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Effects of field-grown transgenic *Cry1Ah1* poplar on the rhizosphere microbiome

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Highlights

- *Cry1Ah1* has no significant effects on the rhizosphere microbiome population.
- *Cry1Ah1* expression does not change the community diversity and structure of rhizosphere bacteria.
- The relative abundances of most rhizosphere microbe are not notably influenced by *Cry1Ah1* expression.

Abstract

Populus is a genus of globally significant plantation trees used widely in industrial and agricultural production. Poplars are easily damaged by *Micromelalopha troglodyta* and *Hyphantria cunea*, resulting in decreasing quality. Because of their strong insect resistance, *Bt* toxin-encoded *Cry* genes have been widely adopted in poplar breeding. Therefore, the potential adverse effects of *Cry1Ah1*-modified (CM) poplars on the ecological environment have been concerned. The Illumina novaseq platform was used to perform high-throughput sequencing. Alpha diversity analyses were performed using the Chao1 index to determine community richness, and the Shannon index analyses were used to determine community richness and evenness. Our analysis of rhizosphere soil chemistry patterns revealed that rhizosphere soil available phosphorus, rhizosphere microbial biomass nitrogen, and rhizosphere phosphorus levels were declined. In contrast, rhizosphere microbial biomass carbon level increased in CM poplar rhizosphere samples. We applied metagenomic sequencing of non-transgenic (NT) and CM poplar rhizosphere samples collected from a natural field; the predominant taxa included Proteobacteria, Acidobacteria, and Actinobacteria. Together, these results showed that the *Cry1Ah1* expression has no significant influences on the community composition of rhizosphere microbiomes. Also, the *Cry1Ah1* expression in poplars has no notable effects on the relative abundances of most rhizosphere bacteria. In addition, there are no significant adverse effects of CM varieties on most rhizosphere fungal abundances. However, only a few rhizosphere fungal abundances differ in NT and CM varieties.

Keywords: *Bt* toxins; *Cry1Ah1*-modified poplar; metagenomic sequencing; Transgenic poplar; rhizosphere

1 Introduction

Poplar (*Populus*) is a genus of globally important plantation trees used widely in industrial and agricultural production (Boerjan, 2005). However, with the deterioration of the global environment, characterized by increasing salt, drought, pest, and disease stresses, the global production of poplar is becoming challenging. One approach to address this challenge is genetic modification. The manipulation of critical genes has been applied to alter poplar characteristics in transgenic lines, resulting in improved traits for better growth in adverse environments (Genissel et al., 2003; Xu et al., 2019; Yang et al., 2015).

Insecticide resistance based on *Bacillus thuringiensis* (*Bt*) has allowed the development of a variety of insect resistance proteins for commercial genetically modified (GM) crops (Beegle and Yamamoto, 1992). In addition, *Bt* toxin-encoded *Cry* genes have been widely applied in commercial GM crops, improving plant resistance to insect pests (Crecchio and Stotzky, 2001; Dohrmann et al., 2013). Despite the many benefits of *Bt*-modified plants, a significant potential disadvantage is their effect on soil chemical properties and the structure and diversity of rhizosphere microorganisms, including bacteria and fungi (Dohrmann et al., 2013). Therefore, rhizosphere microorganisms associated with *Bt*-modified plants are of great interest (Cotta et al., 2014; Zhaolei et al., 2017; Zhaolei et al., 2018). However, regarding the complexity of field conditions and the lack of a unified approach to microorganism analysis, studies exploring the influence of *Bt*-modified plants on soil microorganisms sometimes produce conflicting results (Čerevková et al., 2018; Li et al., 2018a; Liu et al., 2015; Shu et al., 2019). For example, one study showed that soil microorganisms are not adversely affected by the cultivation of *Bt*-modified cotton plants (Li et al., 2018b).

In contrast, another study suggested that exogenous gene products expressed by *Bt*-modified transgenic crops may interact with soil microorganisms and affect their activities and functions (Liu et al., 2005). *Bt*-modified plants have been found to alter bacterial population diversity compared with

non-transgenic (NT) plants (Singh et al., 2013) and may enhance the population size of soil microbial communities (Hu et al., 2018; Velmourougane and Sahu, 2013). Compared with NT rice, *Bt*-modified rice has been found to have a short-term effect on rhizosphere microbial community function (Wei et al., 2018). Thus, there are some differences among studies on the impact of *Bt*-modified plant varieties on the soil microbial community. Further research is needed to evaluate the safety of *Bt*-modified crop plants. Since the commercialization of GM plants, the global planting area of GM crops has been overgrown, and new varieties of GM plants have been emerging continually (Miethling-Graff et al., 2010). GM plants have provided great economic and environmental benefits worldwide, such as increased plant yield and reduced chemical fertilizer and pesticide application. However, the potential impacts of GM plants on the ecological environment have raised concerns (Mina, 2008). Soil microorganisms are an essential part of the soil ecosystem and involve various biochemical processes, including organic matter accumulation, mineralization, nutrient transformation, and circulation (Ali et al., 2009; Innerebner et al., 2011). In addition, GM plants communicate with soil microorganisms during the growth process; therefore, research on the potential effects of GM plants on the soil microbial community is of great significance in evaluating their potential risks (Debruyn et al., 2017; Inceoğlu et al., 2010; Liu et al., 2019b). The study aimed to identify the effects of *Cry1Ah1*-modified (CM) poplar plantation on soil chemical properties and the diversity and structure of the soil microorganism community after three years of growth under field conditions. We applied high-throughput sequencing of 16S rRNA and internal transcribed spacer 1 (ITS1) to evaluate the diversity and composition differences in rhizosphere microorganisms between NT and CM varieties. The results contribute to our knowledge of how CM varieties affect rhizosphere soil microbial community function and provide reference information for evaluating the safety of CM varieties.

2 Materials and Methods

2.1 Plant material and experimental field design

In the previous study, the *Cry1Ah1* gene was cloned into destination vector pH35GS. CM poplars,' Nanlin 895' (*Populus deltoides* × *P. euramericana*), were regenerated by inoculating poplar leaf discs with *Agrobacterium tumefaciens* strain LBA4404, including recombinant plasmid pH35GS-*Cry1Ah1* (Xu et al., 2019). To study the effects of CM poplars on a natural soil ecosystem, we designed a field test in Sihong, Jiangsu Province (118°68′N, 33°72′E). Three-year-old NT and CM varieties marked as A5-0, A4-6, Z1-3, A5-23, and A3-4 were selected, and six plots were established with four replicates per clone (Supplemental Figure 1). To identify the influence of CM varieties on the rhizosphere soil microbiome, we planted NT poplars around the experimental field (Supplemental Figure 1E). An additional 3-m-wide isolation zone was established between communities, with an area of about 676 m^2 (26 m × 26 m). The poplars were cultivated by cutting in March 2017 with permission from the State Forestry Administration; the experimental field was managed conventionally without chemical fertilizers or pesticides. The experimental field also confirmed similar soil characteristics and microenvironment.

There are four poplars in each small line area, including NT and CM varieties (lines A5-0, A4-6, Z1-3, A5-23, and A3-4) (Supplemental Figure 1). The weeds and leaves were removed from the soil surface, and a soil extractor was used to take out a soil column about 50 cm (diameter 8 cm) around every poplar rhizosphere. The fine roots in the 10-30 cm soil column were carefully taken out, and the soil within 3 mm of fine roots was considered the rhizosphere soil. The rhizosphere soil of every four poplar trees in the small area was collected and mixed into one sample. The rhizosphere soil samples were taken out from the entire experimental field through this sampling method, and there were six duplicate rhizosphere soil samples for NT and each CM variety. All rhizosphere soil samples were passed through a 10-mesh sieve, mixed thoroughly, added to sterile centrifuge tubes, and then placed in a liquid nitrogen tank for transport to the laboratory.

2.2 Identification of *Cry1Ah1* expression level and insecticidal activity

158 The fully expanded poplar leaves were collected from NT and CM varieties, and the collected
159 samples were placed in a liquid nitrogen tank for transport to the laboratory. All the collected leaves,
160 including NT and CM varieties, were assayed to detect Cry1Ah1 expression level using an ELISA kit
161 (EnviroLogix, Portland, ME, USA). In addition, pupae of Micromelalopha troglodyta were collected
162 from poplars cultivated in the field and hatched in a culture room at 27 ± 2°C and 74% humidity with
163 a 14-h light/10-h dark photoperiod. The eggs were collected from a female adult, and instar larvae were
164 fed with NT and CM varieties. Larval mortality of M. troglodyta was counted on the 6th and 12th days.
165 Three independent biological samples have been performed to determine rhizosphere soil physical and
166 chemical indices. Chloroform fumigation was performed to obtain the rhizosphere soil microbial
167 biomass nitrogen (MBN), carbon (MBC), and phosphorus (MBP) (Čerevková et al., 2018; Cui et al.,
168 2020; Franzluebbers et al., 1996; Witt et al., 2000). The rhizosphere soil samples treated with
169 chloroform fumigation and non-chloroform fumigation were extracted with K_2SO_4 solution. The
170 rhizosphere MBC, MBN, and MBP were measured using a Vario TOC cube analyzer (Elementar,
171 Langenselbold, Germany). Briefly, fresh rhizosphere soil samples were dissolved in chloroform, and
172 the mixtures were boiled for 5 minutes in a vacuum. Then, 0.5 mol/L K_2SO_4 was added to the mixtures
173 and subjected to fumigation in the dark at 25°C for 24 h. The resulted mixture was then filtered with a
174 quantitative filter paper. Simultaneously, the control groups were performed similarly, except that the
175 rhizosphere soil samples were added to the reaction mixture for detection.

176 The rhizosphere soil alkaline nitrogen was determined using the Conway method (i.e., the alkali
177 hydrolysis diffusion method). Briefly, 10 mL of NaOH was used to dissolve air-dried soil samples set
178 in the outer chamber of a diffusion dish, and 2 mL of boric acid (an indicator solution) was placed in
179 the inner chamber. After incubation at 40°C for 24 h, NH_3 in the inner chamber absorption solution
180 was titrated with 0.005 mol/L H_2SO_4 as a standard solution. Simultaneously, the control groups were

181 performed similarly, except that the rhizosphere soil samples were added to the reaction mixture for
182 detection.

