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Effect of transgenic cotton continuous cropping on soil bacterial community

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Abstract

Purpose: In agricultural practices, continuous cultivation of genetically modified crops with high commercial value has a definite impact on soil microbial diversity. Soil microorganisms directly define the operational degree and function realization of the soil ecosystem. To understand the safety of environmental release, we studied the effects of continuous cropping of transgenic cotton on the diversity of bacterial communities in the rhizosphere soil.

Methods: We have applied a high-throughput sequencing method and compared the bacterial community structure as well as diversity of rhizosphere soil of the transgenic cotton line (25C-1) and its parent cotton line (TH2).

Result: Structural analysis of the bacterial community showed that *Arthrobacter* and *Sphingomonas* are significantly enriched after continuous cropping of transgenic cotton lines and had a positive impact on the soil's ecological environment. Interestingly, parameters of the physical and chemical properties of soil used for the continuous cropping of the two cotton lines for 3 consecutive years show no detectable change, other than total nitrogen. Notably, Spearman's correlation analysis suggests that total nitrogen is the key environmental factor that affects the bacterial community of the soil used to cultivate the transgenic cotton.

Conclusion: We did not find a notable difference in species diversity between the two samples. However, the proportions of beneficial bacteria (*Arthrobacter* and *Sphingomonas*) increased and the total nitrogen content has changed in 3 years. These results provide necessary insights into the function and role of bacteria in transgenic cotton. This study will help future investigators assess the potential ecological risks of genetically modified plants.

Keywords: Transgenic cotton rhizosphere soil, High-throughput sequencing, Bacterial community, Environmental safety assessment of transgenic crops

Introduction

The global production of genetically modified crops has grown profoundly from 1.7 million hectares in 1996 to 191.7 million hectares in 2018 (Babar et al. 2019). The agriculture practices with commercial transgenic crops, such as cotton (a leading cash crop) and maize (Trivedi et al. 2012), have brought enormous economic benefits worldwide (Guo et al. 2018). However, world average cotton yield in 2005 was estimated at 650 kg lint ha^{-1}, a 73% loss to various stresses (comparable to those values published for other crops). Among all stresses, the main factors affecting cotton yield are drought and salt stress, and improving the tolerance of cotton to these two abiotic stresses is currently the most urgent task (Saranga et al. 2009). A recent report demonstrates that under stress conditions, the *Arabidopsis* transcription factors *CBF/DREB1* bind to *CRT/DRE* to activate the transcription of its downstream genes, *COR* (Randall and Yamasaki 2016). This enhances the ability of the plants to resist various abiotic stress such as low temperature, drought, and high salt (Verslues et al. 2006).

Like the advancement of any other technology, the question of environmental risks associated with transgenic plants remains to be answered. The increased worldwide commercial cultivation of genetically modified crops in

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the past 20 years is likely to have a profound impact on soil microbial communities (Lilley et al. 2006; Hutchison et al. 2010; Lu et al. 2012). Multiple lines of evidence have shown that transgenic plants affect the soil microbial communities, especially rhizosphere bacteria (Koskella and Stotzky 1997; Knox et al. 2007; Jepson et al. 2010; Trivedi et al. 2012). The foreign genes in transgenic crops may remain in the soil through root exudates or defoliation. This may lead to the change of soil microbial communities, and the physical and chemical properties of soil will change accordingly. Moreover, inflicted changes may also affect the availability and release of soil nutrients (DeAngelis et al. 2009; Uroz et al. 2010). Therefore, the effect of transgenic plants on soil microbial communities is of great significance for scientific assessment to evaluate its potential ecological risks in the future.

Soil microorganisms play a central role in the biogeochemical cycle of nutrient and organic matters. This is mainly caused by decomposition of the organic materials in the soil that essentially maintains a stable agricultural ecosystem (Van der Heijen et al. 2008). Most plants have symbiotic relationships with soil microbes (bacteria and fungi) during their growth and development. Soil bacteria are the most abundant and widely distributed among soil microorganisms. The plant grows in close association with a bacterial community that lives and thrives in soil around the surface (rhizosphere) or inside their roots (endosphere) (Berg et al. 2014). In particular, plants are known to define the composition of their rhizospheric bacterial microbiome (Berendsen et al. 2012). Thus, alteration in the composition and structure of the soil microbial communities or composition is reflected as deteriorating soil quality, and thus also plant health (Raza et al. 2016).

The rhizosphere was defined over 100 years ago as the zone around the root where microorganisms and processes important for plant growth and health are located (Bakker et al. 2013). Any changes in the physical and chemical properties of rhizosphere soil can reflect the influence of plants on it (Yang et al. 2017). Root morphology and distribution of crops affect the structure of the soil microbial community, while root exudates directly affect the soil microbial community abundance (Nie et al. 1997). The quality and growth pattern of the plants indirectly reflect the quality of the soil (Qiao et al. 2017). Therefore, it is more important to investigate the effect of transgenic crops on the structure and diversity of soil microbial communities.

