The influence of genetically modified glyphosate-tolerant maize CC-2 on rhizosphere bacterial communities revealed by MiSeq sequencing

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Abstract: Genetically modified (GM) crops have brought huge economic benefits to mankind, however, at the same time, their safety issues are drawing growing attention. This investigation was conducted to assess whether the long-term cultivation of GM glyphosate resistant maize CC-2 effects bacterial communities in the rhizosphere soil. A 2-year follow-up trial was conducted, and soils were sampled at various plant developmental stages. The bacterial community structure of the rhizosphere soil was analysed by the high-throughput sequencing and compared with the near-isogenic non-GM maize Zheng 58. We showed here that long-term cultivation of CC-2 has no significant effect on the structure and diversity of bacterial communities, while different growth stages had significant effect. These results provided a reliable theoretical basis for the future cultivation and increased commercialisation of CC-2.

Keywords: *Zea mays* L.; soil-plant ekosystem; soil microbiota; soil microorganisms; 16S rRNA gene sequencing

The global acreage of genetically modified (GM) crops has increased dramatically ~112-fold from 1.7 million ha in 1996 to 191.7 million ha in 2018, while 58.9 million ha of field was planted maize crop, and which the herbicide tolerant maize NK603 got the most approvals (61 approvals) (ISAAA 2018). Despite of the extension of GM crops planting areas, the safety of GM crops is still a controversial issue worldwide.

Soil microorganisms play an important role in the soil-plant ecosystem. In agricultural soil, bacteria take part in the carbon cycle through fixation and decomposition (Bhatti et al. 2017). Plant growth-promoting rhizobacteria (PGPR) stimulate plant growth in multiple ways, including nutrient fixation and producing plant growth regulators (Bhattacharyya and Jha 2012). The jasmonic acid signalling pathway induced by plant defences can suppress bacteria for plant development and enrich bacteria for biological control agents (Carvalhais et al. 2013). Given their abundance and important roles in the soil, bacteria are often used as a sensitive indicator for assessing the effects of GM crops (Liang et al. 2018). As soil microorganisms affect plants in many ways, it is also important to know how plants affect soil microorganisms especially GM plants (Mandal et al. 2020).

Plants are the main source of carbon and energy for rhizosphere bacteria (Shtark et al. 2003, Garbeva et al. 2004). Plants can therefore affect the biodiversity of soil bacteria communities through selectively excreting novel proteins that are favoured by cer-

tain bacterial groups (Dunfield and Germida 2004). Changes in the content of plant secondary metabolism compounds or alterations in crop chemistry not directly linked to the particular genes introduced, which might affect the soil microbiota directly or indirectly (Turrini et al. 2015). The quantity of root exudates correlates with plant developmental stages, specifically, plants produce the least root exudates at seedling stage, increasing output until flowering stage then decreasing output in maturity stages (Garbeva et al. 2004). The chemical composition of root exudates also varies with plant developmental stage (Haldar and Sengupta 2015). Root exudates quantity and composition is also affected by environmental factors such as climate, humidity, temperature and light (Haldar and Sengupta 2015). Therefore, annual replication was necessary when analysing the influence of GM plant on rhizosphere bacteria. Though some studies have already focused on the environmental safety of GM plants, especially their effects on rhizosphere bacteria (Liang et al. 2018, Lu et al. 2018). However, the influence of GM plants still needs to be analysed case-by-case for the reason that some GM plants have no influence on overall soil function, but the microbial composition and some key functions mediated by soil microbes were altered (Bai et al. 2019).

As it is possible that the novel proteins from GM plants can transiently influence environment to some extent, safety of GM plant is the urgent concerning before promoting planting. The *EPSPS* gene expressing glyphosate-tolerant maize CC-2 is a cultivar researched and developed by China Agricultural University. According to the above, the effects of GM maize CC-2 on soil bacteria need to be evaluated before commercial planting. Using the MiSeq sequencing approach we try to get information about the environmental safety of GM maize CC-2.