183 The rhizosphere soil available phosphorus was identified by molybdenum–antimony colorimetry.
184 Briefly, air-dried soil samples were mixed with 0.5 mol/L $NaHCO_3$ and activated carbon, shaken for
185 30 minutes, and filtered immediately with phosphate-free filter paper. Then, 1–5 mL of filtrate was
186 extracted, and the absorbance value was determined. Soil pH was also measured using the glass
187 electrode method. Finally, the rhizosphere soil samples were mixed with 2.5 × the water volume, and
188 the suspension pH was determined using a PP-25 Professional Meter electrode (Sartorius, Germany).
189 Simultaneously, the control groups were performed similarly, except that the rhizosphere soil samples
190 were added to the reaction mixture for detection.

191 **2.3 Rhizosphere soil DNA extraction and high-throughput sequencing**

192 Using the Fast DNA Spin kit for soil (MP Biomedicals, United States), 36 independent
193 rhizosphere soil samples were obtained from the NT poplars and five CM varieties (lines A5-0, A4-6,
194 Z1-3, A5-23, and A3-4). Triplicate DNA extractions from each replicate of rhizosphere soil samples
195 were mixed and composited into one DNA sample to overcome the heterogeneity. The quality and
196 integrity of the DNA were determined by electrophoresis on 0.8% agarose gel, and the extracted DNA
197 samples were diluted 10-fold and stored at −80°C for further molecular analyses. Using the extracted
198 genomic DNA as a template, the V3-V4 region (515f/907r) of the 16S rRNA gene and ITS1 region
199 (1737f/2043r) of the ITS1 rRNA gene were amplified to identify the composition and diversity of the
200 microbiological community (Dennis et al., 2013; Xu et al., 2016a). We then performed high-throughput
201 sequencing using the Illumina novaseq platform. The raw data were filtered using the Trimmatic
202 software to obtain high-quality clean paired-end reads, spliced using the FLASH software. The
203 minimum overlap length was set to 10 bp, and the maximum mismatch ratio of the splicing sequence
204 was 0.1. After filtering, influential splicing segment clean tags were obtained. All clean tags were

clustered using the VSEARCH software (https://github.com/torognes/vsearch). The clean tags were denoised in amplicon sequence variants (ASVs), and chimeras were filtered with UNOISE3 (Edgar, 2016). Taxonomic assignment of ASVs was performed in QIIME 2 v2018.2 (Caporaso et al., 2010, https://qiime2.org) using the QIIME 2 feature classifier plugin (Bokulich et al., 2018).

All data analyses were performed using the SPSS 19.0 software (IBM, USA). Differences in the physical and chemical properties between NT and CM varieties were evaluated using one-way analysis of variance (ANOVA) and Tukey's post hoc comparison. The alpha diversity analyses were performed using the Chao1. They observed species indices to determine community diversity of rhizosphere bacteria and the phylogenetic diversity (PD) whole-tree and Shannon indices to determine community richness and evenness (Lozupone and Knight, 2005). According to ASVs clustering results, alpha diversity was calculated in Mothur v.1.30.1 with rarefaction analysis after sub-sampling the libraries to exact size (Amato et al., 2013). UniFrac was conducted with beta diversity analysis and phylogenetic analysis. Bray-Curtis distances between NT and CM varieties were calculated and visualized with principal component analysis (PCA).

3 Results

3.1 Effects of CM varieties on *M. troglodyta*

The Bt-Cry1AELISA kit was used to identify the *Cry1Ah1* expression level in NT and CM varieties. The results showed that *Cry1Ah1* was expressed in CM varieties, and lines A4-6 and A5-0 had the higher *Cry1Ah1* expression level. In comparison, line A3-4 had the lower *Cry1Ah1* expression level (Supplemental Table1). In addition, the insecticidal activities of CM varieties were identified, and the results showed that the CM varieties had higher insecticidal activity to *M. troglodyta* than NT poplars (Supplemental Figure 2). Significantly, lines A4-6 and A5-0 with higher *Cry1Ah1* expression levels exhibited relatively more substantial insecticidal activity than *M. troglodyta*.

3.2 Effects of CM poplars on rhizosphere soil chemistry patterns

During the first three years of poplar establishment, the mean soil pH ranged from 7.73 to 8.23 in rhizosphere soil (Figure 1A). Also, there was no significant change in rhizosphere soil pH between NT and CM varieties by sampling date. For the CM varieties (lines A5-0, A4-6, Z1-3, A5-23, and A3-4), rhizosphere soil alkaline nitrogen ranged from 64.54 to 83.15 mg/kg, with similar values observed for NT poplars, and no significant difference between NT and CM varieties (Figure 1B). However, the CM varieties had significantly lower rhizosphere soil available phosphorus in the field-grown stage than NT poplars (Figure 1C). The rhizosphere MBC contents of NT poplars ranged from 160 to 172 mg/kg and differed significantly from those of the CM varieties (Figure 1D). In addition, CM varieties had significantly lower MBN and MBP contents than NT poplars (Figure 1E and F).

3.3 Data quality control and ASVs analysis

Using the Illumina hiseq, an average of 53,567 and 68,783 16S rDNA tags and 33,750 and 87,922 ITS1 tags were generated from the rhizosphere microbiome. Chimaeras and short tag sequences were removed to obtain high-quality clean tags comprising an average of 33,390 and 86,787 16S rDNA tags and 21,523 and 22,555 ITS1 tags (Supplemental Table 2). Also, clean tag distributions of rhizosphere bacteria were visualized. The results showed that the lengths of clean tags ranged from 200 to 440 bp. Clean tags with the lengths of 420-440 bp occupied the largest proportion (Supplemental Figure 3A), while clean tag distributions of rhizosphere fungi ranged from 200-360bp, and clean tags with the lengths of 200-260 bp occupied the largest proportion (Supplemental Figure 3B). Using the Qiime ver. 2.0 and Vsearch 2.7.1, the chimeric and organelle sequences were removed to produce 10787 rhizosphere bacterial community sequencing ASVs and 7,732 fungal community sequencing ASVs (Supplemental Table 3).

3.4 Rhizosphere bacterial diversity

The mothur (Schloss et al., 2009) has been applied to perform ASV rarefaction analysis based on ASV clustering results to construct alpha rarefaction curves and evaluate the putative differences in the alpha diversity. The rhizosphere bacteria sample rarefaction curves illustrated that most NT and CM varieties saturate around 6500-7000 ASVs, suggesting slight differences in the diversity of rhizosphere bacterial community between NT and CM varieties (Figure 2A). The Shannon wiener curves (Bates et al., 2013) were also constructed to evaluate the rhizosphere bacterial diversity. The results showed that the Shannon curves are flat when the number of reads is 10000, illustrating that the amount of sequencing data is large enough to reflect the vast majority of rhizosphere bacterial information in the 36 samples. In addition, Shannon curves of the 36 samples fitted together (Figure 2B), which suggested that rhizosphere bacterial communities in different sequencing depths share similar diversity.

The alpha diversity analysis was used to reflect the richness and diversity of rhizosphere bacteria. The Chao1 indexes in NT poplars had highly similar results in CM varieties, including A5-23, Z1-3, and A3-4. At the same time, the *Cry1Ah1* expression might increase the community richness of rhizosphere bacteria, which the analysis of Chao1 showed no dominant differences between NT and CM varieties (Figure 3A and Supplemental Table 4). Also, the observed species in NT poplars had no dominant differences compared to that in CM varieties, except line A4-6 (Figure 3B and Supplemental Table 4). The PD whole tree in NT and CM varieties shared similar features with observed species (Figure 3C and Supplemental Table 4). There are no significant differences in the analysis of the Shannon (Figure 3C, D, and Supplemental Table 4), which suggested that the community diversity of rhizosphere bacteria was similar between NT and CM varieties. Based on the alpha diversity, we concluded that *Cry1Ah1* expression slightly influences the rhizosphere bacterial richness but does not affect the community diversity of rhizosphere bacteria.

A Bray–Curtis dissimilarity matrix was calculated on normalized and square-root transformed read abundance data to compare the composition of rhizosphere bacterial members between NT and CM varieties. Based on weighted UniFrac, beta diversity analysis with PCA was applied to analyze the bacterial community structures among NT, A5-0, A4-6, Z1-3, and A5-23, and A5-0, A4-6, Z1-3, A5-23, A3-4, and NT were overlapped together and could not be separated (Figure 4), which indicated that the community structures of NT and CM varieties were similar. The *Cry1Ah1* expression did not affect the bacterial community structures.

3.5 The higher *Cry1Ah1* expression level may have marginal effects on rhizosphere bacteria of field-grown poplars

To determine the effect of *Cry1Ah1* expression on the rhizosphere bacteria, we investigated the taxonomic distinctiveness of poplar rhizosphere bacteria. In addition, the DeSeq2 was used to select the putative statistically differential rhizosphere bacteria. The relative abundances of rhizosphere bacteria of NT and CM varieties at the phylum, class, order, family, and genus levels were identified. At the phylum level, the Firmicutes, Myxococcota, Nitrospirota, Sva0485, Fibrobacterota, Latescibacterota, Desulfobacterota, and Proteobacteria considered as the dominant bacteria were found in the rhizosphere bacterial community (Figure 5A). The DeSeq2 analysis found that the dominant bacteria share similar abundances between NT and CM varieties. In contrast, the relative abundances of Cyanobacteria and Methylomirabilota showed a significant difference between NT and line A3-4 (Figure 6A). Also, the Methylomirabilota, Proteobacteria, Zixibacteria, MBNT15, Dadabacteria, Thermoplasmatota, Cyanobacteria, Chloroflexi, Bacteroidota, Acidobacteriota, Firmicutes, and Myxococcota were present at different abundances between NT and line A4-6 (Figure 6B). In addition, a few rhizosphere bacteria abundances were the difference between NT and A5-0, A5-23 or Z1-3 (Figure 6C-E). According to the above evidence, we concluded that *Cry1Ah1* expression has no influence on most rhizosphere bacteria abundances and only changes a small part of rhizosphere bacteria abundances.

299 At the class level, the rhizosphere bacterial community composition of NT and CM varieties was
300 similar (Figure 5B). The relative abundances of rhizosphere bacteria had no significant difference
301 between NT and line A3-4 except for Cyanobacteria and Methylomirabilia (Figure 7A). In native
302 fields, Alphaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Subgroup6, Blastocatellia, and
303 Thermoleophilia, which account for approximately 60% of the total rhizosphere bacteria, were present
304 at similar relative abundances in NT and CM varieties (Figure 5B). In addition, rhizosphere bacteria
305 with lower relative abundances, such as Actinobacteria, Gemmatimonadetes, Nitrospira, Holophagae,
306 and Acidimicrobiia, were significantly different between NT and line A4-6 (Figure 7B). Compared
307 with NT, the relative abundances of rhizosphere bacteria in lines A5-0, A5-23, and Z1-3 were similar,
308 and only small parts of rhizosphere bacteria abundances displayed differences (Figure 7C-E).
309 Furthermore, at the species level, a minor part of rhizosphere bacteria abundances from NT poplars
310 was slightly lower or higher than that from CM varieties, indicating that the CM varieties had little
311 influence on rhizosphere bacteria with lower relative abundances (Figure 8).