High-throughput sequencing technology can be used to obtain comprehensive information about soil microbial structure, diversity, and function. This can also be used for the in-depth assessment of the changes in soil microbial communities over time. In the present work, the soil physical and chemical properties and soil microbial diversity of transgenic (25C-1, possessing the *CBF1* gene) as well as non-transgenic cotton (TH2) have been studied. The study was aimed to (1) explore the correlation between the changes in physical and chemical properties of soil and the relative abundance of soil microbial communities in soil used to cultivate transgenic cotton, (2) study the effects of continuous cropping of transgenic cotton on the microbial community of plant rhizosphere soil, and (3) identify the dominant bacterial population in the continuous cropping of genetically modified cotton. The study provides considerable insights into the impact of genetically modified cotton on soil microbial communities that may also help policymakers in evaluating environmental safety threats of commercialized genetically modified crops.

Materials and methods

Site description, field experiment, and soil sampling

The experiments in the present article were performed at the Shihezi University Experimental Base (44° 20′ N, 85° 50′ E), in the Xinjiang Uygur Autonomous Region, China. The field site is located in the early maturing or extremely early mature cotton planting ecological zone of the northern foothills of the Tianshan Mountains in Xinjiang. It is located in the North Temperate Zone that has a temperate continental plateau climate. The warmest month of the year is July, with a mean temperature of 25.2 to 26.2 °C, and the maximum goes up to 42.2 °C. The coldest month of the year is January, with a mean temperature of − 18.6 to − 15.5 °C, and a minimum temperature drops down to − 37.8 °C. A total of around 168~171 days in a year are reported frostless in the region. Moreover, average annual rainfall and annual evaporation in the region are recorded around 213 mm and 1537 mm, respectively.

TH2 was used as the recipient cotton cultivar. The transgenic cotton line 25C-1 was developed in the Key Laboratory of Agricultural Biotechnology of Shihezi University using a pollen tube pathway method on TH2. The cotton field adopts a random block design which has 3 rows per plot, and each plot covers an area of 11.25 m^2, with about 300 plants and a spacing of 10 cm. The 3 plots were planted as 3 replicates (Liu et al. 2019).

The rhizosphere soil samples were collected for 3 consecutive years precisely on July 15. The soil samples were collected following Riley and Barber's shake method (Riley and Barber 1970). In 3 repeated random plots planted with different cotton materials, five sample points were selected from each plot using an S-shaped distribution for sampling. A boring auger with an inner diameter of 6.0 cm was vertically inserted into the soil, which collected rhizosphere soil from the bottom of the interplanting lines (5–20 cm). The acquired mixed samples were sieved (4 mm, 5 mesh). The sieved samples

were stored at 4 °C before being used in the laboratory for further experiments.

Soil physical and chemical properties

The properties of the soil samples collected over time were analyzed following the previously described methods (Hungria et al. 2013). Organic matter (OM) and total nitrogen (TN) of the soil samples were quantified using KCr_2O_7 and $HClO_4$-H_2SO_4 digestion methods, respectively. Available phosphorus (AP) was measured by Mo-Sb colorimetric method from the soil samples extracted using $NaHCO_3$. Atomic absorption spectrometry was performed over the samples extracted using NH_4OAc to quantify the available potassium (AK) in the soil. The pH and electrical conductivity (EC) of the soil samples were determined in a 1:5 (w/v) suspension of soil in water.

DNA extraction, PCR amplification, and Illumina MiSeq sequencing

Total genomic DNA was extracted from samples using PowerSoil® DNA Isolation Kit and as instructed by the manufacturer (Mo Bio Laboratories, Solana Beach, CA, USA). The purified DNA was subjected to 1% agarose gels to measure the quality of the soil samples.

The DNA samples were diluted with ddH_2O to obtain dilutions of 1 ng/μl. The 16S rRNA genes of the V4 region were amplified using 515F-806R (5′-GTGCCAGCMGCCGCGGTAA-3′and 5′-GGACTACHVGGGTWTCTAAT-3′) primer pairs having barcodes. All PCR reactions were carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

Sequencing of the DNA samples for further analysis was performed on an Illumina HiSeq 2500. Paired-end reads were assigned to samples based on their unique barcode followed by a truncation of the barcodes and primer sequences (Magoč and Salzberg 2011). The raw sequencing data were merged using FLASH (v1.2.7). Chimeras were removed using Mothur, while the short sequences from the spliced strands, for quality control and filtering, were obtained effectively by splicing fragments (clean tags). Thereafter, based on the valid data, UIIust and Uclust methods (Edgar 2013) were used for the OTU (operational taxonomic unit) clustering and species classification analysis that is based on the 97% sequence similarity (Bokulich et al. 2013). The RDP classifier was used to annotate taxonomic information for representative sequences of each OTU. An in-house Perl script was developed and used to analyze α- and β-diversity (Zhang and Wang 2017).

Statistical analysis

The physicochemical data were analyzed by one-way ANOVA followed by Duncan's multiple range tests using the SPSS Statistical Software Package ver. 26 (SPSS Inc., USA). The significance level of the data was set at $P < 0.05$. In α-diversity analysis, rarefaction curves, Chao1, and Shannon's index were generated to reflect community richness and their diversity respectively, whereas the comparative analysis of microbial community composition of different samples was assessed using β-diversity. The degree of bacterial differences between samples was analyzed based on non-metric multi-dimensional scale (NMDS). We also performed an unweighted UniFrac distance matrix for UPGMA (unweighted pair-group method with arithmetic mean) cluster analysis and integrated the clustering results with the relative abundance of each sample. Spearman's correlation heat map prepared to reflect a relationship between soil fungal community composition and environmental factors.