MATERIAL AND METHODS

Plant materials and field design. The GM glyphosate-tolerant maize CC-2 (CC) and the near-isogenic non-GM maize Zheng 58 (CCCK) were used in this study. Both of cultivars were provided by China Agricultural University. The maize lines were simultaneously planted at the experimental field station of the Jilin Academy of Agriculture Sciences in Gongzhuling City, Jilin province, China (43°19'N, 124°29'E) during 2014–2015. The total area of this experimental field is 900 m^2, and divided into 6 plots

of 10×15 m^2. This consisted of 3 replicate plots for CC and CCCK, respectively, which were randomly distributed (CC, three replicates: CC_1, CC_2 and CC_3; CCCK, three replicates: CCCK_1, CCCK_2 and CCCK_3). Maize was cultivated in accordance with the regular agronomic practices in the northeast China.

Soil type and sampling method. The examined soils belong to Phaeozems, which contains alkaline nitrogen, available potassium, available phosphorus, and organic carbon with 77.54 ± 0.07 mg/kg, 154.10 ± 0.76 mg/kg, 10.68 ± 0.07 mg/kg and 15.71 ± 0.05 g/kg, respectively. The pH of the soil was 5.36 ± 0.02 (IUSS Working Group WRB 2015, Fan et al. 2018). The five-point sampling method was used to sample in a plot. Then five plants from the same plot were put together, the rhizosphere soil was shaken off and 50 g soil collected as a sample, three replicates for each cultivar. These were stored at –20 °C. Samples were taken when the plants were at seedling stage (SS), at anthesis (AS) and at maturity-setting stage (MS) each year.

DNA extraction. DNA was extracted from 0.5 g of rhizosphere soil samples using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, USA). A NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA) was used to evaluated the quality and quantity of DNA. Extracted DNA satisfied the equipment's demand (OD260/280 = 1.8–2.0, c ≥ 20 ng/μL) and was used for PCR amplification.

16S rRNA amplification and sequencing. The amplification and Illumina MiSeq sequencing of the bacterial 16S rRNA gene was performed at BGI (Shenzhen, China). Briefly, the V4 region of 16S rRNA was amplified by PCR using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Kozich et al. 2013). Tags were clustered to operational taxonomic units (OTUs) at 97% similarity of sequence by USEARCH (version 7.0.1090) (Edgar 2013). Ribosomal database project (RDP) classifier version 2.2 was used to compare OTU representative sequences with greengene database, using 0.8 confidence value as cutoff. Sequences were inputed into the NCBI GenBank Sequence Read Archive under BioProject PRJNA549353.

Data analysis and statistics. The α-diversity indices were calculated by Mothur (version 1.31.2). The Wilcoxon Rank-Sum test and Kruskal-Wallis test were used for multi-groups comparison ($P < 0.01$). R version 3.1.1 (R Development Core Team 2011) was used to draw histogram of species at different taxonomic levels, principal component analysis (PCA)
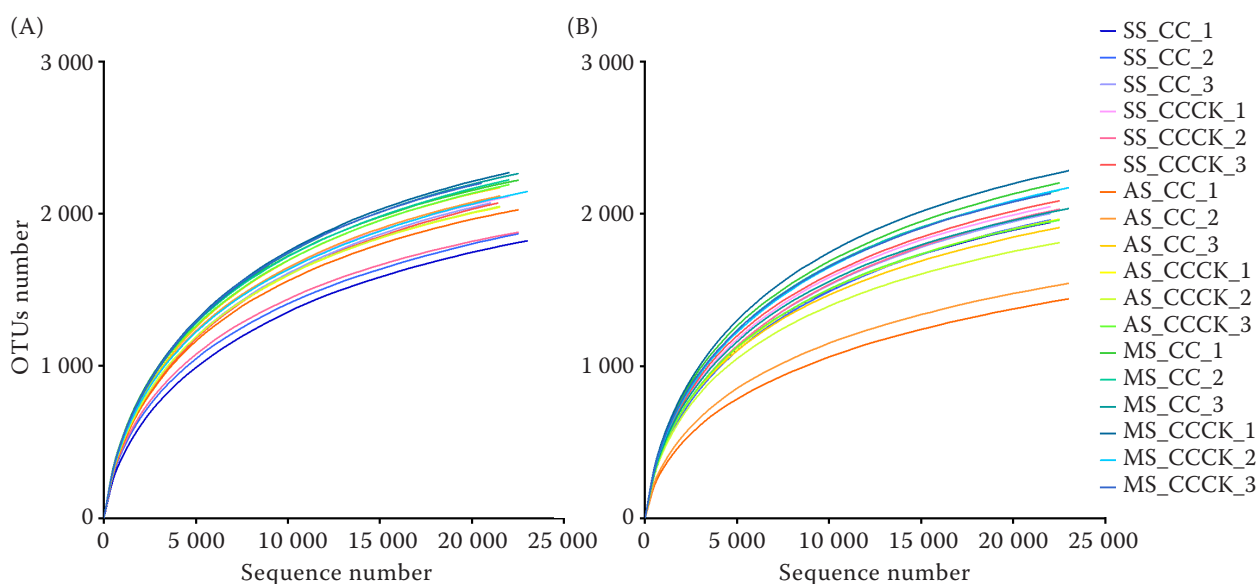
Figure 1. Rarefaction curves of operational taxonomic units (OTUs) clustered at 97% similarity in (A) 2014 and (B) 2015. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58. The sample labeled with CC_1, CC_2 and CC_3 correspond to three replicates of genetically modified cultivar CC; CCCK_1, CCCK_2 and CCCK_3 represent three replicates of cultivar CCCK