312 We investigated the taxonomic distinctiveness of poplar rhizosphere soil fungi to determine
313 whether *Cry1Ah1* expression affected rhizosphere fungus communities. The Chao1 analysis showed
314 that the community richness of rhizosphere fungi in CM varieties shares similar community richness
315 to NT, except line A5-0 (Supplemental Figure 4A). In addition, no significant difference was present
316 at the observed species, PD whole tree, and Shannon between NT and CM varieties. However, the
317 observed species and PD whole tree in line A5-23 had no slight difference compared to that in line Z1-
318 3 (Supplemental Figure 4B-D). The alpha diversity showed *Cry1Ah1* expression may slightly improve
319 the fungal community richness but does not influence the diversity of rhizosphere fungi. PCA evaluated
320 the fungal community structures among NT and CM varieties based on the Bray–Curtis dissimilarity
321 matrix. The results showed that NT and CM varieties are gathered, and the Cry1Ah1 expression does
322 not affect fungal community structures (Supplemental Figure 5). The dominant fungal phyla in poplar

rhizosphere soils included Ascomycota, Basidiomycota, and Mortierellomycota (Supplemental Figure 6A); the sequence load of the six dominant phyla, represented by high sequence numbers, represented more than approximately 80% of the total sequence, whereas that of low-abundance phyla comprised less than 20% of the entire sequence (Supplemental Figure 6A). Except for Ascomycota, there was no significant difference among rhizosphere fungi between NT and CM varieties at the phylum level (Table 1). Based on the analysis of rhizosphere fungal abundance in NT and CM varieties, we concluded that *Cry1Ah1* expression has no significant influence on the relative abundances of most rhizosphere fungi and only affects a few rhizosphere fungal abundances. Besides, we filtered extremely rare ASVs from the dataset to determine relative abundances at the class, order, family, and genus levels. Similar relative abundances of most rhizosphere fungi were observed between NT and CM varieties (Supplemental Figure 6B-E). However, the relative abundances of Archaeosporomycetes, Agaricostilbomycetes, Lobulomycetes, Cystobasidiomycetes, Schizosaccharomycetes, Sordariomycetes, and Ascomycota Incertae sedis at the class level were different between NT and CM varieties (Supplemental Table 5). Compared with NT poplars, only 13 kinds of 148 rhizosphere fungi relative abundances in CM varieties differed (Supplemental Table 6). Also, the major rhizosphere fungi at the family level showed similar abundances between NT and CM varieties. In contrast, only 7.9% of rhizosphere fungi with the lower abundance were present at differences between NT and CM varieties (Supplemental Table 7). In addition, most rhizosphere fungi abundances were found to have no differences between NT and CM varieties at the genus level (Supplemental Table 8). The rhizosphere fungal abundances analysis indicated that most rhizosphere fungi are low and not significantly different between NT and CM varieties. Only relative abundances of a few rhizosphere fungi were different in NT poplars and CM varieties.

4 Discussion

Biological diversity comprises community composition, structure, and function (Clergue et al., 2005). Interactions between soil microorganisms and other organisms influence nutrient cycling, which plays an essential role in soil condition, quality, and health (Čerevková et al., 2018; Ito et al., 2015; Zhao et al., 2014). The rhizosphere is a functional interface for material exchange between plants and soil ecosystems. Plants assimilate CO_2 during photosynthesis and transport some photosynthetic products to their underground parts, promoting the growth and metabolism of soil microorganisms, which transform organic nutrients into inorganic forms for absorption and utilization by plants (Mahnert et al., 2015). With the recent emergence of transgenic plants, the impact of their cultivation on the structure and function of the rhizosphere microbial community has become a concern (Garbeva et al., 2008; Liu et al., 2005; Pickett, 2016). Therefore, the structural diversity of the rhizosphere microbial community is an essential index for evaluating the effects of GM on the soil ecological environment.

4.1 Changes in rhizosphere soil MBC, MBN, and MBP content in NT and CM varieties

The rhizosphere soil microbial biomass is an essential parameter for assessing active soil nutrients and a sensitive indicator of environmental change in terrestrial ecosystems (He et al., 2003; Powlson et al., 1987; Singh and Gupta, 2018). In addition, MBC, MBN, and MBP participate in the ecosystem cycling of carbon, nitrogen, and phosphorus (Chen et al., 2018; Li et al., 2014). However, the effects of GM plants on MBC, MBN, and MBP have not been reported. Therefore, in the present study, we examined the impact of field-cultivated CM plants on MBC, MBN, and MBP content to study the relationship between CM plant growth and carbon, nitrogen, and phosphorus transformation in natural soil.

As a part of active soil carbon, MBC is the driving force of soil organic matter decomposition, closely related to the cycling of soil elements. We found that rhizosphere MBC content is significantly higher in CM varieties than in NT poplars. Conversely, rhizosphere MBN content decreased in CM

varieties grown in the field, affecting rhizosphere microorganisms growth, metabolism, and structure. As an essential source of active soil nitrogen, MBN plays a vital role in regulating soil nitrogen supply (Xu et al., 2013). In addition, MBP is the most active component of soil organic phosphorus, which governs the mineralization and fixation of soil phosphorus. Thus, MBP is an essential source of available soil phosphorus, reflecting active soil phosphorus capacity and turnover intensity (Chen and He, 2004; Sharma et al., 2004). Our results showed that CM varieties alter the rhizosphere MBN and MBP contents, at least during the study period, affecting the capacity of soil microorganisms to metabolize carbon, nitrogen, and phosphorus.

The rhizosphere MBC/MBN ratio can reflect rhizosphere microbial community structure. The MBC/MBN ratio is about 5:1 for bacteria, 6:1 for actinomycetes, and 10:1 for fungi (Liu et al., 2019a; Lopes and Fernandes, 2020; Lovell et al., 1995; Wardle, 1998; Wu et al., 2013). Based on our results, the MBC/MBN ratio for NT poplars in our study site was about 4.6 ± 0.3, indicating that rhizosphere bacteria may play a dominant role in determining rhizosphere MBC and MBN contents. However, the MBC/MBN ratio for CM varieties was about 9.2 ± 1.7, suggesting that rhizosphere microbial fungi in CM varieties participate widely in rhizosphere soil microenvironment regulation. Xu et al. (2016b) performed a systematic analysis of MBC, MBN, and MBP in the global terrestrial ecosystem. They reported mean values of MBC/MBN, MBC/MBP, and MBN/MBP ratios of 7.6, 42.4, and 5.6. In the present study, the rhizosphere MBC/MBP and rhizosphere MBN/MBP ratios for NT poplars were 63.2 ± 3.1 and 13.6 ± 0.9, respectively, higher than those reported for the global terrestrial ecosystem. This discrepancy maybe because of the low nitrogen and phosphorus content in the experimental field, resulting in lower rhizosphere MBN and rhizosphere MBP contents and lower rhizosphere MBC/MBP and rhizosphere MBN/MBP ratios. Compared with NT poplars, CM varieties showed higher rhizosphere MBC/MBN, rhizosphere MBC/MBP, and rhizosphere MBN/MBP ratios; thus, *Cry1Ah1*

393 transformation may directly affect the growth of rhizosphere microorganisms or inhibit rhizosphere
394 microbial activity, affecting the metabolism of MBC, MBN, and MBP.

395 **4.2 Effects of *Cry1Ah1* expression on native rhizosphere communities**

396 Bt protein confers strong insecticide resistance as a dominant trait of GM crops; it has been widely
397 used in transgenic breeding to achieve insecticide-resistant plants. Xu et al. (2019) showed that field-
398 planted CM poplars had strong insecticide resistance. With increasing GM crops worldwide, GM crop
399 cultivations' environmental and ecological impact has raised concerns globally. Some studies have
400 shown that GM crops have serious adverse effects on biodiversity and threaten the environment (Angle,
401 2008). Whether GM crops affect rhizosphere microbial composition, structure, and function has
402 become a widely studied question in oncology and food safety (Dunfield and Germida, 2004). The
403 plant rhizosphere is a dynamic microenvironment in which many factors, such as plant species
404 (Dohrmann and Tebbe, 2005), soil type (Buee et al., 2009), and root location (Watt et al., 2006), affect
405 the composition and structure of microbial communities around plant roots (Yang and Crowley, 2000).
406 Therefore, to avoid the interference of these factors on the examination of rhizosphere microbial
407 communities in the present study, we selected poplar trees planted in a single location and collected
408 samples simultaneously. Such a design can effectively avoid the influence of other factors on the
409 results, focusing only on the effects of poplar type (NT or CM) on the rhizosphere microbial
410 community.

411 Many studies have explored the relationship between soil biodiversity and the ecological safety
412 of transgenic plants. The high insect resistance to *Bt*-maize makes it an essential transgenic crop with
413 no effect on soil microbial communities (Oliveira et al., 2008) or rhizosphere communities (Dohrmann
414 et al., 2013). Field-cultivated *Bt* transgenic cotton has also been found to have no significant effect on
415 rhizosphere communities other than WT cotton (Zhang et al., 2015). In the present study, the
416 community diversity and rhizosphere bacterial abundances in NT and CM varieties showed no

417 significant difference. Similarly, *Cry1Ac*-sugarcane was found to have no impact on rhizosphere
418 microbial diversity or enzyme activity than NT sugarcane within a single crop season (Zhou et al.,
419 2016). Furthermore, no persistent or adverse effects on the rhizosphere bacterial community population
420 were detected between NT and *Bt*-modified rice (Wu et al., 2009). In addition, Li et al. (2018b)
421 reported that *Bt* transgenic rice could change bacterial community composition but not fungal
422 abundance or community structure. Some communities contained a few dominant taxa, whereas others
423 contained many taxa of low abundance. According to the alpha diversity, we found the observed
424 species and PD whole tree differ between NT and line A4-6, which suggested a difference in species
425 richness and community diversity between NT and line A4-6. Concerning the subsequent analysis of
426 relative abundances of rhizosphere bacteria in NT and CM varieties, we concluded that differences in
427 species richness and community diversity might be originated from the rhizosphere bacteria with low
428 abundance. Weinhold et al. (2018) performed a similarity percentage analysis to identify major
429 differences in abundance within groups and found that highly abundant families contributed
430 significantly to dissimilarities. Therefore, according to the alpha diversity, *Cry1Ah1* expression had no
431 significant influence on the rhizosphere bacterial richness and community diversity of rhizosphere
432 bacteria. Based on identifying relative abundances of rhizosphere bacteria in NT and CM varieties, we
433 found no significant difference in the abundances of major rhizosphere bacteria between NT and CM
434 varieties. In contrast, only the differences were found in the minor rhizosphere bacteria between NT
435 and CM varieties. We also concluded that the *Cry1Ah1* expression does not affect the relative
436 abundances of major rhizosphere bacteria in native fields and rhizosphere fungal abundances. Also,
437 the taxonomic diversity and structure of rhizosphere fungal communities and the relative abundances
438 of most rhizosphere fungi were similar among NT and CM varieties. However, a small fraction of
439 rhizosphere fungal abundances in special CM varieties differed from NT poplars. Based on these
440 findings, we concluded that *Cry1Ah1* expression has no effects on the rhizosphere microbial
441 community composition and large numbers of rhizosphere microbial abundances.