Results

Soil physical and chemical properties

The physical and chemical properties of soil are shown in Table 1. The parameters, namely pH, EC, OM, AP, and AK, of the soil samples from the two cotton lines were not significantly different ($P > 0.05$). Interestingly, a significant difference was observed in the TN values of the soil samples. After the first year of planting, the TN values of TH2 and 25C-1 were 0.33 ± 0.06 and 0.27 ± 0.12, and then increased to 0.61 ± 0.10 and 0.44 ± 0.04 in the second year ($P < 0.05$).

Soil bacteria community and diversity

A total of 544,645 high-quality reads were obtained from six samples using Illumina MiSeq sequence analysis. A total of 32,820 OTUs were also observed at a 97% similarity (rhizosphere soil sample of transgenic cotton is denoted by SS1, while the recipient cotton rhizosphere soil sample by SS2). The bacterial OTUs were assigned with 54 phyla, 110 classes, 211 orders, 364 families, and 516 genera.

Annotation of the soil microbial data obtained suggests that 10 predominant phyla are predominantly present in the sample. Subsequent analysis of the data indicates that Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes, Bacteroidetes Planctomycetes, Chloroflexi, Verrucomicrobia, Nitrospirae, and Firmicutes precisely share 26.53, 22.34, 13.41, 13.54 5.51, 4.14, 3.57, 2.91, 1.47, and 1.20% of the total microbial predominance respectively (Fig. 1). Furthermore, a heat map was prepared for the abundance in each sample (Fig. 2). It should be noted that the top 35 genera were selected for clustering from the two levels of species and samples. *Arthrobacter*, *Blastocatella*, *unidentified_Gemmatimonadetes*, *Sphingomonas*, *Gemmatimonas*, *Massilia*, *Haliangium*, *Gaiella*, *Solirubrobacter*, and *Bryobacter* presents the first 10 bacterial genera that precisely share 1.66, 1.39, 1.69, 1.33, 1.20, 0.73, 0.83, 0.75, 0.69, and 0.81% of the total abundance (Fig. 2).

Table 1 The basic physical and chemical properties of the soil

Years[a]	Treatment[b]	PH	EC (ms m^{-1})	OM (g kg^{-1})	TN (g kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})
1	TH2	7.88 ± 0.08 a	0.21 ± 0.10 a	8.74 ± 1.17 a	0.33 ± 0.06 bc	15.51 ± 9.35 a	286.16 ± 47.16 a
	25C-1	7.84 ± 0.32 a	0.22 ± 0.10 a	8.68 ± 5.17 a	0.27 ± 0.12 c	13.22 ± 4.25 a	276.36 ± 77.25 a
2	TH2	7.94 ± 0.24 a	0.42 ± 0.47 a	12.22 ± 2.56 a	0.61 ± 0.10 a	20.22 ± 3.71 a	322.05 ± 37.59 a
	25C-1	7.84 ± 0.28 a	0.44 ± 0.39 a	12.33 ± 2.39 a	0.44 ± 0.04 b	13.90 ± 5.45 a	302.57 ± 48.22 a
3	TH2	7.50 ± 0.07 a	0.19 ± 0.06 a	11.09 ± 0.39 a	0.39 ± 0.05 bc	11.08 ± 2.71 a	285.48 ± 41.41 a
	25C-1	7.67 ± 0.38 a	0.15 ± 0.05 a	10.72 ± 1.45 a	0.28 ± 0.03 c	10.10 ± 4.32 a	248.57 ± 126.77 a

The data have been shown as mean ± SE. Letters following the data indicate significant differences between samples during different sowing years of each cotton line (Duncan's multiple range test was employed, $P < 0.05$)
[a]1, 2, and 3 represent continuous cropping during the 1st, 2nd, and 3rd year, respectively
[b]Acceptor cotton rhizosphere soil (TH2) and rhizosphere soil of transgenic cotton (25C-1)

All the sparse curves obtained using QIIME pipeline with a 97% sequence similarity tend to be close to the saturation platform. This indicates that the number of sequencing reads in each sample was reasonable; SS1 and SS2 are equally rich in terms of the species diversity and uniformity that is also apparent in terms of their high coincidence values (Fig. 3).

Beta diversity analysis

Non-metric multi-dimensional scale (NMDS) is a classification method suitable for ecological research that can be explained by a nonlinear model. It reliably reflects the nonlinear trend of data that help to overcome the shortcomings of linear models (e.g., PCA, PCoA). The NMDS model relies on the distance between points in multi-dimensional space to reflect the degree of difference between individual samples (Fig. 4). A clustering tree can be constructed to show the similarity between different samples by cluster analysis over them. UPGMA cluster tree structure is denoted on the left, while the species relative abundance distribution map of each sample at the gate level is shown on the right (Fig. 4).

The NMDS variation analysis suggests that SS1 and SS2 are mainly different on the NMDS scale of A-3 and B-3 points (Fig. 4). Unweighted UniFrac cluster analysis of six soil samples exhibited that SS1.1, SS1.2, SS2.1, and SS2.2 were relatively similar. On the contrary, SS1.3 and SS2.3 relate to different branches and show no significant difference in the classification levels of the 10 phyla (Fig. 5).