and rarefaction curves. The minimum difference was tested by one-way ANOVA in software SPSS 17.0 (SPSS Inc., Chicago, USA) ($P < 0.01$).

RESULTS AND DISCUSSION

Analysis of rhizosphere bacterial communities sequencing data. Overall, there were 809 999 high-quality sequences obtained and grouped into 5 277 OTUs at 97% similarity without singleton in 36 samples. The number of DNA sequences per sample ranged from 20 750 to 23 465, with a mean of 22 500 ± 649 sequences per sample. The OTUs were identified with

an average of 2 045 ± 183, ranging from 1 451 to 2 283. The rarefaction curves did not approach the asymptote as sequence number increased, which indicated that more OTUs can be identified in further sequencing. Besides, no significant difference of rarefaction curves was observed between CC and CCCK (Figure 1).

Analysis of 5 277 OTU showed that 4 714 OTUs were observed in 2014 and 4 417 OTUs were observed in 2015, which shared 3 854 OTUs (73%) together. 563 OTUs (10.7%) only existed in 2015 while 860 OTUs (16.3%) only existed in 2014 (Figure 2A). As shown in the Venn diagram (Figure 2B), 2 709 OTUs (57.5%) exited in all the growth stages of plants in
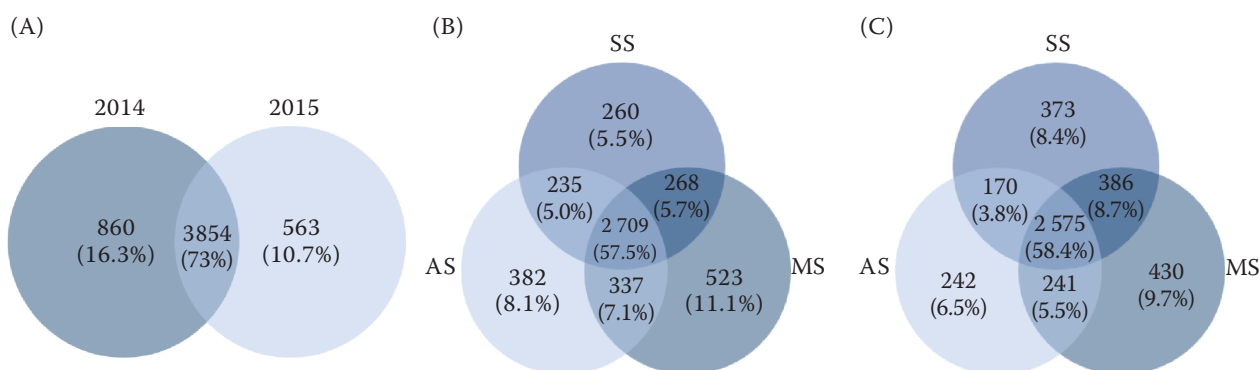

Figure 2. Venn diagram showing (A) variable overlaps between the year 2014 and 2015; (B) variable overlaps between three growth stages in year 2014, and (C) variable overlaps between three growth stages in year 2015. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58

2014. And 260 OTUs (5.5%) only observed in SS, 382 OTUs (8.1%) in AS, 523 OTUs (11.1%) in MS, which indicated that the number of OTUs existing in various developmental stages increased with plant maturity. In 2015, however, 373 OTUs (8.4%) only existed in SS, 242 OTUs (5.5%) in AS, 430 OTUs (9.7%) in MS. And 2 575 OTUs (58.3%) were shared among the three stages (Figure 2C).