5 Conclusion

This study evaluated the ecological risk and potential effects of insect-resistant poplar cultivation by comparing the effects of NT and transgenic varieties on rhizosphere microbial communities. We detected no significant effects of NT and CM cultivation on microbial population and community structure; meanwhile, most rhizosphere bacteria shared similar relative abundances between the NT and CM varieties, suggesting that *Cry1Ah1* expression has no effects on the major rhizosphere bacteria abundances. In conclusion, we found that *Cry1Ah1* expression has no change in microbial population and community structure and does not impact most rhizosphere bacterial abundances.

6 Conflict of Interest

The authors declare that the research was conducted without any commercial relationships construed as a potential conflict of interest.

7 Author Contributions

JZ and AM designed and funded experiments. HW wrote the first draft of the manuscript. AM and JZ revised the manuscript. HW, AM, GYL, YHC, CMY, and FZ performed the experiments. All authors read and approved the final version of the manuscript.

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Abbreviations

NT: non-transgenic; Bt: Bacillus thuringiensis; GM: genetically modified; CM: Cry1Ah1-modified; ITS: internal transcribed spacer; MBN: microbial biomass nitrogen; MBC: microbial biomass carbon; MBP: microbial biomass phosphorus; ASVs: amplicon sequence variants; PD: phylogenetic diversity; PCA: principal component analysis; ELISA enzyme-linked immunosorbent assay

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11 Figure Captions

Figure 1: Evaluation of rhizosphere soil physical and chemical properties in non-transgenic (NT) and *Cry1Ah1*-modified (CM) poplar varieties. Analysis of rhizosphere soil pH (A), rhizosphere soil alkaline nitrogen (B), rhizosphere soil available phosphorus (C), rhizosphere microbial biomass carbon (D), rhizosphere microbial biomass nitrogen (E), and rhizosphere microbial biomass phosphorus (F) in NT and CM poplar varieties. Data were analyzed using one-way variance (ANOVA) analysis and Tukey's post hoc comparison. ***$P < 0.001$.

Figure 2: Analysis of amplicon sequence variants (ASVs) rarefaction curves and Shannon-wiener curves between NT and CM varieties.

Figure 3: Analysis of the taxonomic distinctiveness of rhizosphere bacteria based on alpha diversity. (A) Chao1 index. (B) Observed species index. (C) Phylogenetic diversity (PD) whole-tree index. (D) Shannon index. Data were analyzed using one-way ANOVA and Tukey's post hoc comparison. Significant differences ($P < 0.05$) are stated in lowercase letters.

Figure 4: Principal component analysis (PCA) of rhizosphere bacterial communities at the ASVs level. ASVs were defined at a 97% sequence similarity cut-off in mothur. The differences and distances among NT, A5-0, A4-6, Z1-3, A5-23, and A3-4 can be visualized based on analysis of ASVs composition.

Figure 5: Overall composition of rhizosphere bacterial communities and the relative abundances of rhizosphere bacteria at the phylum or class level between NT and CM varieties. Phylum-level (A) and class-level (B) taxonomic analysis of bacterial distribution in rhizosphere soil samples of the NT and CM varieties based on 16S amplicon and metagenomic data.

Figure 6: The DeSeq2 analysis for the selection of statistically differential rhizosphere bacteria at phylum level between NT and lines A3-4 (A), A4-6 (B), A5-0 (C), A5-23 (D), and Z1-3 (E).

Figure 7: The DeSeq2 analysis for the selection of statistically differential rhizosphere bacteria at a class level between NT and lines A3-4 (A), A4-6 (B), A5-0 (C), A5-23 (D), and Z1-3 (E).

Figure 8: The DeSeq2 analysis for the selection of statistically differential rhizosphere bacteria at species level between NT and lines A3-4 (A), A4-6 (B), A5-0 (C), A5-23 (D), and Z1-3 (E).

12 Supplemental Figures

Supplemental Figure 1: Poplar growth and experimental design for poplar field trials. Diagram of the experimental design for poplar field trials, the NT and CM varieties were arranged randomly. The CM varieties are A5-0, A4-6, Z1-3, A5-23, and A3-4.

Supplemental Figure 2: Insecticidal activity of NT and CM varieties against *Micromelalopha troglodyta*. The larval mortality of *M. troglodyta* on days 6 (A) and 12 (B) of feeding on NT and CM varieties. Data were analyzed using one-way ANOVA and Tukey's post hoc comparison. *P < 0.05 and **P < 0.01, respectively.

Supplemental Figure 3: The distribution of rhizosphere microbe clean tags. The clean tags distribution of rhizosphere bacteria (A) and rhizosphere fungi (B).

Supplemental Figure 4: Analysis of the taxonomic distinctiveness of rhizosphere fungi based on alpha diversity. (A) Chao1 index. (B) Observed species index. (C) Phylogenetic diversity whole-tree index. (D) Shannon index. Data were analyzed using one-way ANOVA and Tukey's post hoc comparison. Significant differences (P < 0.05) are stated in lowercase letters.

Supplemental Figure 5: PCA of rhizosphere fungal communities at the ASVs level. ASVs were defined at a 97% sequence similarity cut-off in mothur. The differences and distances among NT, A5-0, A4-6, Z1-3, A5-23, and A3-4 can be visualized based on analysis of ASVs composition.

Supplemental Figure 6: Overall composition of rhizosphere fungal communities and the relative abundances of rhizosphere bacteria between NT and CM varieties. Phylum-level (A), class-level (B), order-level (C), family-level (D), and genus-level (E) taxonomic analysis of fungal distribution in rhizosphere soil samples of the NT and CM varieties based on ITS1 amplicon and metagenomic data.

13 Table Captions

Table 1: The relative abundances of rhizosphere fungi at the phylum level between NT and CM varieties. Data were analyzed using Kruskal-wallis comparison. "P < 0.05" indicates a significant difference between NT and CM varieties.

14 Supplemental Tables

Supplemental Table 1: *Cry1Ah1* expression level in NT and CM varieties.

Supplemental Table 2: The 16S rDNA tags and ITS1 tags generated from the rhizosphere microbiome.

Supplemental Table 3: ASVs for bacterial and fungal community sequencing.

Supplemental Table 4: The alpha diversity analysis of rhizosphere bacteria in NT and CM varieties.

Supplemental Table 5: There are relative abundances of rhizosphere fungi between NT and CM varieties at the class level. Data were analyzed using Kruskal-wallis comparison. "P < 0.05" indicates a significant difference between NT and CM varieties.

Supplemental Table 6: The relative abundances of rhizosphere fungi at the order level between NT and CM varieties. Data were analyzed using Kruskal-wallis comparison. "P < 0.05" indicates a significant difference between NT and CM varieties.

Supplemental Table 7: The relative abundances of rhizosphere fungi at the family level between NT and CM varieties. Data were analyzed using Kruskal-wallis comparison. "P < 0.05" indicates a significant difference between NT and CM varieties.

Supplemental Table 8: The relative abundances of rhizosphere fungi at the genus level between NT and CM varieties. Data were analyzed using Kruskal-wallis comparison. "P < 0.05" indicates a significant difference between NT and CM varieties.

