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Complete List of Authors:	<p>Pan, Jiangang; Inner Mongolia University of Science and Technology, School of Life Science and Technology; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences; University of the Chinese Academy of Sciences, College of Resource and Environmental Science</p> <p>Lv, Xin; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences</p> <p>Jin, Decai; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences</p> <p>Bai, Zhihui ; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences</p> <p>Qi, Hongyan ; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences</p> <p>Zhang, Hongxun; University of Chinese Academy of Sciences, College of Resources and Environment</p> <p>Zhuang, Guoqiang; Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences</p>
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Developmental stage has a greater effect than Cry1Ac expression in transgenic cotton on the phyllosphere mycobiome

Jiangang PAN ^{1,2,3*}, Xin LV ², Decai JIN ², Zhihui BAI ², Hongyan QI ², Hongxun ZHANG ³, Guoqiang ZHUANG ²

1. School of Life Science and Technology, Inner Mongolia University of Science & Technology, Baotou 014010, China

2. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

3. College of Resource and Environmental Science, University of the Chinese Academy of Sciences, Beijing 100049, China

* Corresponding author Tel.: +86-472-5951944, E-mail: jgpan@aliyun.com

ABSTRACT: Transgenic Bt cotton is widely cultivated, yet its impact on the phyllosphere mycobiome is poorly understood. Hence, the objective of this study was to investigate the effects resulting from the planting of Bt cotton on fungal diversity composition. The alpha diversity for the Bt cotton line SGK321 was lower than that of control plants at the budding stage and blossoming and boll-forming stage, while an obvious increase in diversity for Bt cotton XP188 was observed at the same stage. The Cry1Ac levels were higher at the seedling stage than at the budding stage and blossoming and boll-forming stage. There was no direct relationship between the expression of the Bt protein and variation in the fungal community for Bt cotton. However, PCoA and PCA results indicated that community structure differed among developmental stages. These results indicated that developmental stage, rather than Cry1Ac expression, was the key factor shaping the phyllosphere mycobiome in transgenic cotton.

Key Words: transgenic Cry1Ac cotton; phyllosphere; fungal diversity; ELISA; 454 pyrosequencing

Introduction

Transgenic Bt cotton is one of four current large hectareage biotech crops, with a planting area that reached 45.8 million hectares in 2017 (ISAAA 2017). Bt cotton not only has low insecticide requirements, but also contributes to the alleviation of poverty and hunger. Bt cotton is particularly important in developing countries. Although the majority of farmers benefit from planting Bt cotton, the potential ecological risk is non-negligible. The *Bacillus thuringiensis* (Bt) Cry1Ac protein is a delta-endotoxin that targets insects in the orders Lepidoptera, Coleoptera, and Diptera (Grossi-De-Sa et al. 2007; Nariman 2007). Its adverse effects on non-target organisms have been studied extensively, particularly its effects on microbial diversity owing to the key role of the microbiome in whole plant ecosystems (Lundberg et al. 2012).

Despite many studies of the effects of transgenic plants on the soil microbial community (Kapur et al. 2010; Lamarche et al. 2011; Li et al. 2011; Pasonen et al. 2009), their effects on the phyllosphere microbiome are unclear. Although the phyllosphere, i.e., the aboveground portion of plant, is not conducive to microbial colonists, many microbes representing wide taxonomic diversity colonise this habitat (Lindow and Brandl 2003), including abundant fungi, many of which have important functions (Kemen 2014). For example, *Pseudozyma flocculosa* can be harmful to powdery mildews (Avis and Bélanger 2002) and *Aureobasidium pullulans* Y-1 can inhibit *Aspergillus carbonarius* growth (Dimakopoulou et al. 2008). Owing to nutrient limitation

on leaf surfaces, colonisation with nonpathogenic fungi can effectively inhibit the growth of pathogenic fungi (Kapooria and Sinha 1969; Skidmore and Dickinson 1976), and some epiphytic fungal pathogens also activate the plant defense pathways in response to fungal pathogen attack or other biological stresses (Oliveira et al. 2018). Hence, phyllosphere mycobiota possess not only ecological importance, but also exhibit biocontrol activity, which can promote disease resistance.