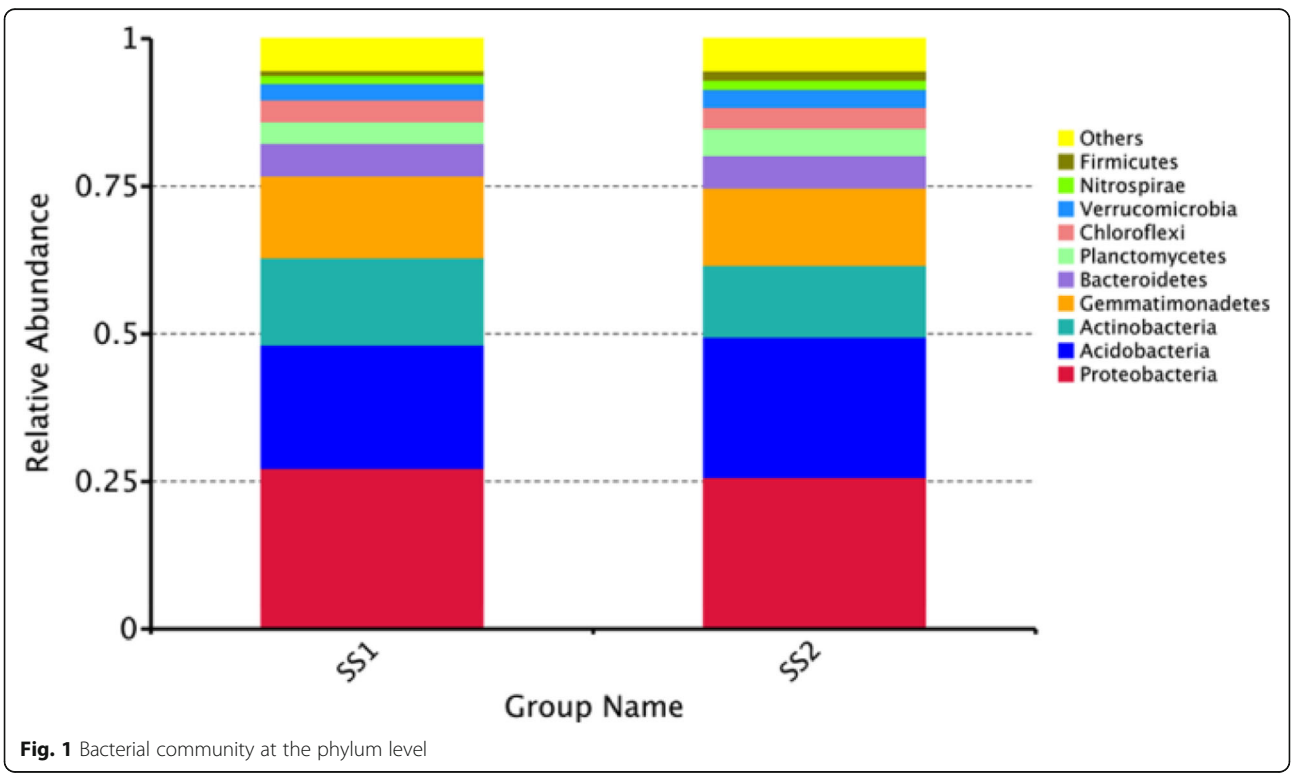

Fig. 1 Bacterial community at the phylum level

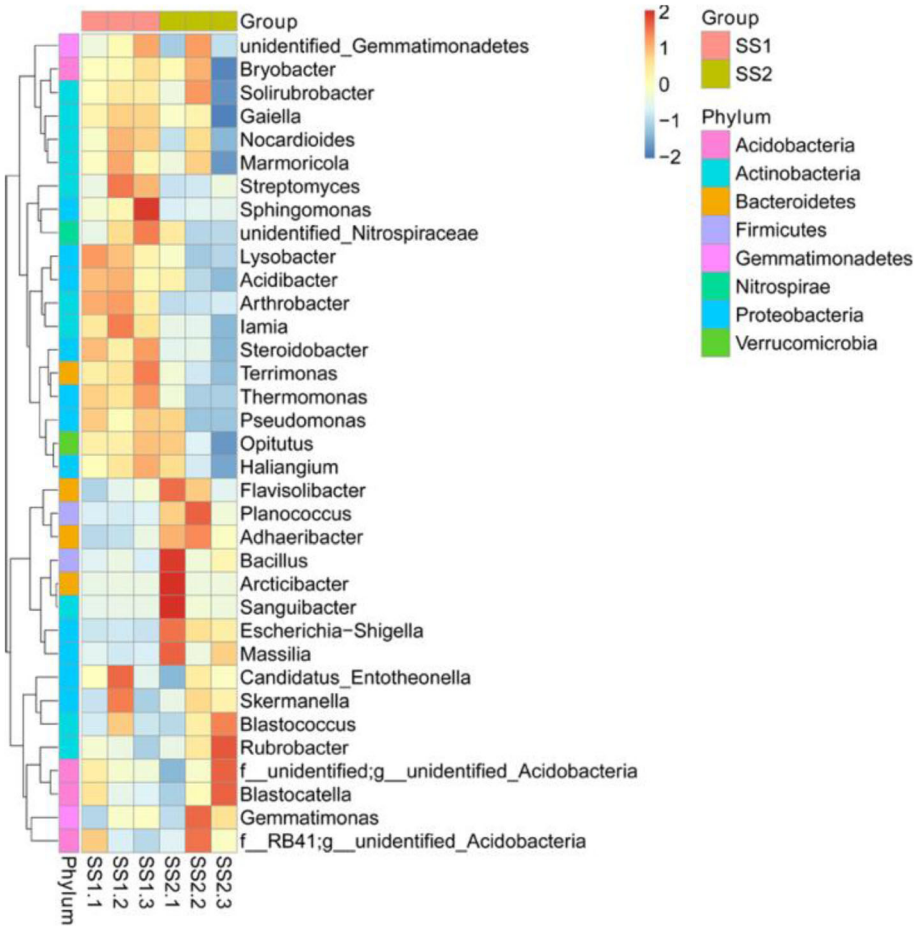

Fig. 2 A generic horizontal heat map of the relative abundance of bacterial communities in six indicated samples

Correlation between environmental data and microbial communities

We used Spearman's correlation analysis to access the abundance of the top 35 genera identified based on the heat maps (Fig. 6). The results suggest that TN is the most critical environmental factor that significantly affects the soil bacterial community of transgenic cotton. More specifically, TN was correlated negatively with *Acidibacter*, *Lysobacter*, and *Iamia*, while positively with *Planococcus* and *Adhaeribacter* bacteria. In addition to the TN, AP was also negatively correlated with *Blastococcus* and *Candidatus Entotheonella*. Moreover, the correlation data did not suggest any definitive link between other environmental factors and bacterial communities. Taken these results together, it was confirmed that the microbial communities namely *Acidibacter*, *Lysobacter*, *Iamia*, *Planococcus*, *Adhaeribacter*, *Blastococcus*, and *Candidatus Entotheonella* were not significantly different after 3 years of continuous cropping of both the transgenic cotton line 25C-1 and its parent cotton line TH2. Moreover, they do not belong to the dominant genus.

Discussion

Transgenic plants that show herbicide tolerance, salt tolerance, drought tolerance, disease resistance, and insect resistance improved product quality and superior agronomic properties (Liu et al. 2005). Considering the possible environmental consequences of growing genetically modified crops, evaluating the impact of genetically modified crops on soil microorganisms will become an important issue. In this study, the effects of continuous cropping of genetically modified cotton on its soil bacterial community structure and diversity were investigated by high-throughput sequencing technology.

Based on the studies conducted at Central Institute for Cotton Research, Nagpur, it was found that growing *Bt* cotton does not affect the soil biological properties (Velmourougane and Sahu 2013). In this study, our analysis showed that soil TN content significantly differs ($P < 0.05$) (Table 1). Soil TN plays a vital role in controlling soil fertility and crop yield (He et al. 2009). The TN content is a dynamic parameter, the level of which in the soil changes with deposition and consumption of nitrogen (Batista et al. 2015). This depends on multiple