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Figure 1

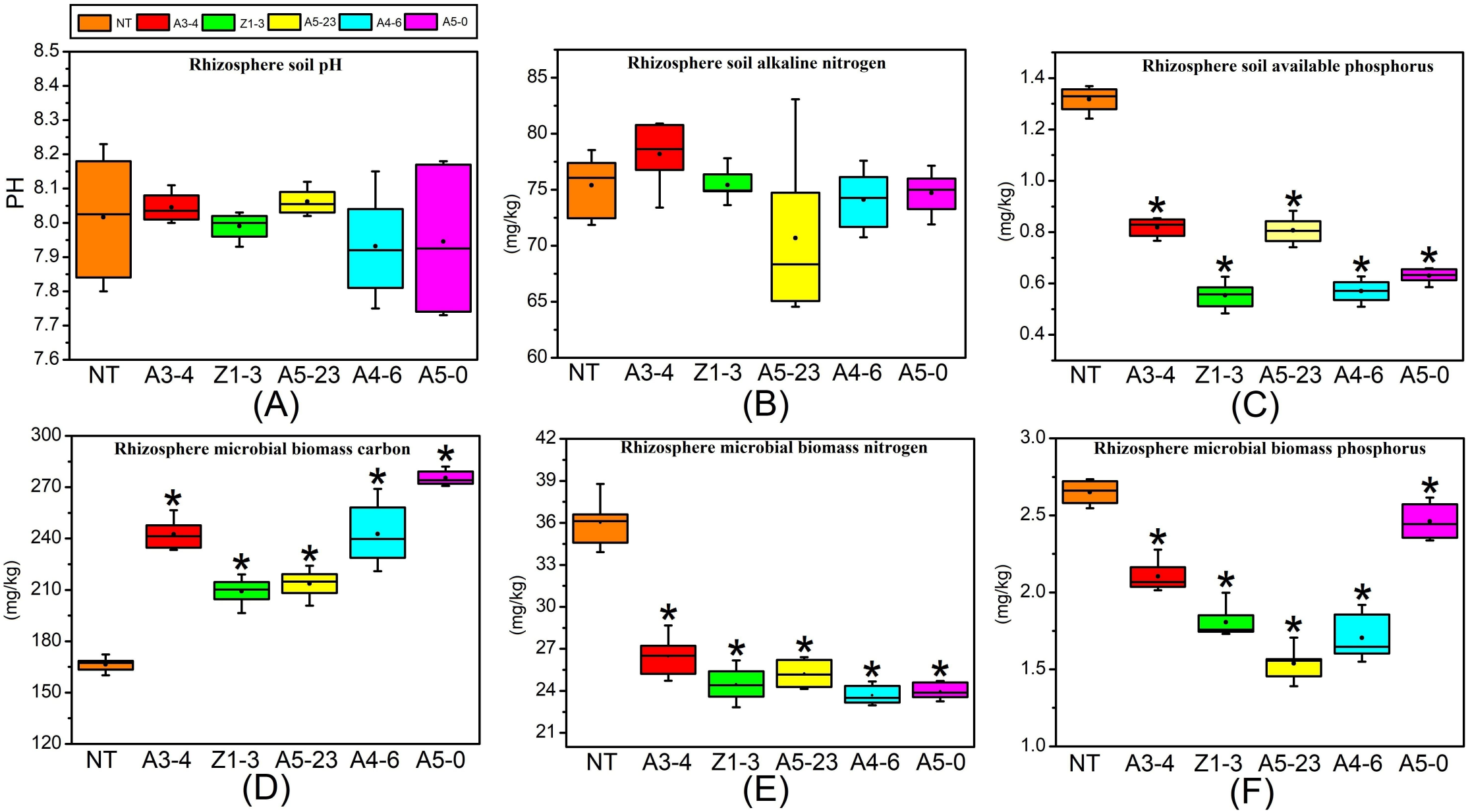
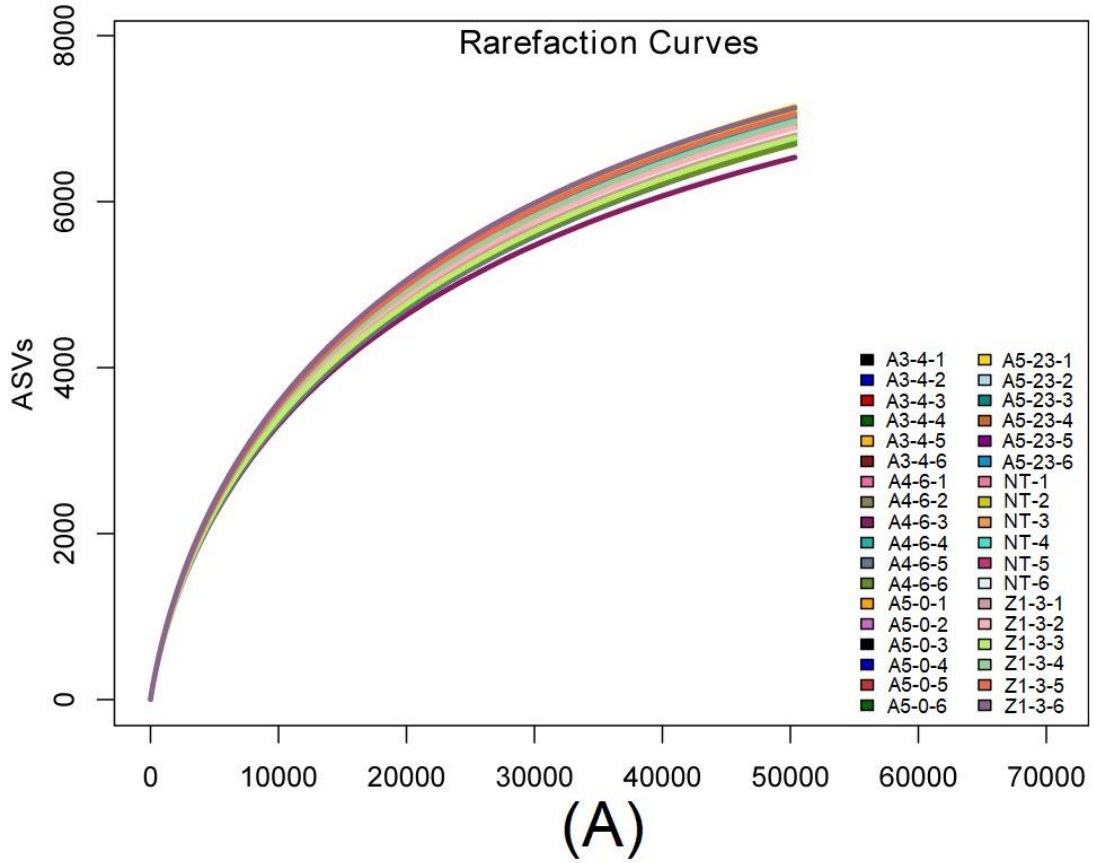
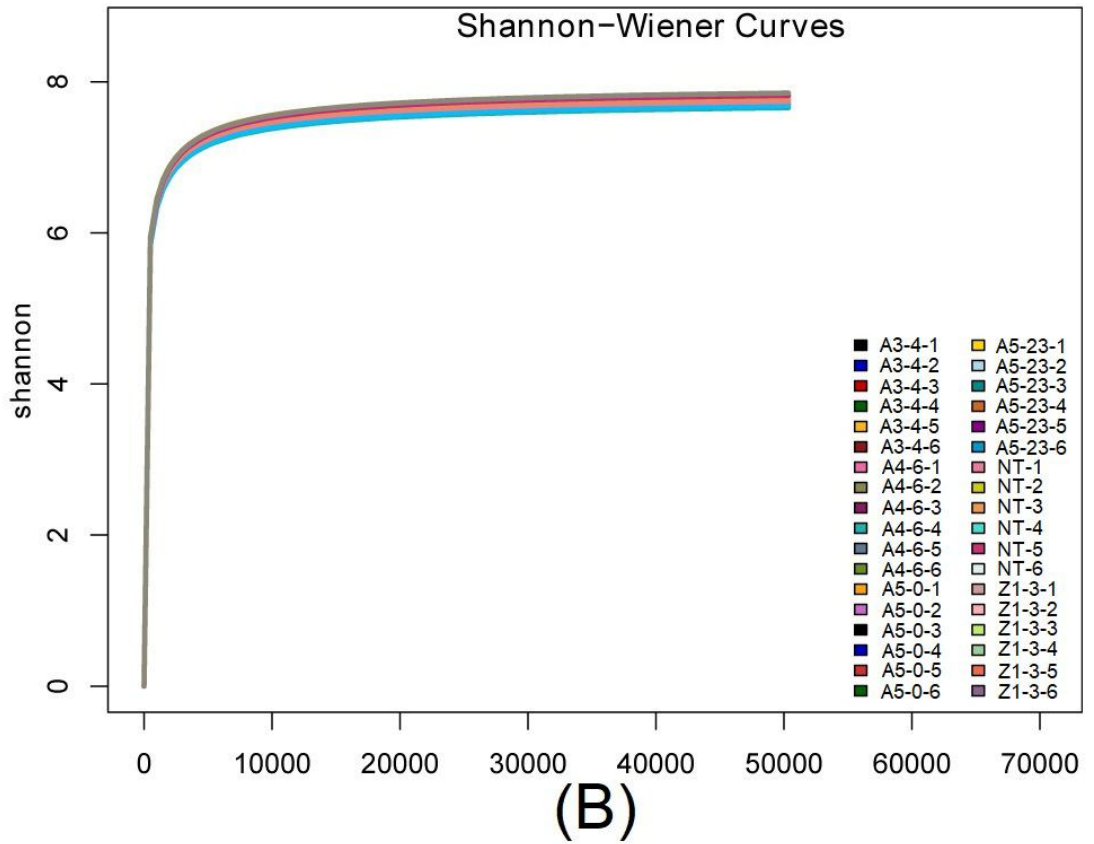

NT
A3-4
Z1-3
A5-23
A4-6
A5-0
Rhizosphere soil pH
PH
(A)
Rhizosphere soil alkaline nitrogen
(mg/kg)
(B)
Rhizosphere soil available phosphorus
(mg/kg)
(C)
Rhizosphere microbial biomass carbon
(mg/kg)
(D)
Rhizosphere microbial biomass nitrogen
(mg/kg)
(E)
Rhizosphere microbial biomass phosphorus
(mg/kg)
(F)

Figure 2


Rarefaction Curves
ASVs
0
2000
4000
6000
8000
0
10000
20000
30000
40000
50000
60000
70000
A3-4-1
A3-4-2
A3-4-3
A3-4-4
A3-4-5
A3-4-6
A4-6-1
A4-6-2
A4-6-3
A4-6-4
A4-6-5
A4-6-6
A5-0-1
A5-0-2
A5-0-3
A5-0-4
A5-0-5
A5-0-6
A5-23-1
A5-23-2
A5-23-3
A5-23-4
A5-23-5
A5-23-6
NT-1
NT-2
NT-3
NT-4
NT-5
NT-6
Z1-3-1
Z1-3-2
Z1-3-3
Z1-3-4
Z1-3-5
Z1-3-6
(A)


Shannon−Wiener Curves
shannon
0
2
4
6
8
0
10000
20000
30000
40000
50000
60000
70000
A3-4-1
A3-4-2
A3-4-3
A3-4-4
A3-4-5
A3-4-6
A4-6-1
A4-6-2
A4-6-3
A4-6-4
A4-6-5
A4-6-6
A5-0-1
A5-0-2
A5-0-3
A5-0-4
A5-0-5
A5-0-6
A5-23-1
A5-23-2
A5-23-3
A5-23-4
A5-23-5
A5-23-6
NT-1
NT-2
NT-3
NT-4
NT-5
NT-6
Z1-3-1
Z1-3-2
Z1-3-3
Z1-3-4
Z1-3-5
Z1-3-6
(B)