Effects on the structure of bacterial communities. There were 34 phyla of bacteria identified in the 36 samples (Figure 3). Among them, Proteobacteria was the dominant phyla with an average of 33.3% relative abundance, followed by Actinobacteria (17.3%), Acidobacteria (13.6%), Bacteroidetes (9.0%), Verrucomicrobia (8.4%), Planctomycetes (5.6%), Gemmatimonadetes (4.4%), and Chloroflexi (3.8%). These phyla accounted for > 95% of relative bacterial abundance in total. 0.7% of relative abundance belonged to unclassified phyla. As shown in Figure 4, the eight bacterial phyla of CCCK were not significantly different from that of CC in 2014 (Figure 4A). However, the relative abundance of Proteobacteria, Planctomycetes, Gemmatimonadetes, Chloroflexi, Bacteroidetes and Acidobacteria was found to be distinctly different between cultivars at SS in 2015, and this significant difference disappeared later, which also emphasise the importance of annual replication (Figure 4B). Considering that no significant difference of main bacterial phyla was observed between cultivars in 2014 as well as at AS and MS in 2015, the difference at SS in 2015 may be caused by other factors. The seedlings rhizosphere was possibly altered by root depth, in turn

accounting for the differences observed in bacterial phyla (Haldar and Sengupta 2015). Alternatively soil pH is known to have a large influence on bacterial community composition. For example, Acidobacteria is more abundant in acidic soil, and Actinobacteria and Bacteroidetes more abundant in neutral soil (Aguirre-von-Wobeser et al. 2018). Overall, the unexplained variance is most likely to be caused by accidental error caused by some other factors. A three-year study assessing the impact of GM maize MON810 on the soil microbiome also emphasizes the importance of annual replication (Szoboszlay et al. 2019).

In the analysis of bacterial structure at phylum level, Proteobacteria, Acidobacteria and Actinobacteria accounted for the most relative abundance, which indicated the importance role of three phyla playing in maize growing processes. Proteobacteria was the most abundant bacteria in soil (Kolton et al. 2011, Lu et al. 2018). The rapid growth rate of Proteobacteria is hypothesised to be the primary reason for this dominance (García-Salamanca et al. 2013). Proteobacteria's function is hypothesised to be related to mediation of nitrogen transformation in root-associated soil (Li et al. 2014a). In previous studies, Proteobacteria and Acidobacteria have been found to take part in the soil restoration processes where nutrient is limited (Li et al. 2014b). The proportion of Actinobacteria was found to be independ on the type of crop (García-Salamanca et al. 2013).

Effects on the diversity of bacterial communities. The diversity of bacterial communities was assessed by α-diversity and β-diversity. Five indices (observed
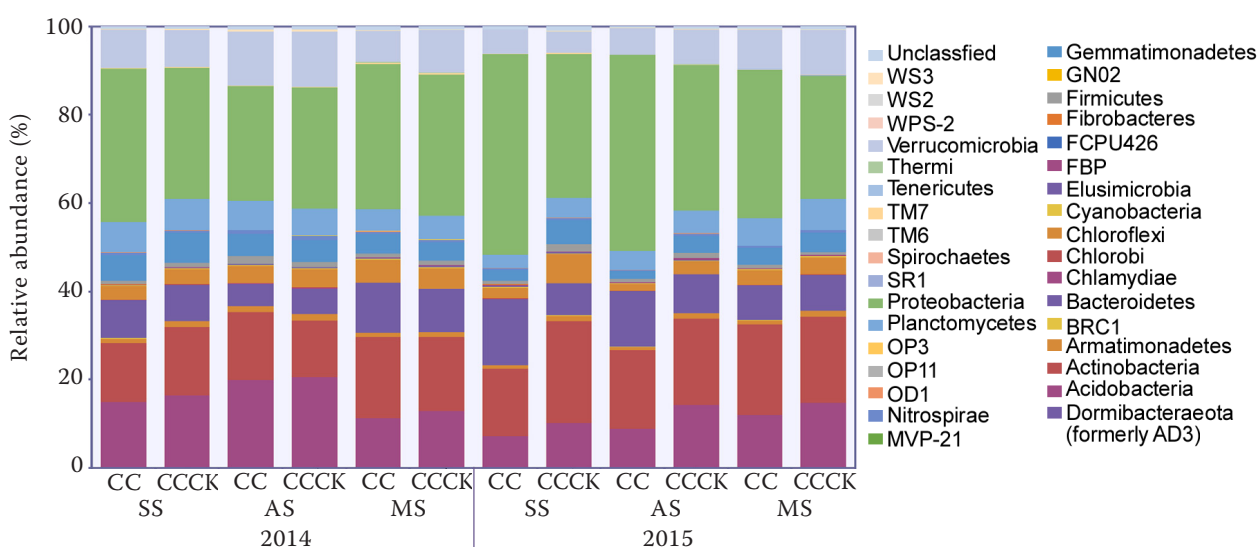

