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No significant differences in rhizosphere bacterial communities between *Bt* maize cultivar IE09S034 and the near-isogenic non-*Bt* cultivar Zong31

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ABSTRACT

Liang J.G., Luan Y., Jiao Y., Xin L.T., Song X.Y., Zheng X.B., Zhang Z.G. (2018): No significant differences in rhizosphere bacterial communities between *Bt* maize cultivar IE09S034 and the near-isogenic non-*Bt* cultivar Zong31. Plant Soil Environ., 64: 427–434.

The release of genetically modified (GM) crops has potential to alter the bacterial population within rhizosphere. Here, the potential effect of GM maize cv. IE09S034 containing the *Cry1Ie* toxin gene from *Bacillus thuringiensis* (*Bt*) was investigated under the field conditions. The community composition and the relative abundance of the bacteria in rhizosphere soil were estimated by analysing 16S rRNA PCR amplicons. Our results indicated that *Bt* maize IE09S034 has no significant effects on the rhizosphere bacterial community. Instead, it was found that factors such as plant growth stage and year have a stronger effect on the bacterial population dynamics. Our findings therefore provide reliable evidence supporting the potential commercial cultivation of the cv. IE09S034.

Keywords: *Zea mays* L.; soil microorganisms; 16S rRNA gene sequencing

Despite the achievement that GM (genetically modified) crops helped to meet the nutritional need of humans and farm animals, they also sparked fierce debates regarding their safety (Cui and Shoemaker 2018). With the increased numbers of commercial application and larger cultivation areas of GM crops, their biosafety to soil microorganisms has become a major issue, specifically, the effect on the rhizosphere bacterial community that plays an important role in promoting plant growth (Turrini et al. 2015, Guan et al. 2016, Lu et al. 2018a). Previous studies have revealed little effects of GM crops on the rhizosphere microbial community (Guan et al. 2016, Singh and Dubey 2017, Tundup et al. 2017). However, it is believed that the effects of GM crops should be assessed on a case-by-case basis (Liang et al. 2016, 2018).

The GM maize cv. IE09S034 has been genetically modified to express an insecticidal protein using the *Cry1Ie* gene, which was initially identi-

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fied in the *Bacillus thuringiensis* isolate Btc007 (Song et al. 2003). To date, several studies have attempted to determine the potential aboveground adverse effects of the GM maize cv. IE09S034 (Guo et al. 2016, Liu et al. 2016). In contrast, the belowground effects of IE09S034 have not been examined. Here, the effects of IE09S034 on the rhizosphere bacterial community were tested during three different growth stages in two experimental years. By using the Illumina MiSeq technique, it is possible to examine the response of rhizosphere bacteria to the GM maize cv. IE09S034 (Lu et al. 2018b). Understanding of the interactions between IE09S034 and the rhizosphere bacterial community would provide the evidence supporting the commercial cultivation of the cv. IE09S034.

MATERIAL AND METHODS

Plant materials and field trials. The *Bt* maize cv. IE09S034 (IE) and the near-isogenic non-GM cv. Zong31 (IECK) were used in this study. IE09S034 expresses the *Cry1Ie* gene under the control of the ubiquitin promoter from maize (Guo et al. 2016). The seeds of IE and IECK were provided by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences.

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. Briefly, each line occupied three blocks (IE, three replicates: IE_1, IE_2 and IE_3; IECK, three replicates: IECK_1, IECK_2 and IECK_3) (Li et al. 2017). Maize was maintained following the agronomic practices typical of northeast China but without applying insecticides. The soil was the black soil typical of the north eastern China, containing 15.71 ± 0.04 g/kg organic carbon, 77.54 ± 0.07 mg/kg alkaline nitrogen, 10.68 ± 0.07 mg/kg available phosphorus, and 154.10 ± 0.76 mg/kg available potassium. The pH of this experimental field was 5.36 ± 0.02 (Liu et al. 2016).