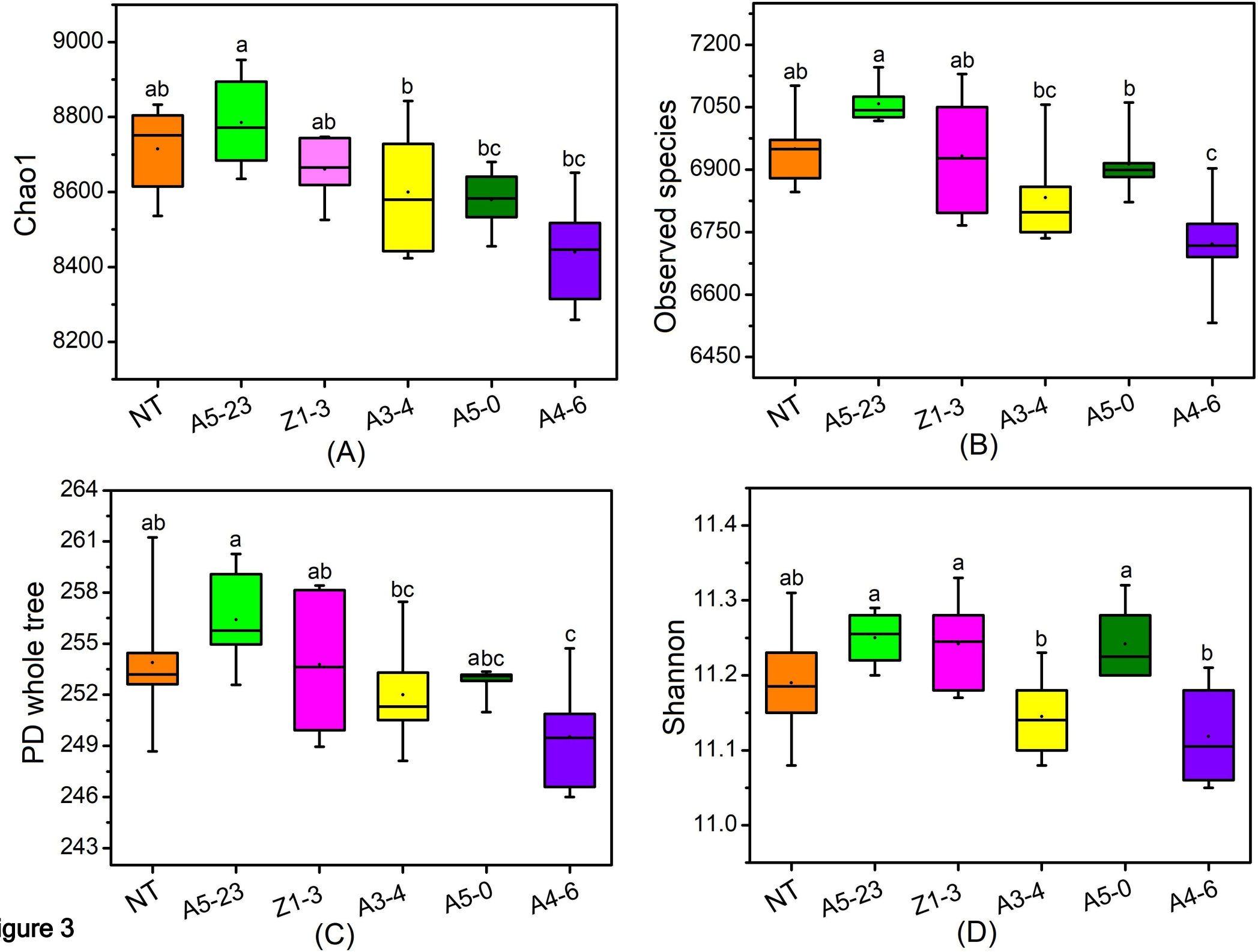
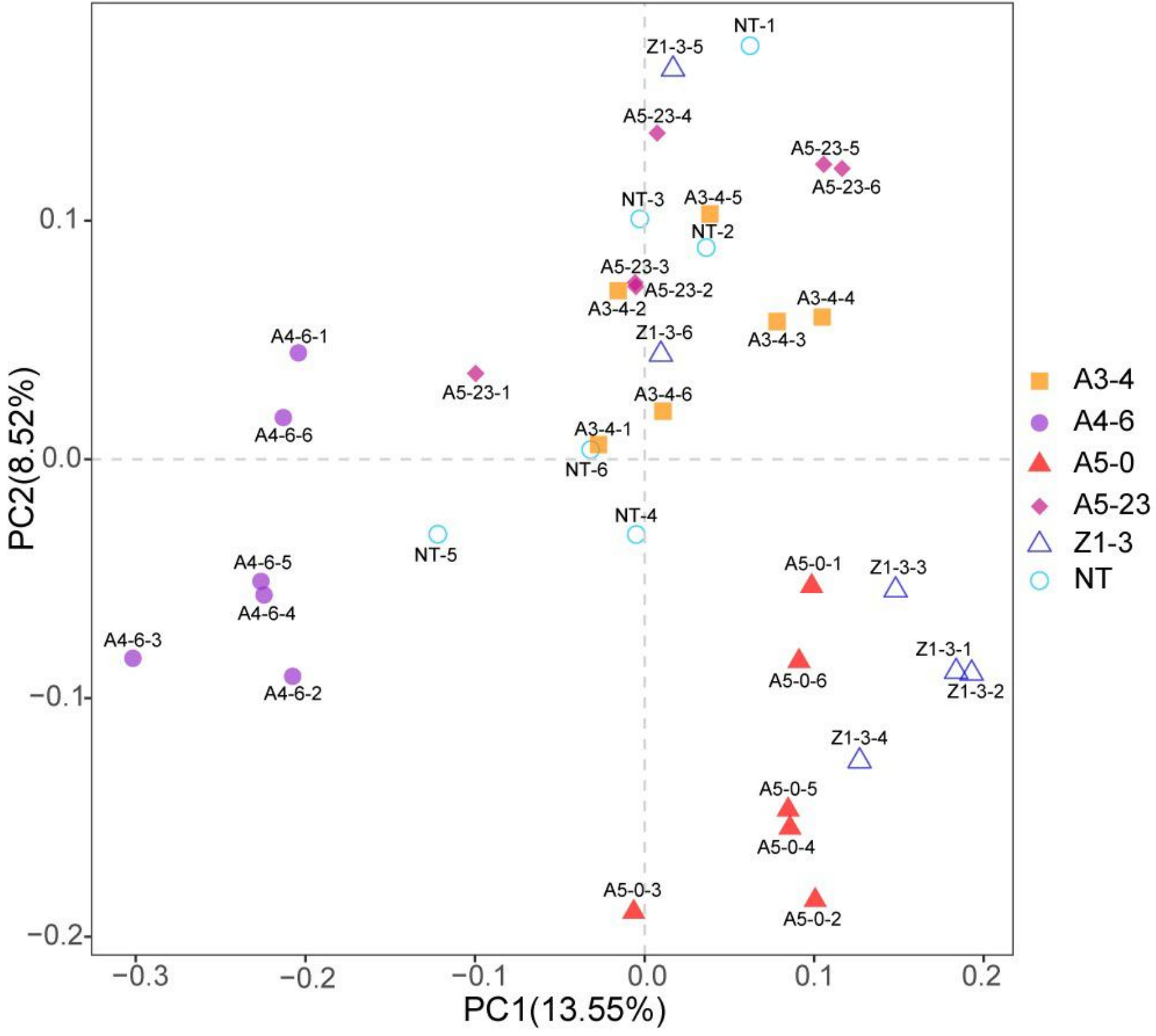
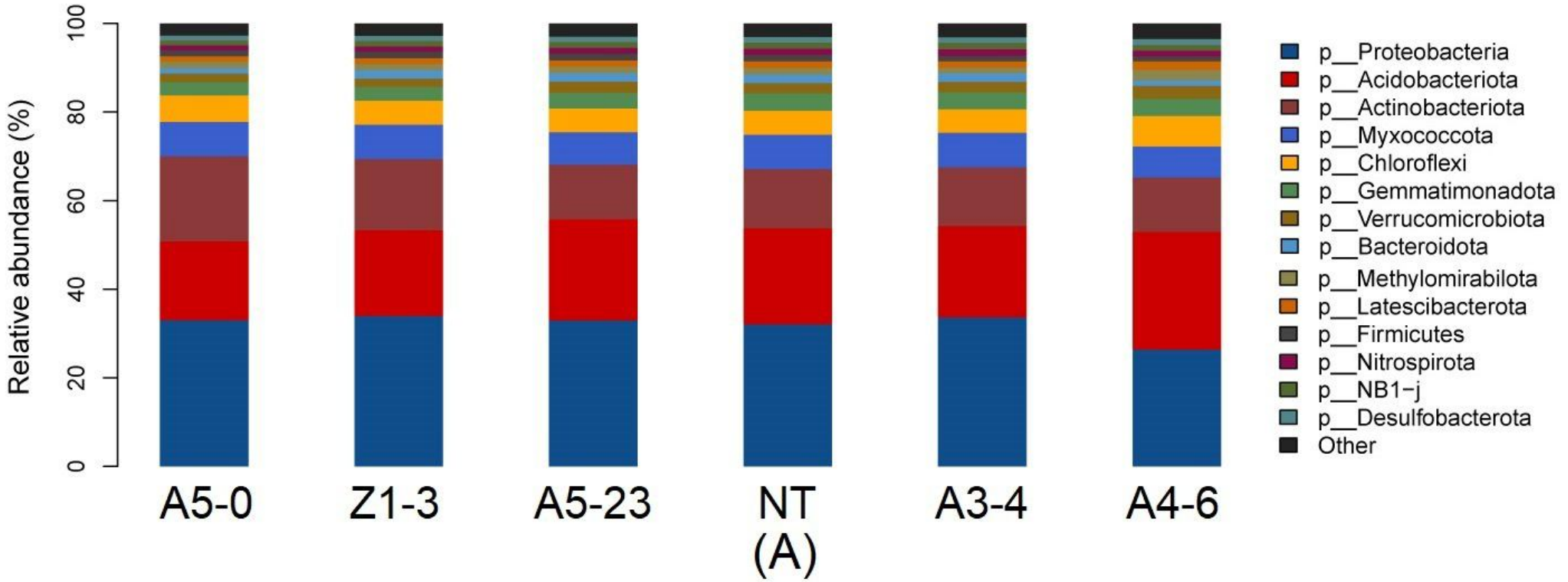

Chao1
9000
8800
8600
8400
8200
ab
a
ab
b
bc
bc
NT
A5-23
Z1-3
A3-4
A5-0
A4-6
(A)
Observed species
7200
7050
6900
6750
6600
6450
ab
a
ab
bc
b
c
NT
A5-23
Z1-3
A3-4
A5-0
A4-6
(B)
PD whole tree
264
261
258
255
252
249
246
243
ab
a
ab
bc
abc
c
NT
A5-23
Z1-3
A3-4
A5-0
A4-6
(C)
Shannon
11.4
11.3
11.2
11.1
11.0
ab
a
a
b
a
b
NT
A5-23
Z1-3
A3-4
A5-0
A4-6
(D)

Figure 3

Figure 4


NT-1
Z1-3-5
A5-23-4
A5-23-5
A5-23-6
NT-3
A3-4-5
NT-2
A5-23-3
A5-23-2
A3-4-2
A3-4-4
A3-4-3
Z1-3-6
A4-6-1
A5-23-1
A3-4-6
A4-6-6
A3-4-1
NT-6
NT-4
NT-5
A4-6-5
A4-6-4
A5-0-1
Z1-3-3
A4-6-3
A4-6-2
A5-0-6
Z1-3-1
Z1-3-2
Z1-3-4
A5-0-5
A5-0-4
A5-0-3
A5-0-2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2
PC2(8.52%)
PC1(13.55%)
A3-4
A4-6
A5-0
A5-23
Z1-3
NT


