volume across all slopes by 11.7~26.5 m, 171.7~324.2 cm^2, and 1.8~3.1 cm^3, respectively. In addition, our results showed that changes in imazethapyr could better explain the significantly larger variance in root length, surface area, and volume than slope, indicating that imazethapyr was the main factor influencing soybean root growth (Figure 2D–F). This result is inconsistent with the result of Qu et al. [1], who found that application of herbicide decreased wheat roots' fresh weight in an incubation experiment. The reason for this phytotoxic effect of herbicide was probably that the experiment was an incubation experiment without weed. Similarly, Zheng et al. [39] also found that the application of imazethapyr limited the growth of *Arabidopsis thaliana* in an incubation experiment. In contrast, a probable explanation for increased soybean root growth after using imazethapyr in this study is that the application of imazethapyr suppressed the growth of weeds surrounding crops, which decreased the competition of crops in the uptake of soil nitrogen, potassium, phosphorus, etc. [3,7,8].

Indeed, many studies have demonstrated that the application of imazethapyr or other herbicides increases crop growth in field experiments. For example, Singh and Singh [40] found that optimal application of imazethapyr improved the crop yield in a field scare. However, the microbial mechanisms behind the improvement effects of imazethapyr and other herbicides are not well understood, especially in sloped soils.

To further explore the microbial mechanisms that lead to improved crop growth after the application of imazethapyr on sloped soils, we studied the effects of slope gradients and imazethapyr on the diversity of soil bacterial and fungal communities. Our results showed that the use of imazethapyr generally reduced the diversity and richness of bacterial and fungal communities (Table 1). This result is consistent with those of some previous studies. For example, Pertile et al. [9,41] found that imazethapyr decreased microbial biomass. Liu et al. [17] also found that imazethapyr could decrease the Shannon index of the bacterial community in the *Arabidopsis thaliana* rhizosphere. In addition, at field conditions, the limitation effects of imazethapyr on the bacterial diversity were also found by Singh and Singh [42]. This result is reasonable. Two probable reasons could explain this result. First, imazethapyr suppresses microbe growth while simultaneously suppressing weed growth [1,38]. Second, imazethapyr can prompt crops to recruit some beneficial microbes for resisting harm from imazethapyr, which results in the soil microbes being more specialized but with reduced microbial diversity [17,43,44]. In addition, we also found that the structures of bacterial and fungal communities between the treatments with or without imazethapyr were significantly different, indicating that imazethapyr significantly reshaped the structures of bacterial and fungal communities. This result is reasonable because imazethapyr can suppress some microbes and help crop roots recruit beneficial microbes to improve environmental tolerance [1,45]. In addition, the increased root growth results in increased exudates, which would also result in increased pathogen growth [46]. Singh and Singh [42] also found that the application of imazethapyr had changed the bacterial structure. To determine which specifical microbes were changed in response to imazethapyr, we further explored the compositions of bacterial and fungal communities at the phylum level. We found that imazethapyr did not increase the representation of the most abundant phylum (*Actinobacteriota*) but did increase the relative abundance of the second most represented phylum (*Proteobacteria*) by 28.1~41.7% across the three sloped soils. This result is consistent with the results of Pertile et al. [9], who also found that the application of imazethapyr increased the relative abundance of *Proteobacteria* [9]. Similarly, Liu et al. [17] also found that *R*-imazethapyr increased the relative abundance of *Proteobacteria*. In addition, *Proteobacteria* are beneficial microbes that can improve crop growth and increase the capacity of crops to absorb soil nutrients [47]. The reason for the improved relative abundance of *Proteobacteria* by the application of imazethapyr was probably that the soybean roots recruited the beneficial microbes for improving the absorption of soil nutrients. In fungal communities, imazethapyr increased the representation of the most abundant phylum (*Ascomycota*) by 14.6% ~ 73.2% across the three sloped soils. Previous studies had proved that the *Ascomycota* was one of the most abundant phyla in the soil [48]. Therefore, the reason for increased *Ascomycota* by the application of imazethapyr was probably that imazethapyr limited the growth of weeds and improved soil nutrients for *Ascomycota* growth.

To further clarify how imazethapyr reshaped the structures of bacterial and fungal communities, co-occurrence networks were constructed in this study. The results showed that imazethapyr decreased the number of hubs, connectors, nodes, and links of bacterial and fungal communities, indicating that imazethapyr decreased the scale of co-occurrence networks in these communities. This result is consistent with the above results indicating imazethapyr decreases the diversity of bacterial and fungal communities. Similarly, Qu et al. [1] found that the use of S-metolachlor herbicides altered the structures of bacterial and fungal communities. Zhang et al. [49] also found that the application of imazethapyr clearly changed the soil microbial biomass and shifted the structure of the microbial community in soybean fields. However, we found that imazethapyr increased