Figure 3. Bacterial composition at the phylum level. Relative read abundance of bacterial phyla within the communities. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58
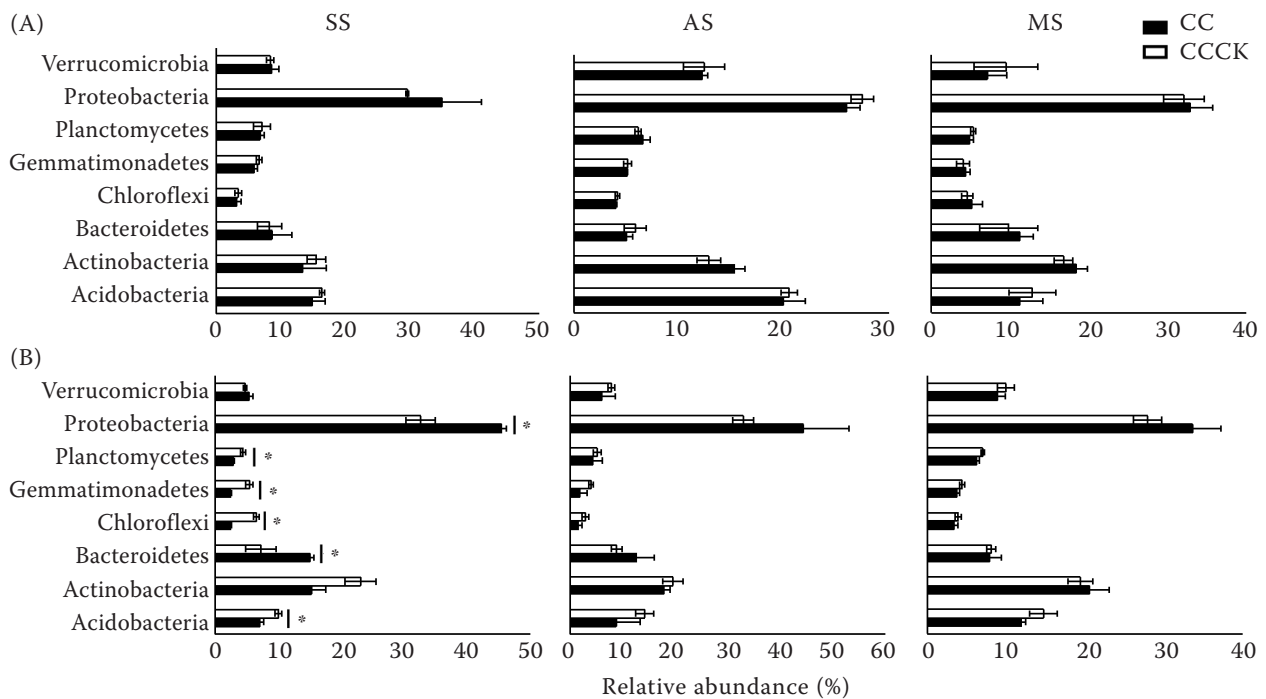
Figure 4. The relative abundance of main phyla in the rhizosphere of CC and CCCK in (A) 2014 and (B) 2015. *Indicates significant difference (*P* < 0.01) according to the ANOVA. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58

species, Chao, ACE, Shannon and Simpson) were selected to describe α-diversity of samples. There were no significant differences in the estimators of community richness (observed species, Chao, and ACE) and diversity (Shannon and Simpson) between CC and CCCK (Table 1). Furthermore, no significant differ-

ence was observed between cultivars, but the growth stage can influence α-diversity significantly (Table 2).