Relative abundance (%)
0
20
40
60
80
100
A5-0
Z1-3
A5-23
NT
A3-4
A4-6
(A)
p__Proteobacteria
p__Acidobacteriota
p__Actinobacteriota
p__Myxococcota
p__Chloroflexi
p__Gemmatimonadota
p__Verrucomicrobiota
p__Bacteroidota
p__Methylomirabilota
p__Latescibacterota
p__Firmicutes
p__Nitrospirota
p__NB1−j
p__Desulfobacterota
Other

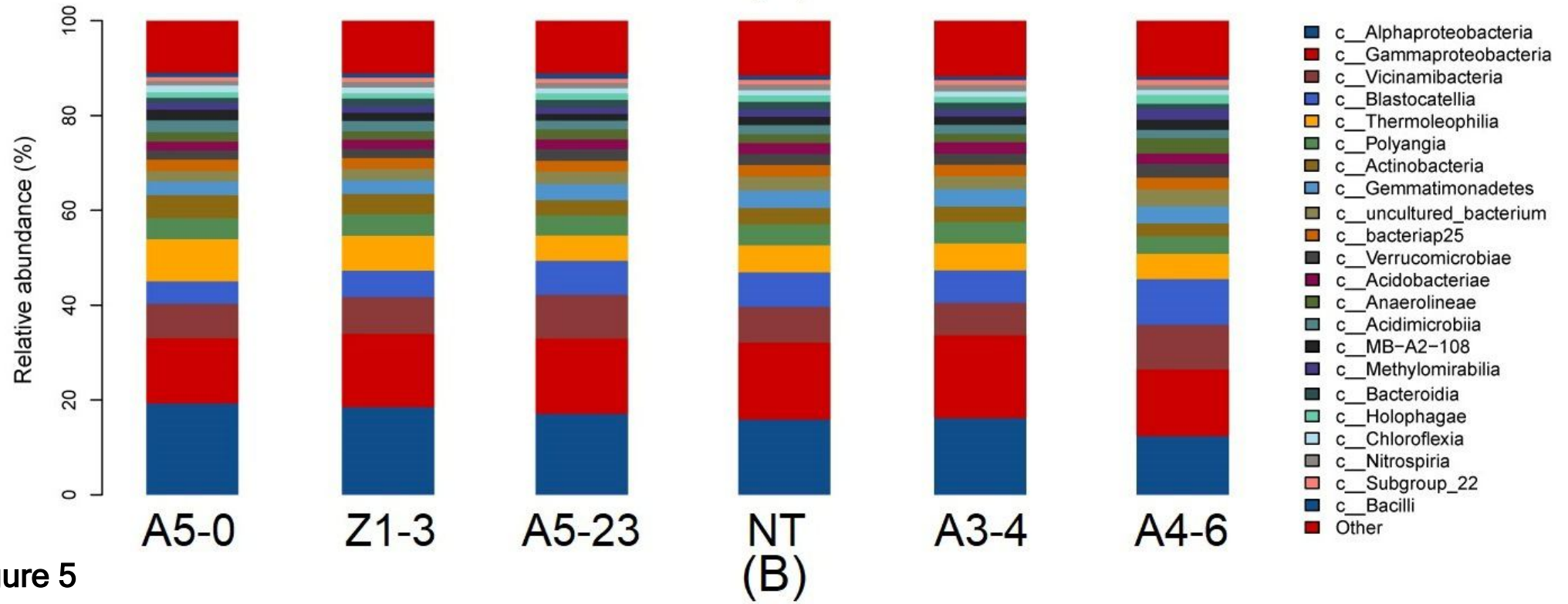

Relative abundance (%)
0
20
40
60
80
100
A5-0
Z1-3
A5-23
NT
A3-4
A4-6
(B)
c__Alphaproteobacteria
c__Gammaproteobacteria
c__Vicinamibacteria
c__Blastocatellia
c__Thermoleophilia
c__Polyangia
c__Actinobacteria
c__Gemmatimonadetes
c__uncultured_bacterium
c__bacteriap25
c__Verrucomicrobiae
c__Acidobacteriae
c__Anaerolineae
c__Acidimicrobiia
c__MB−A2−108
c__Methylomirabilia
c__Bacteroidia
c__Holophagae
c__Chloroflexia
c__Nitrospiria
c__Subgroup_22
c__Bacilli
Other

Figure 5

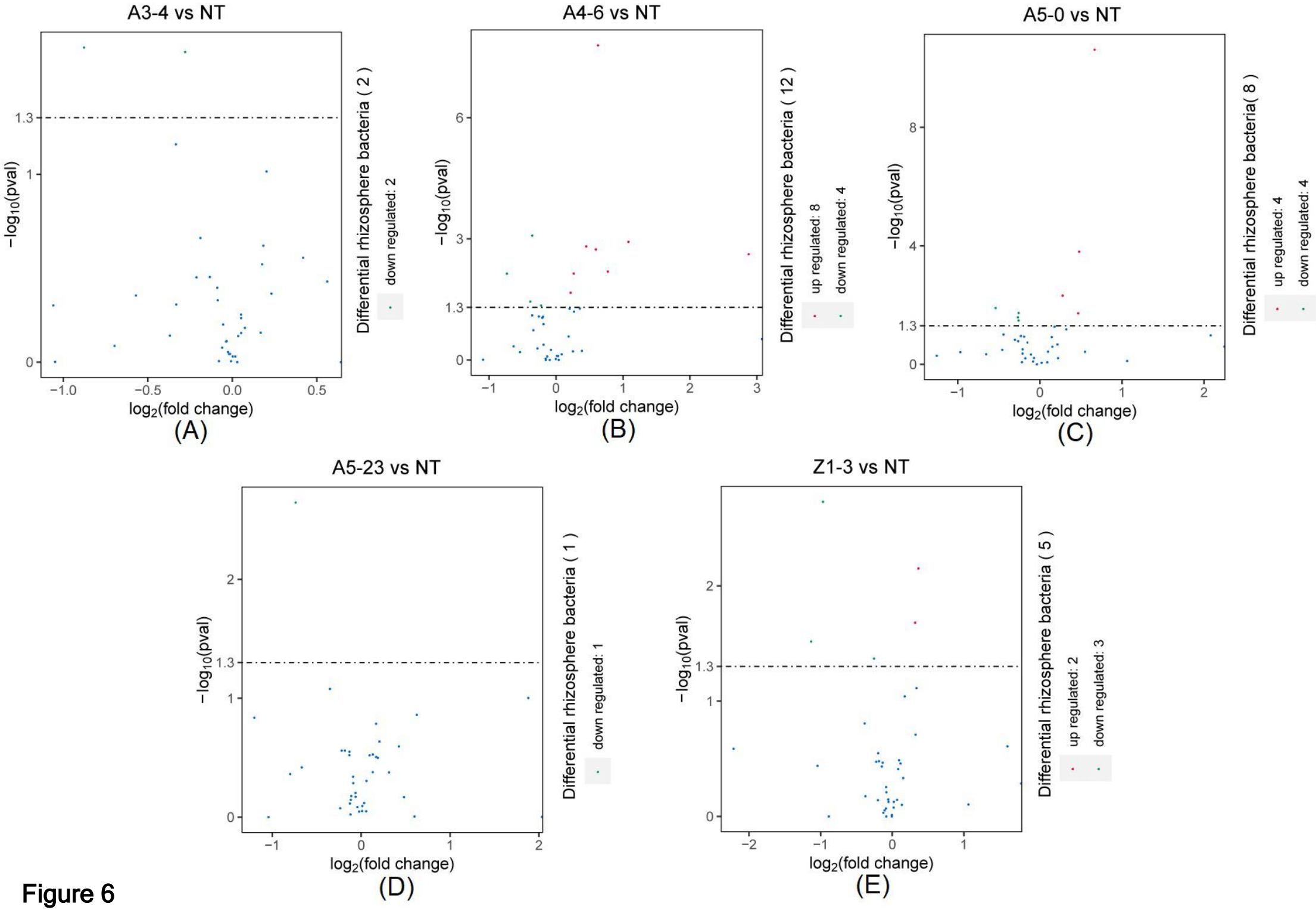

A3-4 vs NT
A4-6 vs NT
A5-0 vs NT
A5-23 vs NT
Z1-3 vs NT
−log10(pval)
log2(fold change)
Differential rhizosphere bacteria (2)
down regulated: 2
Differential rhizosphere bacteria (12)
up regulated: 8
down regulated: 4
Differential rhizosphere bacteria(8)
up regulated: 4
down regulated: 4
Differential rhizosphere bacteria (1)
down regulated: 1
Differential rhizosphere bacteria (5)
up regulated: 2
down regulated: 3
(A)
(B)
(C)
(D)
(E)