Some studies have examined the effects of transgenic plants on phyllosphere microbial diversity (Girlanda et al. 2008; Saha et al. 2007). However, little is known about the effects of transgenic cotton on the phyllosphere mycobiota. Considering Bt protein that is probably released in leaf exudates from Bt plants, it probably has unintended effects on phyllosphere fungal community. Therefore, this will lead to the change of ecological function of phyllosphere mycobiota and host-plant defense. Furthermore, phyllosphere fungal community are also driven by plant traits and taxonomy (Kembel and Mueller 2018). We hypothesized that phyllosphere mycobiome determined by Cry1Ac expression in transgenic cotton, rather than developmental stage. In order to identify this hypothesis, using 454 pyrosequencing, PCR-denaturing gradient gel electrophoresis (PCR-DGGE), and ELISA, we investigated the phyllosphere fungal community on Bt cotton and non-transgenic control plants in the greenhouse.

Materials and methods

72 **Plant material, glasshouse trial, microorganism isolation, and DNA extraction**

73 Transgenic Bt cotton (SGK321 and XP188) and their non-transgenic controls (SHIYUAN321
74 and JM20, respectively) were potted in a glasshouse at Beijing Forestry University on 6 May
75 2011. Each cultivar were planted in 35 pots, each pot contained 2 plants of the same cultivar. The
76 soil was composed of vermiculite, peat, and cultivated soil and was fully blended before use.
77 Sampling was performed four times from June to September of 2011 at major growth stages,
78 including the seedling (6 June), budding (6 July), blossom and boll-forming (6 August) and boll-
79 opening (6 September) stages. A leaf sample from the middle and upper leaf sides was collected,
80 placed in sterile plastic bags, transported to the laboratory rapidly on ice, and transiently stored at
81 -4°C in the laboratory. The samples were named G6, Y6, J6, P6, G7, Y7, J7, P7, G8, Y8, J8, P8,
82 G9, Y9, J9, and P9, respectively (where G, Y, J, and P indicate the genotype SGK321,
83 SHIYUAN321, JM20, and XP188, respectively; and 6, 7, 8, and 9 indicate seedling stage,
84 budding stage, bollosom and flowering stage, and boll opening stage, respectively.). Owing to
85 the high temperature from June to September and the inability to precisely control the
86 temperature in the glasshouse, the windows were opened to modulate the indoor temperature.
87 The methods for microorganism isolation were described by Gu et al. (2010). Briefly, leaf
88 samples were aseptically transferred using flame-sterilised tweezers into a flask containing
89 washing buffer (0.1 M sodium phosphate buffer, pH 7.0) and sonicated for 7 min in an ultrasonic

cleaning bath (frequency 40 kHz) to dislodge the microorganisms from leaves. After slow-speed centrifugation ($4000 \times g$, 4°C) to remove leaf debris, the remaining supernatant was centrifuged at $9000 \times g$ (Hitachi CR21G II; Tokyo, Japan) for 15 min at 4°C . The upper supernatant was discarded and the pellet was collected for DNA extraction. Total DNA was extracted from microbes using the Fast DNA SPIN Kit for Soil (Q-Biogene, Irvine, CA, USA).

The protocol for DNA extraction was conducted according to the manufacturer's instructions, the pellet was subjected to a Lysing Matrix E tube, in which contained a mixture of ceramic and silica particles designed to efficiently lyse all microorganisms. The Sodium Phosphate Buffer and MT Buffer was selected to enable complete sample homogenization and protein solubilization. Homogenization was performed in the FastPrep® Instrument for 45 seconds at speed 5.5. The Binding Matrix Suspension was used to allow binding of DNA and eliminate contaminants that inhibit subsequent reactions. The DNA was treated with 50 μL of of DES (DNase/Pyrogen-Free Water) after washing by way of SEWS-M (Salt Ethanol Wash).