Rhizosphere sampling and soil DNA extraction. Sampling was carried out at various stages of the growth cycle, corresponding to the seedling stage (SS); flowering stage (FS); maturity-setting stage (MS) each growing season according to the previous reports (Liang et al. 2014, 2015). Briefly, the loosely adhering soil on the roots was shaken off, soil tightly adhering to the roots constituting the rhizosphere soil was brushed off and collected (constituting rhizosphere soil) (Liang et al. 2014, 2017).

DNA was extracted from 0.5 g of the rhizosphere soil samples using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, USA). The final quantity and quality of DNA were evaluated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA) (Liang et al. 2015). DNA of high-quality (OD260/280 = 1.8–2.0, c ≥ 20 ng/μL) was adjusted to a concentration of 20 ng/μL and used for PCR amplification.

16S rRNA gene sequencing and analysis. Bacterial 16S rRNA gene amplification and sequencing were performed at BGI (Shenzhen, China). In brief, amplification of the 16S V4 region was accomplished using a dual index paired-end sequencing strategy in an Illumina MiSeq platform as described previously (Caporaso et al. 2011). Each primer consisting of an Illumina adapter, an 8-nt index sequence, 10-nt pad sequences, a 2-nt linker, and gene-specific primers F515/R806. Amplification was performed on a 96-well plate using AccuPrime Pfx SuperMix reagents, and library clean-up and normalization were performed using the Invitrogen SequalPrep Plate Normalization Kit. The library QC was performed using a KAPA Biosystems Q-PCR kit and by obtaining a bioanalyser trace using the Agilent Technologies HS DNA kit (Penton et al. 2016).

16S rRNA gene sequences were analysed using Mothur v1.31.2 (Schloss et al. 2009). Briefly, after removal of barcode and primer sequences, reads were truncated to a length of 250 bp, and only those reads with a quality score (Q) > 15 and no ambiguous bases were retained for further analysis. Chimeras were removed using UCHIME v4.2 (Edgar et al. 2011). Operational taxonomic units (OTUs) were defined at 97% sequence identity. OTU-representative sequences were taxonomically classified using the Ribosomal Database Project classifier trained on the Greengenes-formatted databases. The resulting OTU table was used to determine the relative abundances of taxa, and for subsequent statistical analysis of α- and β-diversity. Paired-end reads of FASTQ files for all samples are available in the Sequence Read Archive under BioProject PRJNA401395.

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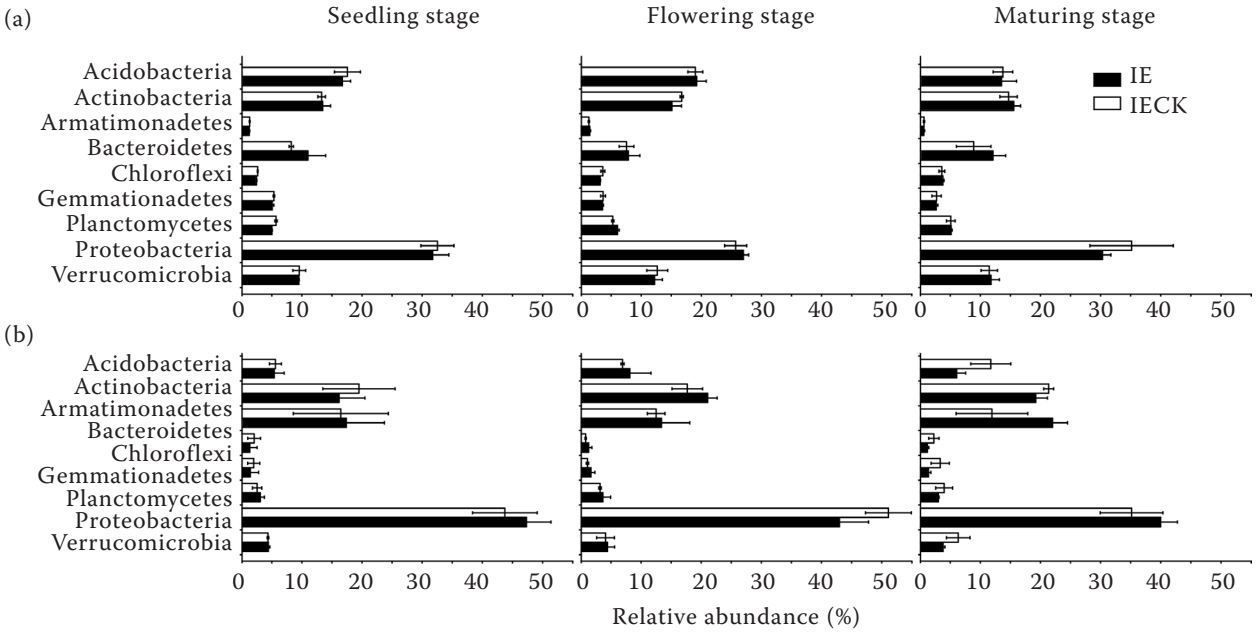