Figure 6

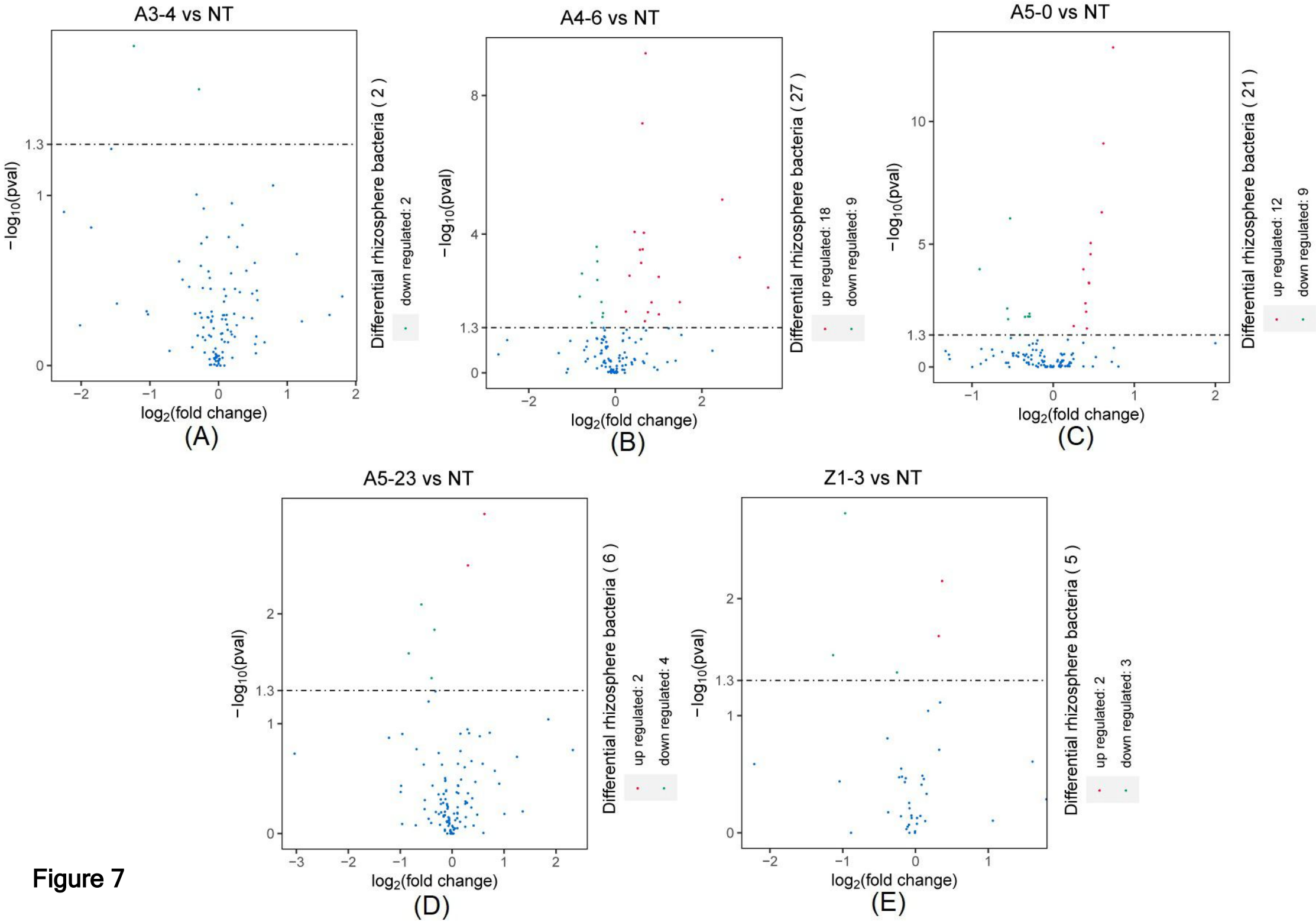

A3-4 vs NT
A4-6 vs NT
A5-0 vs NT
A5-23 vs NT
Z1-3 vs NT
−log10(pval)
log2(fold change)
Differential rhizosphere bacteria (2)
down regulated: 2
Differential rhizosphere bacteria (27)
up regulated: 18
down regulated: 9
Differential rhizosphere bacteria (21)
up regulated: 12
down regulated: 9
Differential rhizosphere bacteria (6)
up regulated: 2
down regulated: 4
Differential rhizosphere bacteria (5)
up regulated: 2
down regulated: 3
(A)
(B)
(C)
(D)
(E)

Figure 7

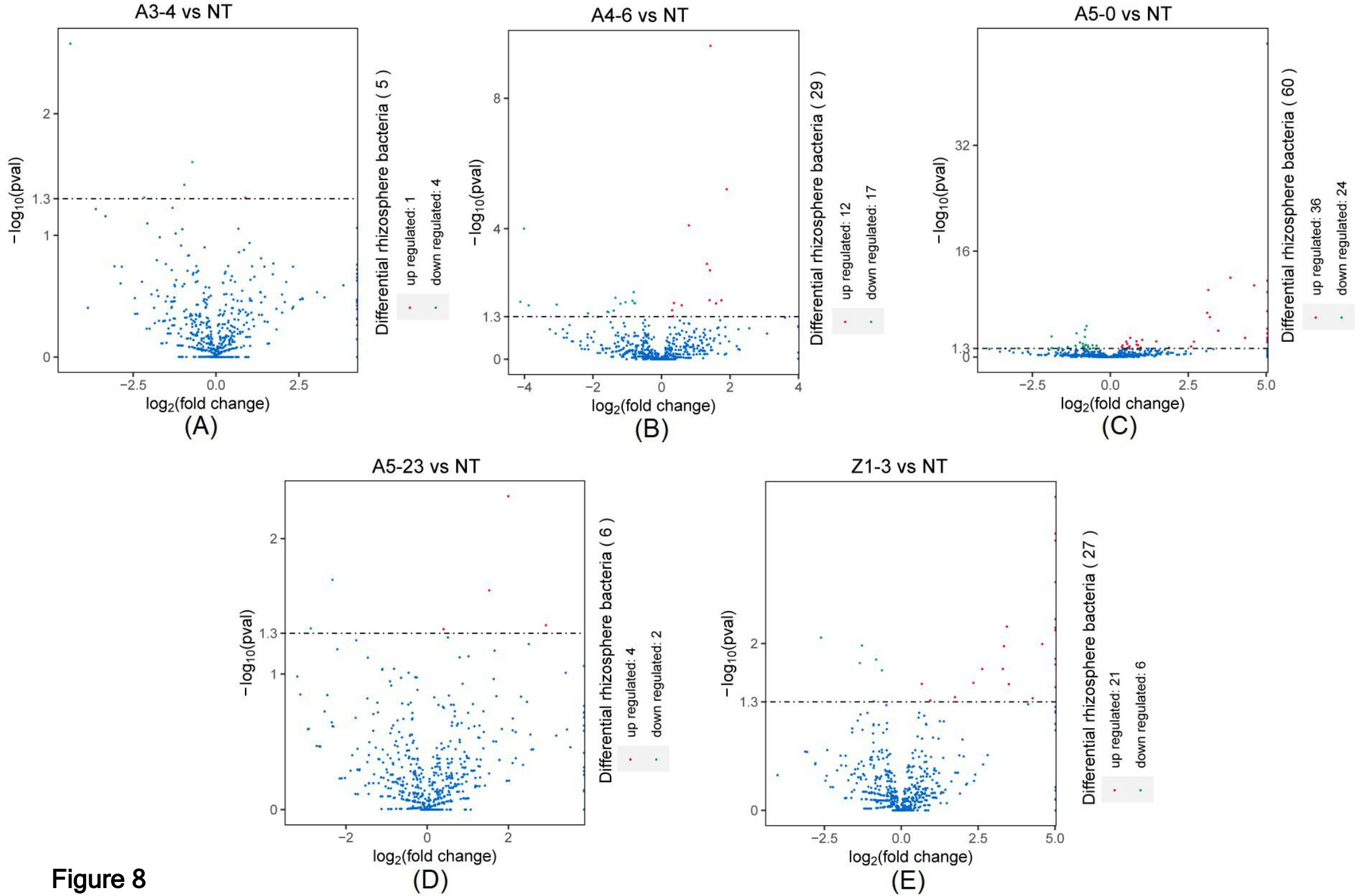

A3-4 vs NT
A4-6 vs NT
A5-0 vs NT
A5-23 vs NT
Z1-3 vs NT
−log10(pval)
log2(fold change)
Differential rhizosphere bacteria (5)
up regulated: 1
down regulated: 4
Differential rhizosphere bacteria (29)
up regulated: 12
down regulated: 17
Differential rhizosphere bacteria (60)
up regulated: 36
down regulated: 24
Differential rhizosphere bacteria (6)
up regulated: 4
down regulated: 2
Differential rhizosphere bacteria (27)
up regulated: 21
down regulated: 6
(A)
(B)
(C)
(D)
(E)

Figure 8

Table 1

ASV	Test-Statistic	P	FDR P	Bonferroni P	A5-0 mean
p__Ascomycota	13.56156156	0.018647881	0.354309737	0.354309737	0.38735852
p__Basidiomycota	10.16216216	0.070768701	0.391712711	1	0.227967163
p__Chlorophyta	10.15615616	0.070929628	0.391712711	1	0.01805686
p__Chytridiomycota	9.092451776	0.105432904	0.391712711	1	0.001884535
p__Cercozoa	8.697065259	0.121774576	0.391712711	1	0.045888695
p__unidentified	8.156156156	0.147836947	0.391712711	1	0.159939382
p__Entomophthoromycota	7.806407963	0.167232549	0.391712711	1	0.000117434
p__Rozellomycota	7.579196217	0.181002706	0.391712711	1	0.000380262
p__Mortierellomycota	7.507507508	0.185548126	0.391712711	1	0.13757102
p__Blastocladiomycota	6.846918489	0.232276395	0.44132515	1	0.003265781
p__Olpidiomycota	5.728450555	0.333544833	0.537634093	1	0.000866774
p__Zoopagomycota	5.322944896	0.377751263	0.537634093	1	0.000715788
p__Glomeromycota	5.162162162	0.396412153	0.537634093	1	0.01409766
p__Entorrhizomycota	5	0.415880187	0.537634093	1	0
p__Monoblepharomycota	4.930281072	0.424447968	0.537634093	1	0.000352302
p__Ciliophora	3.628726784	0.60400532	0.61504672	1	0.000363486
p__GS19	3.627922155	0.604125841	0.61504672	1	2.80E-05
p__Kickxellomycota	3.615658975	0.605963711	0.61504672	1	0.00096184
p__Mucoromycota	3.555235853	0.61504672	0.61504672	1	0.000184539

NT mean	A5-23 mean	A4-6 mean	A3-4 mean	Z1-3 mean
0.61138326	0.61042142	0.243904621	0.484990829	0.392447323
0.135322999	0.253847358	0.417125218	0.331415246	0.198798819
0.011899969	0.006559522	0.009774974	0.01853778	0.021434483
0.003696372	0.001263812	0.000542433	0.000810853	0.002152955
0.017676598	0.006710509	0.007348007	0.007040442	0.00904241
0.07572809	0.076952758	0.25286315	0.111400036	0.232972084
0.000849998	0.000995392	6.15E-05	6.15E-05	3.36E-05
0.001314141	0.000665459	0.000687827	0.00061513	0.001101642
0.115996734	0.026478549	0.028184136	0.020472643	0.116136536
0.001213484	4.47E-05	0.003282557	0.00246052	0.001302957
5.03E-05	7.83E-05	0.000111842	0.000117434	0.000123026
0.002024337	0.000419407	0.000726972	0.000726972	0.001588154
0.021809153	0.014824632	0.034631817	0.017508836	0.021311457
0	1.12E-05	0	0	0
0.000335525	6.71E-05	4.47E-05	0.000173355	0.000313157
1.12E-05	4.47E-05	5.59E-06	0.002829598	0.000184539
0	2.24E-05	0	3.91E-05	5.59E-06
0.000548025	0.00048092	0.000570393	0.000609538	0.00072138
0.000139802	0.000111842	0.00013421	0.000190131	0.000329933

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- supplementaltable3.xlsx
- supplementaltable4.xlsx
- supplementaltable5.xlsx
- supplementaltable6.xlsx
- supplementaltable7.xlsx
- supplementaltable8.xlsx