Quantitative detection of Cry1Ac by ELISA

Extraction of the Cry1Ac protein was performed according to the methods described by Han et al. (2010). Briefly, 0.2 g of a leaf sample from transgenic cotton (SGK321 and XP188) at various sampling stages was homogenised in 2 mL of extraction phosphate-buffered saline with Tween-20 (PBST: 8.0 g of NaCl, 2.7 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.4 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 20 g of PVPP,

dissolved in 1000 mL of water, pH = 7.4) using liquid nitrogen for complete extraction. Three replicates were performed for each sample type. The homogenised samples were washed with an additional 2 mL of PBST and kept in 10-mL centrifuge tubes at 4°C overnight for the extraction of the insecticidal protein. The tubes were then centrifuged at 8000 *g* for 20 min. The supernatants were used for the detection of target proteins using EnviroLogix QualiPlate ELISA Kits (Portland, ME, USA) according to the manufacturer's instructions.

Pyrosequencing of the fungal 18S rRNA gene

The variable V4 region of the 18S rRNA gene was amplified using the universal forward primer 3NDf (Cavalier-Smith et al. 2009) and the reverse primer V4_euk_R2 (Brate et al. 2010).

PCR conditions were as follows: 95°C for 2 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. Pyrosequencing was conducted using a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Indianapolis, IN, USA).

Pyrosequencing data analysis

After sequencing was completed, the quality of all sequence reads was assessed according to the following criteria. Mismatched reads that did not include 18S rRNA gene information were removed. Reads with any ambiguous base or homologous bases scores >8 and any low-quality reads were removed. Reads that were shorter than 150 bp were removed. The longest reads (>540 bp) were also removed. Sequences were searched using BLAST against the Silva database

(Pruesse et al. 2007) using Mothur (Schloss et al. 2009). Sequences were assigned to operational taxonomic units (OTUs, 97% similarity), and the Chao1 richness index and Shannon index were calculated using Mothur. A rarefaction analysis was also performed using Mothur. A UniFrac-based principal coordinates analysis (PCoA) was performed using the R vegan package. The 18S rRNA gene sequences obtained by 454 pyrosequencing have been deposited in the NCBI Sequence Read Archive under accession numbers SRR1010335, SRR1010336, and SRR1009136.

DGGE fingerprinting of the 18S rRNA gene

The primers NS1 and GCFung were used to amplify the fungal 18S rRNA gene fragment, and the PCR conditions were revised as follows: 94°C for 5 min, 40 cycles at 94°C for 30 s, 50°C for 30s, 72°C for 50 s, and finally 72°C for 8 min (May et al. 2001). DGGE was performed using an 8% acrylamide gel containing a 20–40% linear chemical gradient in 1× TAE electrophoresis buffer and was run for 16 h at 60°C and 50 V. After analysing the profile using QUANTITY ONE-4.62 (Bio-Rad, Hercules, CA, USA), DGGE bands were quantified based on presence/absence and density (Pan et al. 2012). Then, the compositions of fungal communities were analysed by principal component analysis (PCA) using Canoco 4.5 (Hou et al. 2017).

Results

Expression Dynamics of Cry1Ac in the Leaves of Bt Cotton

Cry1Ac protein levels in the leaf tissues of transgenic cotton (SGK321 and XP188) were measured at four growth stages (Fig. 1). Overall, the concentration of Cry1Ac in leaf tissues was higher in SGK321 than in XP188 leaf tissues, with Cry1Ac protein concentrations of 691.32 ± 23.51 and 656.57 ± 23.74 ng g⁻¹, respectively. The concentration of Cry1Ac in the leaf tissues of Bt cotton varieties was highest at the seedling stage, decreased at the budding stage, increased at the blossoming and boll-forming stage, and decreased to the lowest point at the boll-opening stage. Hence, the expression of Cry1Ac was more highly dependent on the developmental stage of cotton than on the genotype of cotton.