Figure 1. Relative abundance of the main phyla in the IE (*Bt* maize IE09S034) and IECK (near isogenic non-GM cultivar Zong31) rhizosphere during (a) 2014 and (b) 2015 growing seasons ($P < 0.01$)

Table 1. Relative abundance of 21 rare phyla in the IE (*Bt* maize IE09S034) and IECK (near isogenic non-GM cultivar Zong31) rhizosphere in 2014 ($P < 0.01$)

	Seedling stage		Flowering stage		Maturing stage	
	IE	IECK	IE	IECK	IE	IECK
AD3	0.027 ± 0.014[a]	0.027 ± 0.011[a]	0.034 ± 0.013[a]	0.031 ± 0.019[a]	0.012 ± 0.003[a]	0.019 ± 0.007[a]
BRC1	0.029 ± 0.018[a]	0.027 ± 0.012[a]	0.035 ± 0.008[a]	0.019 ± 0.010[a]	0.039 ± 0.024[a]	0.034 ± 0.013[a]
Chlamydiae	0.053 ± 0.026[a]	0.020 ± 0.020[a]	0.059 ± 0.026[a]	0.061 ± 0.034[a]	0.052 ± 0.017[a]	0.047 ± 0.009[a]
Chlorobi	0.040 ± 0.017[a]	0.046 ± 0.021[a]	0.076 ± 0.033[a]	0.080 ± 0.023[a]	0.058 ± 0.022[a]	0.047 ± 0.034[a]
Cyanobacteria	0.149 ± 0.054[a]	0.154 ± 0.013[a]	0.145 ± 0.019[a]	0.258 ± 0.134[a]	0.386 ± 0.066[a]	0.231 ± 0.016[a]
Elusimicrobia	0.135 ± 0.029[a]	0.224 ± 0.041[a]	0.176 ± 0.019[a]	0.188 ± 0.065[a]	0.226 ± 0.043[a]	0.159 ± 0.065[a]
FBP	0.112 ± 0.045[a]	0.113 ± 0.020[a]	0.077 ± 0.027[a]	0.059 ± 0.006[a]	0.106 ± 0.005[a]	0.071 ± 0.008[a]
Fibrobacteres	0.032 ± 0.007[a]	0.013 ± 0.004[a]	0.048 ± 0.016[a]	0.030 ± 0.015[a]	0.022 ± 0.000[a]	0.031 ± 0.023[a]
Firmicutes	0.475 ± 0.029[a]	0.552 ± 0.027[a]	0.886 ± 0.211[a]	1.037 ± 0.252[a]	0.932 ± 0.223[a]	0.763 ± 0.244[a]
Nitrospirae	0.573 ± 0.025[a]	0.626 ± 0.051[a]	0.747 ± 0.110[a]	0.702 ± 0.101[a]	0.387 ± 0.169[a]	0.415 ± 0.053[a]
OD1	0.085 ± 0.024[a]	0.113 ± 0.020[a]	0.094 ± 0.044[a]	0.119 ± 0.034[a]	0.153 ± 0.034[a]	0.143 ± 0.068[a]
OP11	0.003 ± 0.005[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.001 ± 0.003[a]	0.000 ± 0.000[a]	0.003 ± 0.005[a]
OP3	0.009 ± 0.012[a]	0.004 ± 0.008[a]	0.012 ± 0.007[a]	0.015 ± 0.007[a]	0.003 ± 0.005[a]	0.003 ± 0.005[a]
SR1	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.001 ± 0.003[a]	0.004 ± 0.008[a]	0.001 ± 0.003[a]
Spirochaetes	0.000 ± 0.000[a]	0.001 ± 0.002[a]	0.004 ± 0.004[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]
TM6	0.006 ± 0.010[a]	0.003 ± 0.005[a]	0.013 ± 0.012[a]	0.027 ± 0.035[a]	0.013 ± 0.008[a]	0.006 ± 0.010[a]
TM7	0.203 ± 0.034[a]	0.181 ± 0.053[a]	0.170 ± 0.033[a]	0.131 ± 0.017[a]	0.396 ± 0.127[a]	0.268 ± 0.072[a]
Tenericutes	0.023 ± 0.003[a]	0.023 ± 0.011[a]	0.041 ± 0.019[a]	0.042 ± 0.024[a]	0.019 ± 0.009[a]	0.028 ± 0.018[a]
Thermi	0.019 ± 0.013[a]	0.001 ± 0.002[a]	0.003 ± 0.005[a]	0.008 ± 0.009[a]	0.031 ± 0.016[a]	0.034 ± 0.048[a]
WPS-2	0.016 ± 0.007[a]	0.039 ± 0.012[a]	0.038 ± 0.017[a]	0.040 ± 0.037[a]	0.013 ± 0.008[a]	0.025 ± 0.014[a]
WS3	0.217 ± 0.066[a]	0.140 ± 0.032[a]	0.241 ± 0.032[a]	0.221 ± 0.046[a]	0.118 ± 0.072[a]	0.121 ± 0.047[a]
Unclassified bacteria	0.628 ± 0.048[a]	0.568 ± 0.054[a]	0.615 ± 0.060[a]	0.601 ± 0.157[a]	0.739 ± 0.092[a]	0.733 ± 0.135[a]