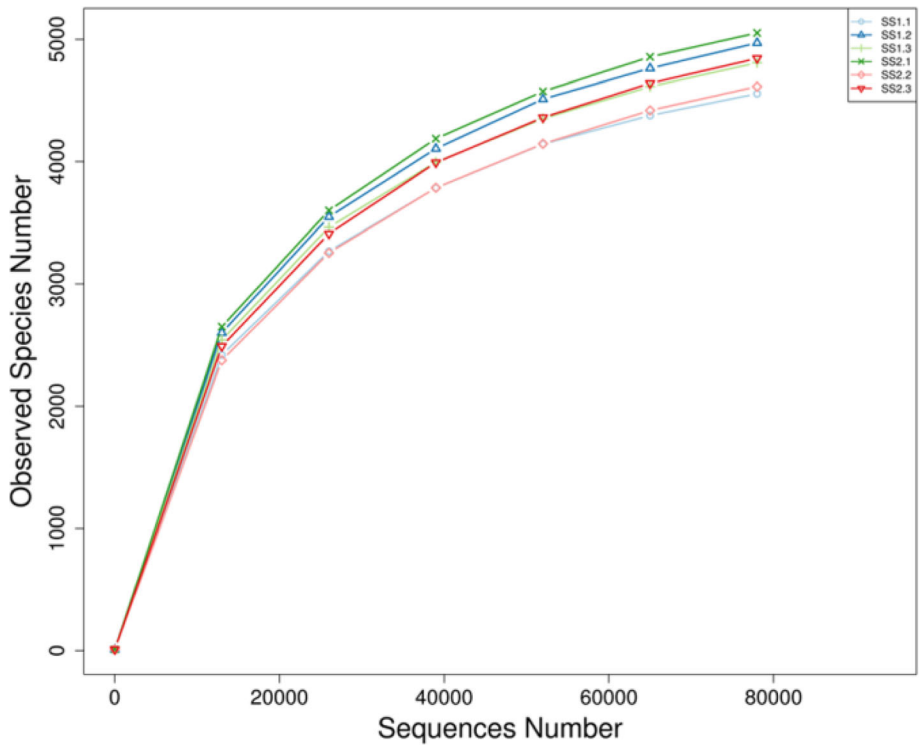

Fig. 3 OTU similarity of each sample with a 97% species diversity curve

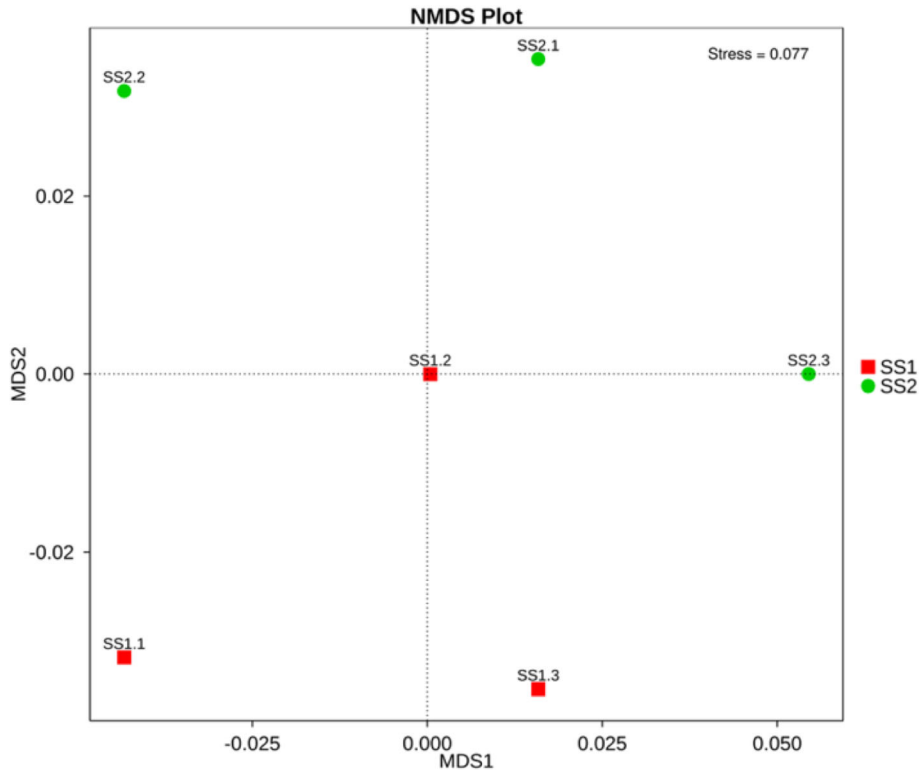

Fig. 4 Non-metric multi-dimensional scaling analysis of species

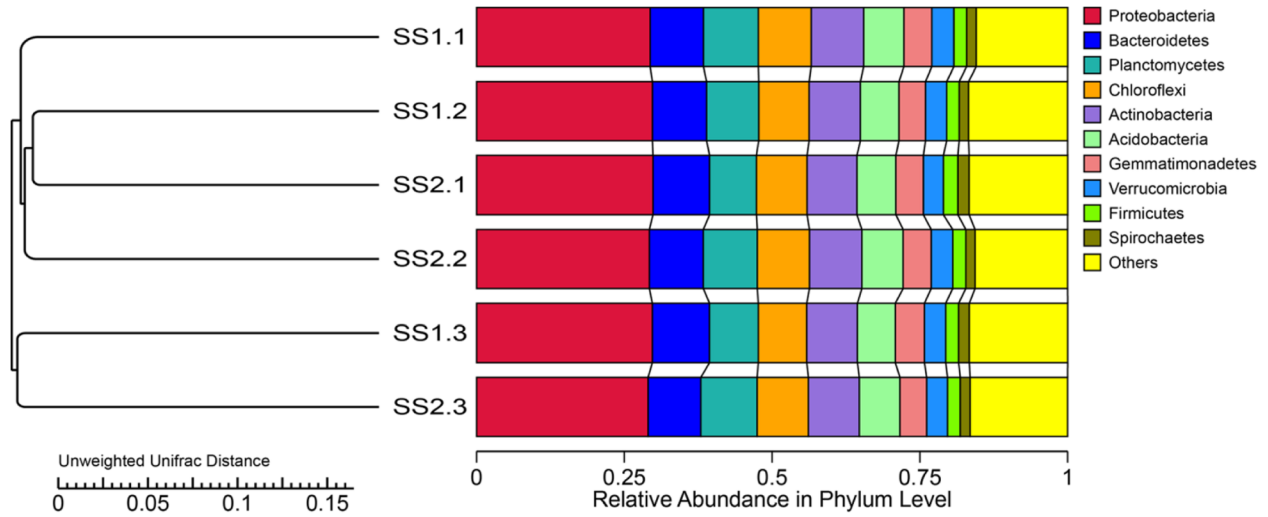

Fig. 5 Unweighted pair-group method with arithmetic mean clustering tree based on unweighted UniFrac distance