Proportion of pyrosequencing reads that were fungal 18S rDNA sequences

By pyrosequencing, we obtained 10716, 4455, 4732, 6296, 5454, 4722, 4499, 4899, 4727, 4093, 5220, 3938, 4968, 5790, 5065, and 5970 sequences of fungal 18S rDNA from G6, Y6, J6, P6, G7, Y7, J7, P7, G8, Y8, J8, P8, G9, Y9, J9, and P9, respectively. Using the samples obtained at the seedling stage (G6, Y6, J6, and P6), the lowest proportion of amplified sequences were fungal 18S rRNA (<5%), which may be explained by DNA impurities that lead to degradation of DNA during the long DNA storage period. Silva alignments indicated that these sequence reads were from plant and insect taxa. For the other samples, we amplified more fungal 18S rDNA sequences ($\geq 90\%$) (Table 1 and Fig. 2). In particular, amplicons from the J7, G8, and Y8 samples included few non-18S rRNA sequences (<1%) (Table 1 and Fig. 2).

Alpha diversity analysis based on pyrosequencing results

The fungal diversity for each sample was evaluated based on rarefaction curves (Fig. 3). The rarefaction curves for G9, Y9, J9, and P9 at the boll-opening stage tended to approach the saturation plateau at a cluster distance of ≥ 0.03 , while the curves for the other samples did not reach saturation. The slopes of the curves for G6, Y6, J6, and P6 at the seedling stage were steepest, which could be attributed to a lower concentration of DNA and longer sample storage. These results indicated that further sequencing is necessary to yield more unique OTUs and to identify more genera or species in the samples.

As summarised in Table 2, the Chao1 abundance indexes were higher for Bt cotton than for their controls in most cases, and phyllosphere fungal abundance was not always lower in Bt cotton than in control cotton. The Chao1 estimates for Bt cotton SGK321 were lower than those of controls at the blossom and boll-forming stage and boll-opening stage, but higher than those of the controls at the seedling stage and budding stage. For Bt cotton XP188, the phyllosphere fungal abundance estimates were higher than those of controls at most stages, except for the boll-opening stage, in which it was slightly lower. Based on the Shannon diversity index, the phyllosphere fungal communities for Bt cotton SGK321 were less complex than those of controls at the budding stage and blossoming and boll-forming stage, but not at the seedling stage and boll-opening stage. There was no obvious decrease in phyllosphere fungal diversity for

the Bt cotton XP188 at the seedling stage, and there was an obvious increase in diversity at the budding stage, blossoming and boll-forming stage, and boll-opening stage compared to diversity estimates in corresponding controls. Accordingly, Bt cotton did not consistently exhibit a negative effect on phyllosphere fungal diversity. The fungal abundance and diversity indexes for every cotton genotype showed the same trends at different sampling stages. In summary, shifts in the developmental stages of cotton were the key factor determining fungal community diversity and abundance, rather than the expression of Cry1Ac in transgenic cotton.

Distribution of fungal 18S rRNA sequences by pyrosequencing

Phyllosphere fungi belonging to the phyla Agaricomycetes, Cystobasidiomycetes, Dothideomycetes, Eurotiomycetes, Glomeromycetes, Leotiomycetes, Microbotryomycetes, Orbiliomycetes, Pezizomycetes, Sordariomycetes, Taphrinomycetes, and Tremellomycetes, in addition to unclassified fungi, were detected by pyrosequencing (Fig. 4). The fungal community composition was similar for Bt cotton and control cotton at the phylum level, with some differences among stages. The fungal communities were dominated by Dothideomycetes and uncultured fungi at the seedling stage and by Dothideomycetes at the budding stage. Uncultured fungi decreased slightly at the budding stage and a slight increase in Agaricomycetes was detected in SHIYUAN321 and SGK321. Dothideomycetes was still the dominant fungal type at the blossoming and boll-forming stage, but an obvious decrease in Agaricomycetes was observed

at this stage. The fungal community detected at the boll-opening stage showed a biased distribution, with more than 97% Dothideomycetes and a sharp decline in other taxa. These results indicated that Dothideomycetes was the dominant group at all developmental stages. At the genus level, 45 different fungal taxa were detected, especially *Cladosporium*, *Lophiostoma*, *Phaeosphaeria*, uncultured Pleosporaceae, and *Pyrenophora* (Fig. 5). Some genera were detected only once in Bt cotton. For example, *Geastrum*, *Pseudocolus*, and *Phallus* were detected in P7, and *Agaricus* was detected in P6.