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RESULTS AND DISCUSSION

Taxonomic structures of the bacterial community. It is suggested that risk assessment studies on the effect of GM crops should be conducted under field conditions over at least two years (Filion 2008). Therefore, a 2-year experiment was carried out to assess the potential impact of the *Bt* maize cv. IE09S034 on rhizosphere bacterial communities in comparison to Zong31. A total of 829 885 16S rRNA raw reads were generated. After removing error-containing sequences and chimeras, 4604 OTUs were identified at a 97% similarity threshold. 30 different phyla were identified in the samples. Among them, Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, Verrucomicrobia, Planctomycetes, Gemmatimonadetes, Chloroflexi and Armatimonadetes accounted for > 90% of the reads. Similar to previous findings, these phyla constituted the dominant taxonomic groups for maize (Schmalenberger and Tebbe 2002, Fang et al. 2005, Miethling-Graff et al. 2010). No significant difference was detected between IE and IECK in the proportions of these phyla (Figure 1).

For rare-to-prevalent community dynamics, both deep sequencing and deep sampling were recommended that could reveal rare microbial taxon (Liang et al. 2014, Lynch and Neufeld 2015). To identify whether there were less common species with lower biomass but may have large effects on soil function, deep sequencing platforms were employed such as Illumina MiSeq that has been shown to reveal more sequences for some rare taxa (Zhan et al. 2014). However, no significant differences were found between IE and IECK for the 21 rare phyla (Tables 1 and 2). Intriguingly, Spirochaetes were in a very low content in 2014 and were not detectable in 2015.