factors, in particular, the nitrogen fixation by plants and hydrolysis of soil organic matter (Wardle 2008). OM is an important constituent of the soil that remains present in trace but is indispensable in maintaining the soil nutrients, physical properties, melting, decomposition, and synthesis of humus (Berg et al. 2014). The results suggest that after the first year of planting, the OM values of TH2 and 25C-1 were 8.74 ± 1.17 and 8.68 ± 5.17, and then increased to 12.22 ± 2.56 and 12.33 ± 2.39 in the second year ($P > 0.05$). The further analysis hinted that the decomposed straw to the cotton field was the main reason for the accumulation of soil organic matter. Interestingly, we did not report any significant difference in PH, EC, OM, AP, and AK in the rhizosphere soil of TH2 and 25C-1 during 3 years of continuous cropping ($P > 0.05$). Taken all these results together, we propose that the transgenic line 25C-1 imposes no considerable impact on the physical and chemical properties of the soil and thus may not have any adverse effects on the plant growth cycle or environment, which agrees with the results of previous *Bt*-cotton research (Sarkar et al. 2009).

The results clearly showed that the main phyla present in the soil samples were Proteobacteria, Acidobacteria, Actinobacteria, and Gemmatimonadetes, among which Proteobacteria was the most important phylum (Fig. 1), which agrees with the results of Fan et al. (2017). Proteobacteria is a gram-negative bacterium containing nitrogen fixation genes in its genome (Delmont et al. 2018). The proportion of Proteobacteria in rhizosphere soil of 25C-1 cotton strain was found to be higher compared to the TH2 cotton strain (Fig. 1). Notably, the proportion of Actinobacteria in rhizosphere soil of 25C-1 cotton strain was also significantly higher compared to the TH2 strain (Fig. 1), which is different from the research results of Wei et al. after continuous cropping of cotton (Wei and Yu 2018). Previous studies have shown that a reduction in the OM in the soil caused by continuous cotton cropping is a major factor leading to a significant reduction in the Actinobacteria population (Zhang et al. 2013). However, our research has shown that the TN, OM, Proteobacteria, and Actinobacteria content in the soil increased after continuous cropping of genetically modified cotton. This may be a different effect of genetically modified cotton on the soil.

Among the top 35 genera identified in the soil samples with an abundance of larger than 1%, five were classified in bacterial genera namely *Arthrobacter*, *Blastocatella*, *unidentified_Gemmatimonadetes*, *Sphingomonas*, and *Gemmatimonas* (Fig. 2). Two (*Arthrobacter* and *Sphingomonas)* of the five among them were significantly different ($P < 0.5$), which was first discovered in the soil of continuous cropping of genetically modified crops. *Arthrobacter* that belong to gram-positive actinomycetes are found in most of the ecological environments including soil. It is reported that some strains of *Arthrobacter* have the ability to degrade pesticides, perform nitrogen fixation, and also produce certain beneficial enzymes (Fu et al. 2014). As an example, *Arthrobacter* spp. can degrade the herbicide atrazine that poses a threat to the environment if discharged to waterways. Jiang et al. isolated an *Arthrobacter* sp. strain HS-G8 that has nitrogen-fixing ability without any nitrogen medium (Jiang et al. 2004). In addition to its nitrogen fixation ability, nitrification and denitrification functions of *Arthrobacter* are also well reported (Fu et al. 2014). Interestingly, a few species of the *Arthrobacter*, such as *Arthrobacter crystallinis* (Camargo et al. 2004) and *Arthrobacter chlorophenolum* (Westerberg et al. 2000), are also capable of biodegradation. This ability has been exploited for bioremediation of contaminated soil for