the nodes from the bacterial phyla of *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* and the fungal phyla of *Ascomycota* and *Basidiomycota*. The nodes from these phyla were evenly scattered throughout the co-occurrence network, indicating that these phyla play a key role in fostering connections in the network and improving its stability. Moreover, imazethapyr improved the positive relations in the co-occurrence network, indicating that imazethapyr also improved the symbiotic relationships in the co-occurrence network, likely because imazethapyr induced soybean roots to recruit beneficial microbes, which resulted in shrinking of the co-occurrence network, though it was more stable and symbiotic. Similarly, Qu et al. [1] found that S-metolachlor strengthened the positive relationships in the co-occurrence network of bacterial communities of wheat root. Overall, although imazethapyr decreased the scale of the co-occurrence network, it improved the stability and symbiotic relationships within. The reason for this result was probably that microbes in co-occurrence networks were recruited by soybean roots [43,44].

We further found that some hubs and connectors (biomarkers) in the co-occurrence networks play a key role in soybean root growth. We defined the biomarkers that were positively or negatively related to soybean root growth as root-promoting or root-limiting microbes, respectively. Interestingly, imazethapyr significantly improved the relative abundance of root-promoting biomarkers and decreased root-limiting biomarkers. This result indicated that imazethapyr improves soybean root growth mainly by increasing the relative abundance of root-promoting biomarkers. This result is reasonable because the application of imazethapyr helps crop roots to recruit beneficial microbes for resisting harm from imazethapyr [43,44]. Liu et al. [17] also found that crops can drive the composition of plant-associated microbiomes and selectively enrich beneficial rhizosphere microbes by releasing root exudates. We further found that most root-promoting biomarkers were from the phyla of *Proteobacteria* and *Bacteroidota*, while most biomarkers in the fungal community were root-limiting biomarkers. Previous studies also reported that most microbes in *Proteobacteria* and *Bacteroidota* phylum were beneficial microbes [17,21]. This result indicated that imazethapyr not only increased the growth of beneficial microbes but also suppressed that of pathogens. The probable explanations for this result are that (1) imazethapyr decreases competition for soil nutrients from weeds, enabling soybean to thrive, which increased root exudations for recruiting beneficial microbes [17], and (2) imazethapyr also causes soybean roots to recruit some microbes that result in increased imazethapyr tolerance [49]. We further found that imazethapyr suppressed nearly all pathways of nitrogen and carbon cycling in the three sloped soils, which likely occurred because imazethapyr decreased the diversity of bacterial and fungal communities. Similarly, Yu et al. [50] explored the effects of the application of herbicides on soil nitrogen cycling and also found that the application of herbicides limited the denitrification processes. This result indicated that imazethapyr increases soybean growth but also suppresses the nitrogen and carbon cycling pathways.

5. Conclusions

To the best of our knowledge, this work is one of the first studies to estimate the influence of imazethapyr on soybean root growth, microbial communities, microbial co-occurrence networks, the interactions between microbes and crop root growth, and nitrogen and carbon cycling pathways in sloped soils. Our results showed that the application of imazethapyr significantly improved soybean root growth (i.e., root length, surface area, and volume). The application of imazethapyr also decreased bacterial and fungal diversity and reshaped the structures of bacterial and fungal communities. In addition, imazethapyr reduced the scale of co-occurrence networks in bacterial and fungal communities (i.e., the number of links in the co-occurrence network). However, imazethapyr also increased nodes that were scattered evenly in the co-occurrence networks and strengthened positive relations in the co-occurrence networks. These results indicated that imazethapyr increased the stability and cooperation of bacterial and fungal communities. We further found that imazethapyr improved the relative abundance of root-promoting biomarkers, most of which were from the bacterial phyla of *Proteobacteria* and *Bacteroidota*. In fungal communi-

ties, most key biomarkers were root-limiting biomarkers, which were suppressed by the application of imazethapyr. Ultimately, the application of imazethapyr increased the levels of root-promoting biomarkers and reduced those of root-limiting biomarkers, in addition to increasing the stability and cooperation of bacterial and fungal communities, thereby enhancing the growth of soybean roots. Nevertheless, the application of imazethapyr also had some negative impacts on soil microbial diversity and the nitrogen and carbon cycling pathways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14063518/s1, Figure S1. The open–closed-type rain-proof shelter for slope soybean field; Figure S2. Daily minimum and maximum temperatures, and precipitation at the experimental site throughout the soybean growing seasons (2018 to 2020); Table S1. The details of soil characteristics; Table S2. The diversity and richness of bacterial and fungal communities; Table S3. The ANOVA result of soybean root traits.

Author Contributions: Data curation, Z.W.; Methodology, Z.W.; Writing—original draft, Z.W.; Writing—review and editing, X.W. and T.W. Conceptualization: methodology, software, Z.W.; validation, formal analysis, X.W.; investigation, resources, T.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly supported by the National Natural Science Foundation of China (No. 31570706) and the Key Research and Development Projects of Liaoning Province, China (2018103007).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all authors involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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