In addition, a small proportion (0.53%) of the bacterial 16S rRNA gene was unclassified due to, to some degree, low reading accuracy and low resolution of short amplicons. This suggests that the high throughput full-length bacterial 16S rRNA gene

Table 2. Relative abundance of 21 rare phyla in the IE (*Bt* maize IE09S034) and IECK (near isogenic non-GM cultivar Zong31) rhizosphere in 2015 ($P < 0.01$)

	Seedling stage		Flowering stage		Maturing stage	
	IE	IECK	IE	IECK	IE	IECK
AD3	0.007 ± 0.009[a]	0.001 ± 0.002[a]	0.010 ± 0.011[a]	0.003 ± 0.003[a]	0.010 ± 0.010[a]	0.004 ± 0.005[a]
Armatimonadetes	0.626 ± 0.216[a]	0.579 ± 0.212[a]	0.817 ± 0.242[a]	0.473 ± 0.223[a]	0.449 ± 0.165[a]	0.774 ± 0.295[a]
BRC1	0.007 ± 0.005[a]	0.013 ± 0.000[a]	0.010 ± 0.003[a]	0.010 ± 0.003[a]	0.035 ± 0.017[a]	0.050 ± 0.006[a]
Chlamydiae	0.030 ± 0.008[a]	0.023 ± 0.009[a]	0.039 ± 0.018[a]	0.023 ± 0.013[a]	0.039 ± 0.002[a]	0.052 ± 0.009[a]
Chlorobi	0.017 ± 0.020[a]	0.035 ± 0.008[a]	0.033 ± 0.008[a]	0.035 ± 0.017[a]	0.031 ± 0.006[a]	0.059 ± 0.026[a]
Cyanobacteria	0.170 ± 0.032[a]	0.222 ± 0.156[a]	0.215 ± 0.081[a]	0.189 ± 0.059[a]	0.154 ± 0.025[a]	0.222 ± 0.037[a]
Elusimicrobia	0.031 ± 0.030[a]	0.051 ± 0.002[a]	0.044 ± 0.044[a]	0.010 ± 0.011[a]	0.037 ± 0.022[a]	0.103 ± 0.037[a]
FBP	0.402 ± 0.226[a]	0.196 ± 0.138[a]	0.267 ± 0.150[a]	0.247 ± 0.112[a]	0.190 ± 0.060[a]	0.197 ± 0.115[a]
Fibrobacteres	0.004 ± 0.07[a]	0.001 ± 0.003[a]	0.025 ± 0.021[a]	0.007 ± 0.013[a]	0.044 ± 0.034[a]	0.077 ± 0.023[a]
Firmicutes	0.529 ± 0.246[a]	0.857 ± 0.439[a]	0.663 ± 0.194[a]	0.329 ± 0.060[a]	1.062 ± 0.267[a]	0.613 ± 0.213[a]
Nitrospirae	0.145 ± 0.136[a]	0.153 ± 0.092[a]	0.164 ± 0.119[a]	0.141 ± 0.025[a]	0.140 ± 0.026[a]	0.328 ± 0.159[a]
OD1	0.021 ± 0.026[a]	0.039 ± 0.014[a]	0.006 ± 0.007[a]	0.010 ± 0.005[a]	0.008 ± 0.004[a]	0.037 ± 0.007[a]
OP11	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.004 ± 0.004[a]	0.003 ± 0.005[a]
OP3	0.000 ± 0.000[a]	0.001 ± 0.002[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]
SR1	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.004 ± 0.004[a]
TM6	0.006 ± 0.010[a]	0.006 ± 0.006[a]	0.004 ± 0.004[a]	0.007 ± 0.007[a]	0.010 ± 0.009[a]	0.019 ± 0.015[a]
TM7	0.141 ± 0.075[a]	0.203 ± 0.188[a]	0.087 ± 0.014[a]	0.104 ± 0.052[a]	0.093 ± 0.024[a]	0.129 ± 0.055[a]
Tenericutes	0.004 ± 0.007[a]	0.003 ± 0.005[a]	0.022 ± 0.024[a]	0.023 ± 0.011[a]	0.008 ± 0.004[a]	0.018 ± 0.012[a]
Thermi	0.021 ± 0.019[a]	0.014 ± 0.013[a]	0.011 ± 0.009[a]	0.020 ± 0.009[a]	0.006 ± 0.002[a]	0.006 ± 0.010[a]
WPS-2	0.011 ± 0.007[a]	0.014 ± 0.003[a]	0.010 ± 0.009[a]	0.016 ± 0.002[a]	0.003 ± 0.002[a]	0.004 ± 0.008[a]
WS3	0.031 ± 0.029[a]	0.044 ± 0.008[a]	0.026 ± 0.018[a]	0.018 ± 0.016[a]	0.027 ± 0.019[a]	0.110 ± 0.097[a]
Unclassified bacteria	0.526 ± 0.274[a]	0.602 ± 0.400[a]	0.302 ± 0.147[a]	0.303 ± 0.035[a]	0.323 ± 0.081[a]	0.470 ± 0.110[a]