The β-diversity was analysed by PCA. The results of PCA showed clearly that there was no significant difference between CC and CCCK both in 2014 and 2015 (Figure 5A, 5C). However, distinct difference

Table 1. Data summary of α-diversity (means ± standard deviation) (*P* < 0.01)

			OTUs	Chao	ACE	Shannon	Simpson
2014	SS	CC	1 927.33 ± 128.14[A]	2 342.18 ± 131.66[A]	2 437.92 ± 141.89[A]	6.11 ± 0.26[A]	0.01 ± 0.00[A]
		CCCK	2 026.00 ± 124.00[A]	2 419.73 ± 161.87[A]	2 497.51 ± 160.67[A]	6.39 ± 0.14[A]	0.01 ± 0.00[A]
	AS	CC	2 108.67 ± 76.25[A]	2 472.32 ± 65.65[A]	2 558.07 ± 88.90[A]	6.39 ± 0.08[A]	0.01 ± 0.00[A]
		CCCK	2 100.33 ± 78.62[A]	2 485.55 ± 89.93[A]	2 557.47 ± 111.45[A]	6.45 ± 0.09[A]	0.01 ± 0.00[A]
	MS	CC	2 239.33 ± 26.63[A]	2 635.31 ± 40.02[A]	2 712.11 ± 45.26[A]	6.56 ± 0.05[A]	0.00 ± 0.00[A]
		CCCK	2 212.67 ± 62.69[A]	2 581.12 ± 53.84[A]	2 681.11 ± 58.50[A]	6.51 ± 0.04[A]	0.01 ± 0.00[A]
2015	SS	CC	1 975.00 ± 27.62[A]	2 382.72 ± 41.14[A]	2 454.93 ± 58.81[A]	6.17 ± 0.04[A]	0.01 ± 0.00[A]
		CCCK	2 062.67 ± 27.61[A]	2 491.31 ± 45.70[A]	2 540.02 ± 40.67[A]	6.34 ± 0.09[A]	0.01 ± 0.00[A]
	AS	CC	1 639.00 ± 244.20[A]	2 061.40 ± 261.78[A]	2 109.93 ± 226.44[A]	5.77 ± 0.46[A]	0.01 ± 0.01[A]
		CCCK	1 918.67 ± 86.33[A]	2 315.06 ± 118.35[A]	2 354.59 ± 106.53[A]	6.23 ± 0.05[A]	0.01 ± 0.00[A]
	MS	CC	2 139.33 ± 90.84[A]	2 570.83 ± 128.88[A]	2 610.99 ± 129.75[A]	6.47 ± 0.12[A]	0.00 ± 0.00[A]
		CCCK	2 197.00 ± 76.39[A]	2 642.86 ± 91.40[A]	2 681.08 ± 71.88[A]	6.55 ± 0.07[A]	0.00 ± 0.00[A]

The number of operational taxonomic units (OTUs) (observed species), richness estimators Chao and ACE, diversity estimators Shannon and Simpson were calculated at 3% distance. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58

Table 2. Comparison of α-diversity by Wilcoxon Rank-Sum test and Kruskal-Wallis test (means ± standard deviation) ($P < 0.01$)

		OTUs	Chao	ACE	Shannon	Simpson
Year	2014	2 102.39 ± 132.66	2 489.37 ± 130.72	2 574.03 ± 135.00	6.40 ± 0.19	0.01 ± 0.00
	2015	1 988.61 ± 211.74	2 410.70 ± 227.07	2 458.59 ± 218.92	6.25 ± 0.31	0.01 ± 0.00
	P-value	0.06	0.31	0.09	0.07	0.23
Cultivar	2014 CC	2 091.78 ± 155.40	2 483.27 ± 148.29	2 569.37 ± 147.27	6.35 ± 0.24	0.01 ± 0.00
	2014 CCCK	2 113.00 ± 114.00	2 495.47 ± 119.32	2 578.70 ± 130.34	6.45 ± 0.10	0.00 ± 0.00
	2015 CC	1 917.78 ± 256.79	2 338.32 ± 267.35	2 391.95 ± 259.23	6.14 ± 0.38	0.01 ± 0.00
	2015 CCCK	2 059.44 ± 134.33	2 483.08 ± 162.17	2 525.23 ± 157.01	6.37 ± 0.15	0.01 ± 0.00
	P-value	0.19	0.43	0.24	0.13	0.27
Growth stage	2014 SS	1 976.67 ± 125.06	2 380.95 ± 138.63	2 467.71 ± 139.44	6.25 ± 0.24	0.01 ± 0.00
	2014 AS	2 104.50 ± 69.42	2 478.94 ± 70.80	2 557.77 ± 90.17	6.42 ± 0.08	0.01 ± 0.00
	2014 MS	2 226.00 ± 45.49	2 608.22 ± 51.78	2 696.61 ± 49.77	6.54 ± 0.05	0.00 ± 0.00
	2015 SS	2 018.83 ± 54.00	2 437.02 ± 71.06	2 497.47 ± 64.94	6.25 ± 0.11	0.01 ± 0.00
	2015 AS	1 778.83 ± 224.27	2 188.23 ± 228.73	2 232.26 ± 207.38	6.00 ± 0.38	0.01 ± 0.00
	2015 MS	2 168.17 ± 81.44	2 606.85 ± 107.43	2 646.03 ± 101.36	6.51 ± 0.10	0.00 ± 0.00
	P-value	0.00	0.00	0.00	0.00	0.00