UniFrac-based principal coordinates analysis based on pyrosequencing data

There was a distinct community structure at each developmental stage based on PCoA, and samples could be separated into four groups (Fig. 6). The samples from G6, Y6, P6, and J6 formed a cluster, the samples from G9, Y9, P9, and J9 formed a cluster, the samples from G7, Y7, P7, and J7 formed a cluster, and the samples from G8, Y8, P8, and J8 formed a cluster. However, the difference between the G7, Y7, P7, and J7 cluster and the G8, Y8, P8, and J8 cluster was not greater than the difference between the other two groups, and the community structure was similar for J7 and Y8.

Fingerprinting of fungal Communities by PCR-denaturing Gradient Gel Electrophoresis

DGGE fingerprints analyses of the 18S rRNA gene fragments were performed to compare the fungal diversity of the phyllosphere from the different development stage. The fungal flora of

216 phyllosphere in transgenic Bt cotton, whether it was XP188 or SGK321, were similar to that in
217 control cotton at the seedling stage and boll opening stage, but some differences between Bt
218 cotton and its control were still be detected at the budding stage and blossoming and boll-
219 forming stage (Fig.7 A and B). At budding stage, Band p1 only appeared in J7 and disappeared
220 in P7 and Y7; Bands p4, p5, and p8 only appeared in Y7 and disappeared in P7 and J7; Band p18
221 only appeared in P7 and disappeared in Y7 and J7; But Band p15 appeared in P7 and J7, and
222 disappeared in Y7 (Fig.7 A). Similarly, Band F2 only appeared in J7 and disappeared in G7 and
223 Y7; Band F5 and F6 only appeared in Y7 and disappeared in G7 and J7; Band F16 appeared in
224 G7 and Y7, and disappeared in J7; Comparing with the budding stage, some differences of
225 phyllosphere fungal communities were also detected among different cotton varieties, Band p7
226 appeared in P8 and J8, and disappeared in Y8; Band p17 appeared in Y8 and J8, and disappeared
227 in P8 (Fig.7 B). Hence, the difference of phyllosphere fungal communities was not only
228 observed between Bt cotton and its control, but also observed between SHIYUAN321 and JM20,
229 between XP188 and SGK321.

230 **Principal component analysis based on 18S rRNA DGGE fingerprint data**

231 Based on the DGGE PCA results (Fig. 8 A and B), the differences among development stages
232 were more obvious than the differences between Bt cotton and unmodified cotton lines. Bt cotton
233 and unmodified cotton lines clustered together at the seedling stage and the boll opening stage,

but exhibited obvious differences at the budding stage and blossom and boll-forming stage. The difference between the unmodified cotton line SHIYUAN321 and JM20 was also obvious at the same stages. These results were similar those of the PCoA using pyrosequencing data.

Discussion

Despite the important ecological function of the phyllosphere mycobiota, relatively little is known about mycobiota compared with knowledge of bacterial communities. Similarly, studies of the effects of transgenic plants on phyllosphere ecology have focused on bacterial diversity, rather than fungal diversity (Cirvilleri et al. 2008; Heuer and Smalla 1999; O'Callaghan et al. 2008). Hence, in this study, we systematically investigated the dynamics of phyllosphere fungal diversity using the Bt cotton varieties SGK321 and XP188 and their corresponding non-transgenic controls at four developmental stages.