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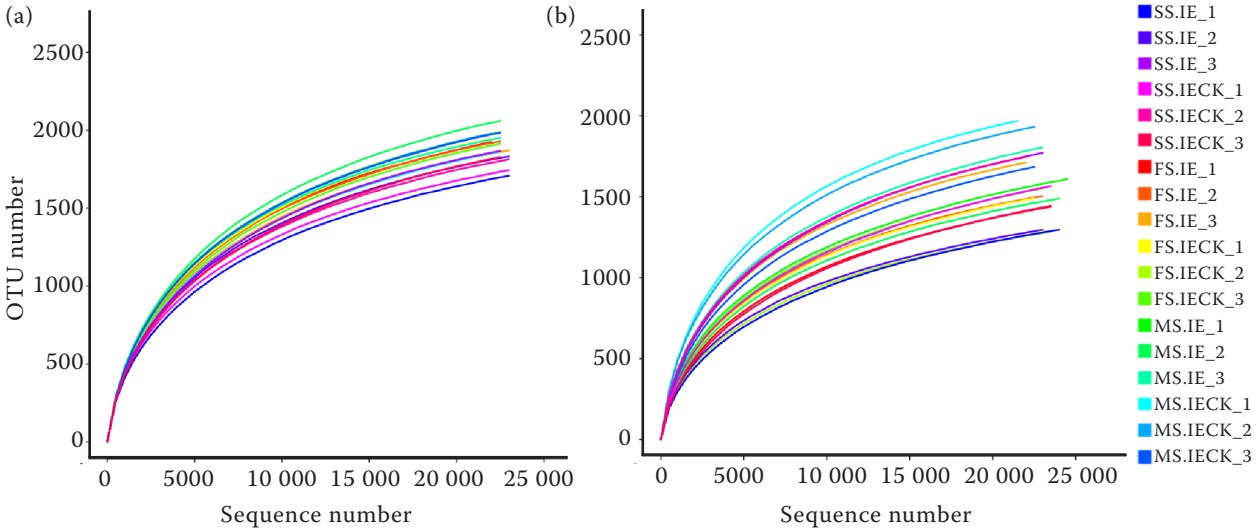

Figure 2. Rarefaction curves of the operational taxonomic units (OTUs) obtained from the maize rhizosphere soil of various cultivars in the year (a) 2014 and (b) 2015. The curves were named in the following form: growth stage – cultivar replicate. SS – seedling stage; FS – flowering stage; MS – maturity-setting stage. IE – *Bt* maize IE09S034; IECK – near isogenic non-GM cultivar Zong31

sequencing methodologies with reduced biases are needed (Wagner et al. 2016). Another possibility is that these unclassified sequences could either be novel and therefore cannot be classified at the present, or that they belong to less well-studied lineages (Yang et al. 2015).

Effects on α-diversity and β-diversity. To accurately assess the changes in the bacterial community due to the introduction of GM crops, it is important to know to what extent microbial communities need to be sampled. Lundin et al. (2012) found that 1000 denoised sequences per sample explain up to 90% the trends in β-diversity. Similarly, 5000 denoised sequences were sufficient to describe trends in α-diversity. Since an average of 23 052 sequences was obtained per sample in our study, it suffices to say that our data are sufficient to describe the patterns in the bacterial α- and β-diversity.

It was found that the rarefaction curves did not differ between IE and IECK in both years, but the data were sufficient for revealing the differences if there were (Figure 2). In addition, there were also no significant differences in the estimators of community richness (observed species) and diversity (Shannon index) between IE and IECK cultivars (Figures 3 and 4).