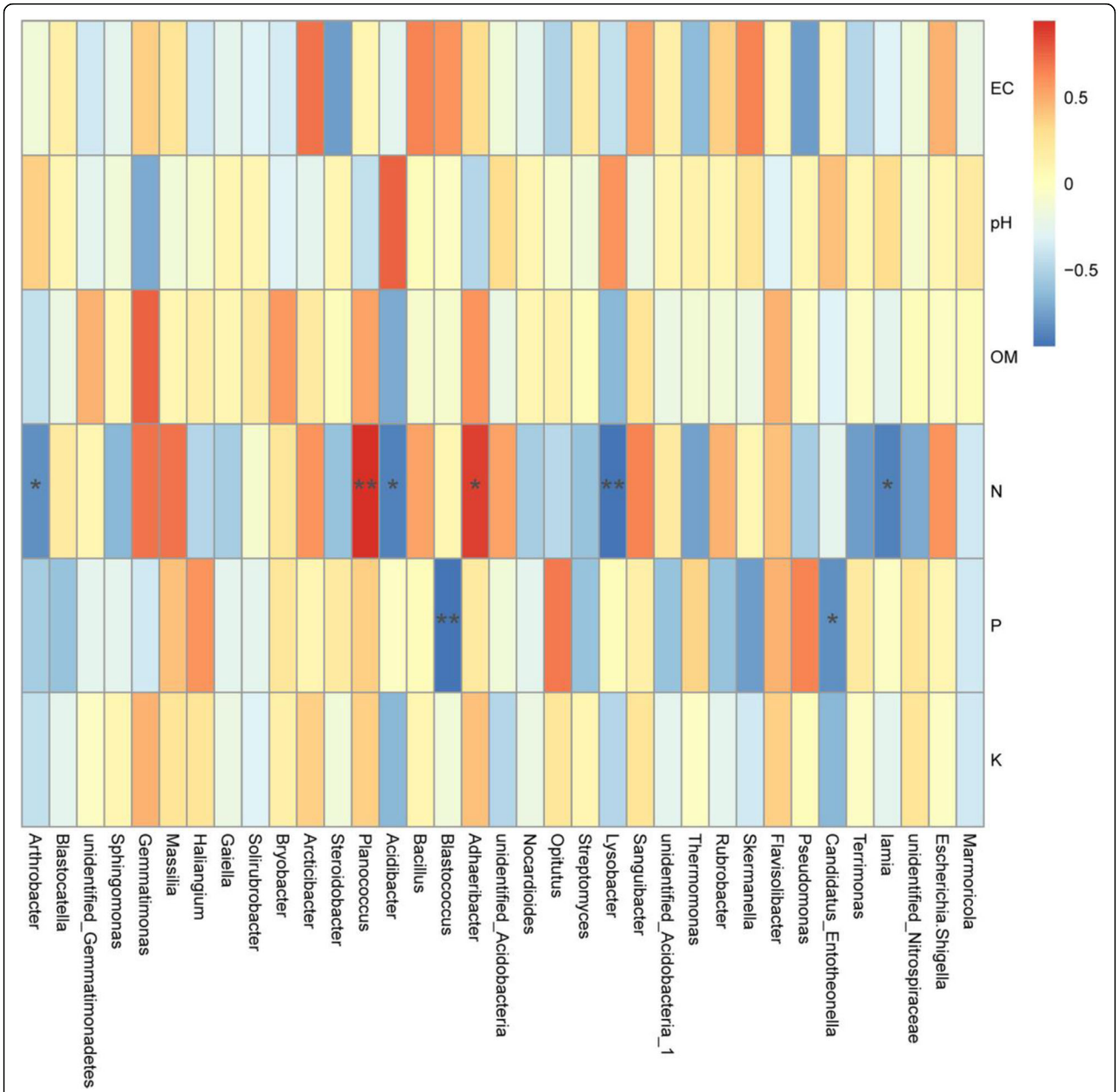

Fig. 6 Correlation heat map of population abundance and environmental variables

chromium and 4-chlorophenol (Ashvini et al. 2018). *Sphingomonas* is a gram-negative bacterium that has a special cellular structure. The unique metabolic mechanism and ability to tolerate poor nutrition facilitate their survival to the harshest natural conditions. It has many potential biotechnological applications such as microbe-induced corrosion, production of valuable extracellular polysaccharide polymers, and degradation of refractory organic compounds (White et al. 1996). Previous studies have shown that *Sphingomonas* can degrade polyethylene glycol (PEG-4000). In combination with other bacteria (known as dual cultures), *Sphingomonas* can also degrade PEG6000 (Takeuchi et al. 1993).

According to the UPGMA cluster tree structure and species relative abundance map in the β-diversity analysis (Fig. 5), there is no significant difference in soil microbial abundance between transgenic cotton and non-transgenic cotton, which is consistent with the results of Shahmoradi et al. (2019). Similarly, many other previous studies revealed that the effect of transgenic crops was minor, transient, or not significant on microbial populations in rhizosphere soil (Turrini et al. 2004; Shen et al. 2006; Sarkar et al. 2009; Velmourougane and Blaise

2014; Zaman et al. 2015). However, the effects of genetically modified plants on soil microorganisms can be divided into direct effects and indirect effects (Liu et al. 2005). Direct impact will depend on the accumulation of genetically modified protein (Oger et al. 1997). For example, transgene proteins for pest and disease resistance can involve the production of chemical substances that are potentially toxic to non-target soil organisms. In contrast, indirect effects are mediated by changes in plant protein and root exudate composition that arise as a result of modifying the metabolic pathways in the plant tissues. Therefore, the potential risk assessment of transgenic plants still needs a lot of research.

Conclusion

High-throughput sequencing analysis of rhizosphere soil samples of cotton lines, TH2 and 25C-1, provided insights about the impact of bacterial communities at the genus level. The results show that *Arthrobacter* and *Sphingomonas* might play a role and impact the soil environment positively during continuous cropping of transgenic cotton. According to the physical and chemical properties of the soil, the significant accumulation of total nitrogen in the soil may also be the result of 25C-1 continuous cropping. Furthermore, the microbial community structure in soil was not affected by the cropping of genetically modified cotton and the total microbial population and diversity of experimental fields remain quite similar during the cropping of both genetically modified cotton and non-genetically modified cotton. Though it is difficult to understand the intricate interactions between plant roots and bacteria in the soil environment, this study provides necessary insights into the indirect effects of continuous cropping of genetically modified cotton on soil microorganisms. Cultivation of *CBF1* cotton may not pose ecological or environmental risk.

Authors' contributions

Wenhui Tian, Xiaolong Yi and Shanshan Liu contributed equally to this work. Aiying Wang and Xiaolong Yi designed the experiment, and Shanshan Liu assisted Xiaolong Yi in completing the experiment. Wenhui Tian , Shanshan Liu and Chao Zhou conducted data analysis. Wenhui Tian wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethics approval and consent to participate is not applicable in this study.

Consent for publication

Consent for publication is not applicable in this study.

Competing interests

The authors declare that there are no conflicts of interests.

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