At seedling stage, the proportion of fungal 18S rRNA sequences was lowest, which may be explained by high storage period of DNA on freeze before performed the analysis. However, fungal diversity and colonization level increased as the season advanced and host tissues age (Tadych et al. 2012), it was probably resulted by low diversity of epiphytic fungi on seedling stage. The Shannon index showed that the Bt cotton line SGK321 had negative effects on phyllosphere fungal diversity at the budding stage and blossoming and boll-forming stage; this finding was not consistent with the results of a field study (Pan et al. 2012). At these stages, the

252 concentrations of Cry1Ac in leaves were lower than those at the seedling stage and higher than
253 those at the boll-opening stage. Furthermore, no obvious negative effect on phyllosphere fungal
254 diversity was observed for Bt cotton XP188 at any developmental stage. Accordingly, there was
255 no obvious direct relationship between variation in phyllosphere fungal community and the
256 expression of the Bt protein in cotton. Based on the field results, the concentrations of Cry1Ac in
257 the leaves of SGK321 reach a maximum at the budding stage and subsequently decrease (Pan et
258 al. 2012), similar to the results of Zaman et al. (2015), but different from the results of this study.
259 This difference may be explained by differences in plant growth in the glasshouse and the field.
260 Cheeke et al. (2015) also found that spatial soil heterogeneity has a greater effect on arbuscular
261 mycorrhizal fungal communities and plant growth than that of Bt genetic modification.
262 The Chao1 index showed that Bt cotton had no obvious effect on fungal abundance. However,
263 obvious differences in fungal abundance and diversity were observed among developmental
264 stages of cotton, and the PCoA and PCA results were consistent. Lundberg et al. (2012) detected
265 quantitative variation in bacteria among plants at different developmental stages and with
266 different genotypes. Chen et al. (2014) reported that warming and CO₂ increases could
267 significantly improve microbial biomass carbon and nitrogen. The differences in phyllosphere
268 mycobiota do not necessarily reflect differences in Cry1Ac protein expression in cotton, but
269 rather are more closely related to growth stage. According to another study, seasonal variation in

the number of soil fungi and bacteria was greater than the variation explained by the expression of the Bt protein, similar to our results (Li et al. 2011) and to the results of Becker et al. (2008). Xie et al. (2016) showed that Bt cotton does not have a significant effect on the fungal community structure in rhizosphere soil. Song et al. (2014) reported that rice growth can affect the fungal community, but the community does not differ significantly between Bt rice and non-transgenic control rice. Sapkota et al. (2015) found that the host genotype is more important in shaping phyllosphere fungal communities than fungicide treatment and location, but our PCoA and PCA results showed that phyllosphere fungal community was more weakly influenced by genotype than by plant developmental stage. Stanwood and Dighton (2011) reported leaf age influenced the community structure of phylloplane fungal communities, similar to this study. Using 454 pyrosequencing, we detected twelve phyla, in addition to many uncultured fungi. Ustilaginomycetes fungi, which were detected in the field, were not detected in the glasshouse, indicating that the environmental conditions greatly influence phyllosphere fungal diversity, consistent with the results of Sapkota et al. (2015).

Conclusion

In summary, we characterised the effects of Bt and developmental stage on fungal communities in the cotton phyllosphere. We found that developmental stage was the most important factor shaping the mycobiota. A comprehensive understanding of the effect of Bt cotton on the

mycobiome will accelerate the sustainable development of agriculture.

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List of figure captions

Figure 1. Dynamics of Cry 1Ac protein content ($\pm$ SEM) in the leaves of Bt cotton SGK321 and XP188 during the different sampling stages.

Figure 2. Composition of pyrosequencing obtained from phyllosphere sample of Bt cotton and their control varieties.

Figure 3. Rarefaction analyses of fungal 18S rRNA gene data from phyllosphere samples of Bt cotton and their control varieties. Operational Taxonomical Units (OTU) were defined at 3% dissimilarity.

Figure 4. Phylogenetic classification in phylum level for the 454 pyrosequencing analysis with phyllosphere fungal composition.

Figure 5. Phylogenetic classification in genus level for the 454 pyrosequencing analysis with phyllosphere fungal composition.

Figure 6. Principal Coordinates Analysis based on the UniFrac metric comparing the phylogenetic composition of the 454 pyrosequencing analysis with phyllosphere fungal composition.