The number of operational taxonomic units (OTUs) (observed species), richness estimators Chao and ACE, diversity estimators Shannon and Simpson were calculated at 3% distance. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58

was observed between plant developmental stages both in 2014 and 2015 (Figure 5B, 5D). The difference of β-diversity between years was not significant (Figure 5E). Based on the results, our data showed that the diversity of rhizosphere bacterial communities was influenced by plant developmental stages rather than cultivars and years, which was possibly caused by root exudation changing with plant maturity (Chaparro et al. 2014). Yang et al. (2017) found that the main phyla associated with growth-related dynamic changes were Bacteroidetes, Proteobacteria and Actinobacteria, indicating that different growth

Table 3. Plant growth stages affecting relative abundance of eight bacterial phyla compared by ANOVA ($P < 0.01$)

	2014					2015				
	SS	AS	MS	*F*-value	*P*-value	SS	AS	MS	*F*-value	*P*-value
Acidobacteria	15.62 ± 1.55	20.22 ± 1.43	12.06 ± 2.76	24.919	0.000	8.60 ± 1.69	11.51 ± 4.26	13.34 ± 1.93	4.156	0.037
Actinobacteria	14.45 ± 2.73	14.06 ± 1.66	17.61 ± 1.46	5.536	0.016	19.19 ± 4.76	18.70 ± 1.74	19.99 ± 1.98	0.225	0.778
Bacteroidetes	8.45 ± 2.31	5.42 ± 0.89	10.54 ± 2.69	8.913	0.003	11.15 ± 4.55	10.73 ± 3.08	7.97 ± 1.05	1.716	0.213
Chloroflexi	3.31 ± 0.53	4.05 ± 0.16	4.88 ± 1.02	8.261	0.004	4.51 ± 2.26	2.24 ± 0.97	3.63 ± 0.52	3.728	0.048
Gemmatimonadetes	6.28 ± 0.62	5.10 ± 0.23	4.23 ± 0.65	22.073	0.000	3.98 ± 1.68	2.89 ± 1.51	4.06 ± 0.51	1.436	0.269
Planctomycetes	6.94 ± 0.95	6.35 ± 0.54	5.12 ± 0.45	10.986	0.001	3.67 ± 0.88	4.72 ± 1.31	6.64 ± 0.51	14.882	0.000
Proteobacteria	32.25 ± 4.86	26.72 ± 1.34	32.49 ± 2.48	6.084	0.012	39.02 ± 7.21	38.69 ± 8.43	30.79 ± 4.03	2.804	0.092
Verrucomicrobia	8.48 ± 0.83	12.30 ± 1.29	8.32 ± 3.25	7.062	0.007	5.06 ± 0.57	6.96 ± 1.97	9.43 ± 1.11	15.812	0.000