Principal coordinate analysis (PCoA) of unweighted (sensitive to rare taxa) UniFrac distance was performed to assess how β-diversity could be partitioned into variations attributable to cultivar, growth stage and year (Peiffer et al. 2013). Our PCoA revealed significant differences in the

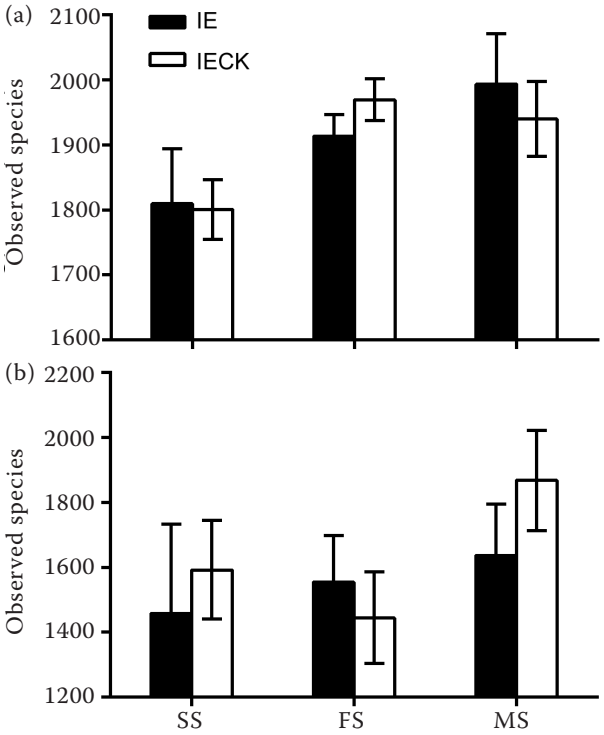

Figure 3. Comparison of the observed species between IE (*Bt* maize IE09S034) and IECK (near isogenic non-GM cultivar Zong31) in the year (a) 2014 and (b) 2015 ($P < 0.01$). SS – seedling stage; FS – flowering stage; MS – maturity-setting stage

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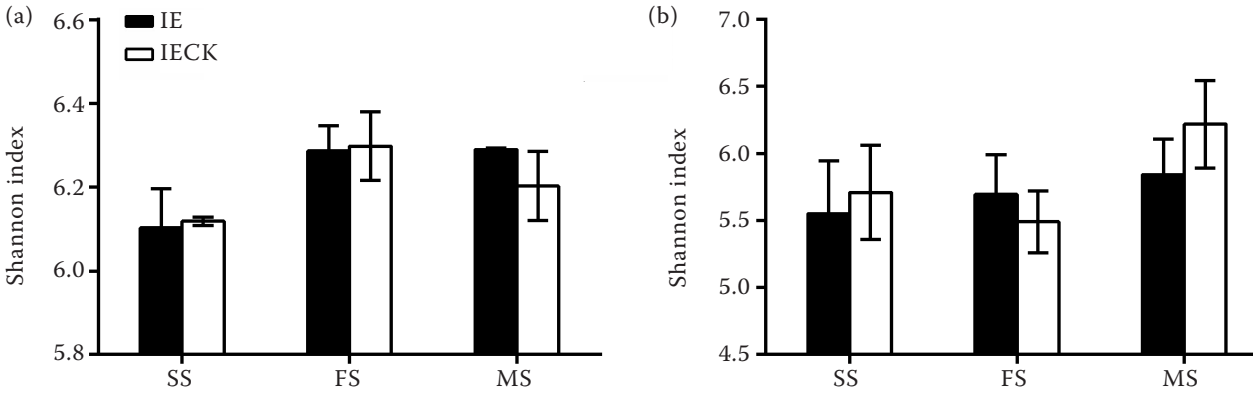

Figure 4. Comparison of the Shannon index between IE (*Bt* maize IE09S034) and IECK (near isogenic non-GM cultivar Zong31) in the year (a) 2014 and (b) 2015 ($P < 0.01$). SS – seedling stage; FS – flowering stage; MS – maturity-setting stage