Figure 7. DGGE profile for PCR-amplified fragments of the 18S rRNA gene for transgenic cotton expressing Bt-protein and his control variety at different sampling times. The letter G,Y, J and P in the lane denote Bt cotton SGK321,SHIYUAN321,JM20 and XP188, respectively. The number 6,7, 8 and 9 in the lane denote the four different sampling stages. The letter A and B denote the Bt cotton XP188 and SGK321, respectively

Figure 8. Principal component analysis on phyllosphere mycobiome: (A) Bt cotton SGK321 and unmodified line control (SHIYUAN321, JM20) at the different sampling stages, in which the number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 represent G6, Y6, J6, G7, Y7, J7, G8, Y8, J8, G9, Y9, and J9, respectively. (B) Bt cotton XP188 and unmodified line control (SHIYUAN321, JM20) at the different sampling times, in which the number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 represent P6, Y6, J6, P7, Y7, J7, P8, Y8, J8, P9, Y9, and J9, respectively.

1 **Table 1. Summary of pyrosequencing**

Sample ID	Total sequences	Non-fungal 18SrRNA sequences	Fungal 18SrRNA sequences	Percentage of Fungal 18SrRNA sequences
G6	10716	10377	339	0.0316
Y6	4455	4324	131	0.0294
J6	4732	4622	110	0.0232
P6	6296	6168	128	0.0203
G7	5454	85	5369	0.9844
Y7	4722	489	4233	0.8964
J7	4499	19	4480	0.9957
P7	4899	252	4647	0.9485
G8	4727	14	4713	0.9970
Y8	4093	18	4075	0.9956
J8	5220	76	5144	0.9854
P8	3938	287	3651	0.9271
G9	4968	244	4724	0.9509
Y9	5790	104	5686	0.9820
J9	5065	58	5007	0.9885
P9	5970	457	5513	0.9235

2

3

4

1 **Table 2. Summary of reads, operational taxonomic units (OTUs), diversity and richness estimates**

Sample ID	Reads	0.03			
		OTU	chao 1 ^a	coverage	Shannon ^a
G6	339	64	112 (84,181)	0.9027	2.94 (2.77,3.11)
Y6	131	40	86 (57,167)	0.8016	2.74 (2.48,2.99)
J6	110	38	76 (52,141)	0.7727	2.84 (2.58,3.10)
P6	128	41	88 (59,166)	0.7813	2.83 (2.58,3.07)
G7	5369	306	609 (500,777)	0.9706	2.39 (2.33,2.46)
Y7	4233	289	583 (475,753)	0.9646	2.78 (2.71,2.85)
J7	4480	270	545 (444,705)	0.9672	2.13 (2.06,2.2)
P7	4647	329	572 (488,702)	0.9660	2.94 (2.88,3.01)
G8	4713	193	299 (254,378)	0.9813	1.34 (1.27,1.41)
Y8	4075	260	434 (368,541)	0.9698	2.38 (2.31,2.46)
J8	5144	233	363 (312,445)	0.9784	1.64 (1.57,1.70)
P8	3651	275	446 (383,546)	0.9644	2.81 (2.73,2.88)
G9	4724	84	138 (110,197)	0.9901	0.48 (0.43,0.52)
Y9	5686	81	234 (148,433)	0.9912	0.4 (0.36,0.43)
J9	5007	71	153 (107,257)	0.9914	0.42 (0.38,0.46)
P9	5513	86	142 (112,209)	0.9922	0.46 (0.42,0.50)

2 a Confidence intervals for the Shannon’s indices and the Chao1 estimates are shown in parentheses.

3

FIG.1

28x20mm (300 x 300 DPI)

FIG.2

36x30mm (300 x 300 DPI)

FIG.3

84x85mm (300 x 300 DPI)

FIG.4

296x209mm (300 x 300 DPI)

FIG.5
269x206mm (300 x 300 DPI)

FIG.6

272x208mm (300 x 300 DPI)

FIG.7

279x215mm (300 x 300 DPI)

FIG.8

239x98mm (300 x 300 DPI)