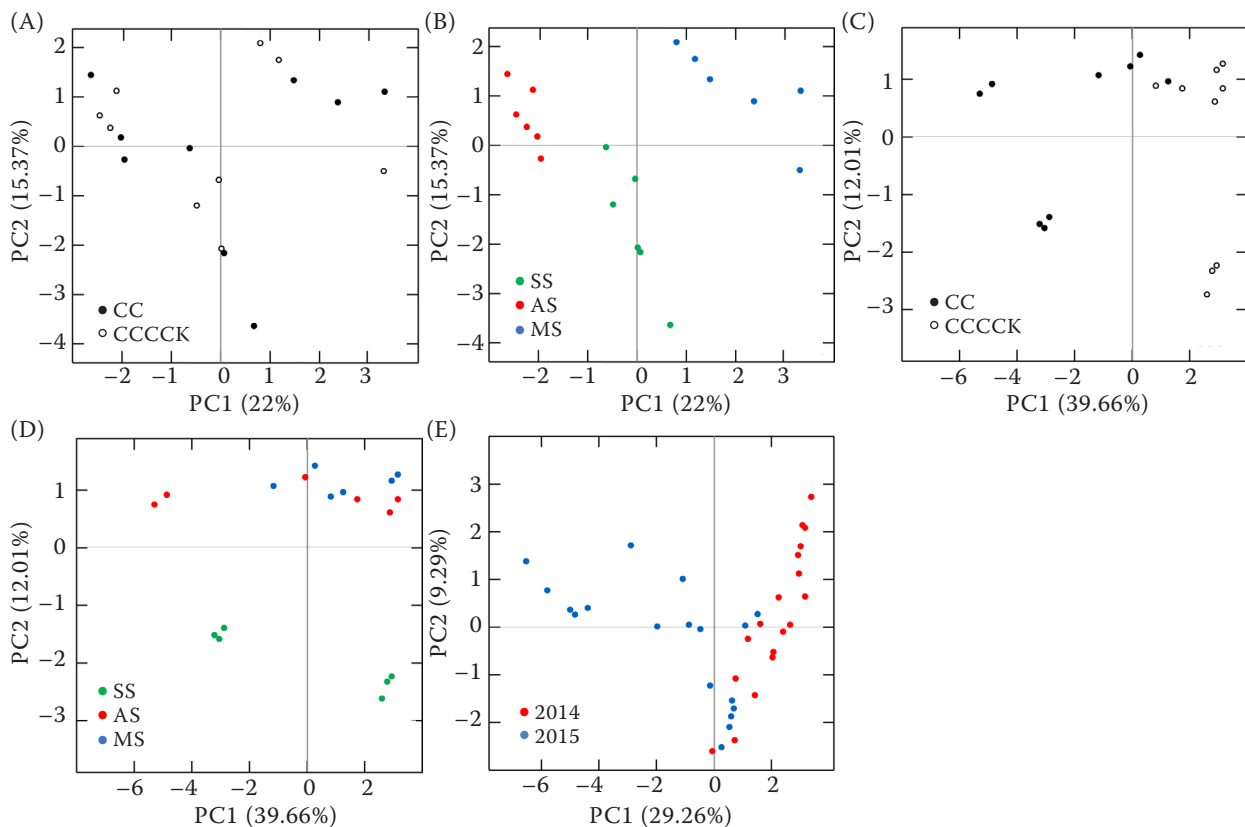SS – seedling stage; AS – at anthesis; MS – maturity-setting stage

Figure 5. Principal component analysis (PCA) of bacterial communities. (A) Bacterial community structure between cultivars in 2014; (B) bacterial community structure of different growth stages in 2014; (C) bacterial community structure between cultivars in 2015; (D) bacterial community structure of different growth stages in 2015, and (E) bacterial community structure between various growth years. The eigenvalues displayed on the diagram axes refer to the percentage variation of the respective axis. SS – seedling stage; AS – at anthesis; MS – maturity-setting stage; CC – CC-2; CCCK – Zheng 58

stages affected the bacterial community composition in maize soil. In our study, when comparing the effects of plant growth stages on relative abundance of eight phyla by ANOVA, relative abundance of Planctomycetes, Gemmatimonadetes, Chloroflexi, Bacteroidetes, Verrucomicrobia, Acidobacteria had significant difference between plant developmental stages in 2014; relative abundance of Planctomycetes and Verrucomicrobia changed significantly with plant development in 2015. Both Proteobacteria and Actinobacteria did not changed their relative abundance significantly during plant growth (Table 3). Therefore, Proteobacteria and Actinobacteria were two important bacterial communities in the whole life of maize, and did not change their relative abundance as the maize grow. Wang et al. (2011) also reported that Proteobacteria and Actinobacteria were the important components of the core microbiome in the rhizosphere.

In conclusion, we found that GM maize CC-2 did not significantly affect the rhizosphere bacterial community dynamics. Our studies provide reliable scientific data to support consideration of CC-2 commercial cultivation.

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