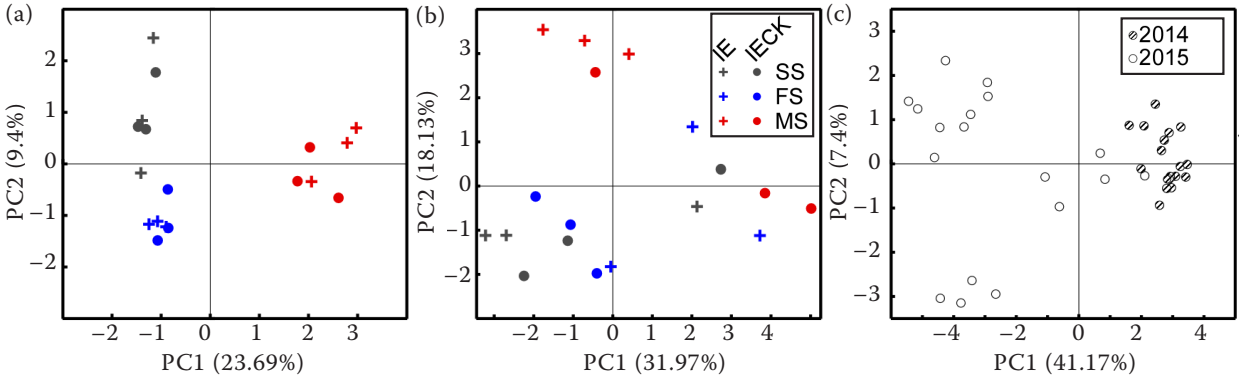

Figure 5. Principal coordinate analysis (PCoA) analysis among cultivars and stages in (a) 2014 and (b) 2015, and (c) PCoA analysis of two experimental years. The variance explained by each principal coordinate axis is shown in parentheses. SS – seedling stage; FS – flowering stage; MS – maturity-setting stage. IE – *Bt* maize IE09S034; IECK – near isogenic non-GM cultivar Zong31

bacterial community structure among the three growth stages during 2014 (Figure 5a), whereas there were no significant differences among the three growth stages in 2015 (Figure 5b). Using cultivar as an explanatory variable, no significant differences were found in the rhizosphere bacterial community between IE and IECK cultivars during the same year.

The ADONIS differences were also calculated among the effects of cultivar, growth stage and

Table 3. ADONIS analysis of effects of cultivar, growth stage and year on the bacterial community structure in rhizosphere soil

		Df	Sums of squares	Mean squares	*F* Model	R^2	*P* (> F)
Cultivar	qiime.data$map[[opts$category]]	1	0.1572	0.15717	1.4163	0.09990	0.070
	residuals	34	3.7732	0.11098		0.96001	
	total	35	3.9304			1	
Growth stage	qiime.data$map[[opts$category]]	2	0.2640	0.13200	1.1882	0.06717	0.140
	residuals	33	3.6664	0.11110		0.93283	
	total	35	3.9304			1	
Year	qiime.data$map[[opts$category]]	1	0.5414	0.54138	5.4314	0.13774	0.001
	residuals	34	3.3890	0.09968		0.86226	
	total	35	3.9304			1	

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year, and no significant effects on the bacterial community structure were found either for cultivar or growth stage (Table 3). Both PCoA and ADONIS analyses revealed a significant difference between the two experimental years (Table 3, Figure 5c). Together, these findings indicated that the growing year was the major factor that contributed to the differences in the community structure. Furthermore, the bacterial community structure in the soil was marginally related to the growth stage. This is consistent with the previous findings that year-to-year variations in soil bacterial communities are not uncommon and plant growth stages have a strong influence in the soil microbiome composition (Cotta et al. 2014, Zhang et al. 2015, DeBruyn et al. 2017).

In conclusion, our results showed that *Bt* maize cv. IE09S034 did not significantly affect the rhizosphere bacterial community dynamics during 2014 and 2015 growing seasons. The bacterial community structure was markedly affected by natural variations relevant to the growing year and growth stage. This finding contributes to our understanding of how GM crops might impact on the ecosystem and the safety concerns posed by the cultivation of GM crops. These studies provide reliable scientific data to support consideration of GM IE09S034 cultivar commercial cultivation.

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