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Research Article

RESEARCH ARTICLE

Microbiota in non-flooded and flooded rice culms

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One sentence summary: The phyllosphere bacteria on flooded and non-flooded rice culms had distinct bacterial communities, and flooded rice culms showed higher richness that non-flooded culms due to having more rare taxa.

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ABSTRACT

Rice plants are the habitat for large and diverse populations of microbes, which play important roles on rice health and productivity. However, the response of microbiome on rice culm to water flooding is poorly understood. In this study, the bacterial community on non-flooded (RSA) and flooded (RSB) rice culms was investigated through 16S rRNA gene sequencing. The results showed that RSA and RSB had significantly distinct bacterial communities. In RSA, *Gammaproteobacteria* and *Pantoea* were the most abundant class (57%), genus (37.06%), respectively, while in RSB, the most abundant phylum and genus was *Firmicutes* (54%) and *Bacillus* (52.63%), respectively. Compared with RSA, the abundance of 27 genera significantly increased and 21 genera significantly decreased in RSB, and some remarkably changed species, such as *Aeromonas*, *Bacillus* were identified, which are sensitive to non-flooded or flooded conditions. In addition, rare operational taxonomic units (OTUs) was much more than abundant OTUs in all samples, and RSB had significantly higher bacterial richness than RSA due to having more rare taxa. Our study would advance the insights into the microbiome of rice culms and its response to flooding, which would help to identify potential beneficial bacteria for improving crop health and sustainable productivity in agroecosystems.

Keywords: Rice culms; phyllosphere microbiome; flooded; abundant taxa; rare taxa

INTRODUCTION

Phyllosphere represents one of the important habitats for microbial colonization on the Earth, which refers to all aerial tissues of plants, mainly including leaves, but also stems, flowers and fruits (Muller *et al.* 2016). The total phyllosphere microbiome (including bacteria, fungi, archaea, protists and other microbes) might greatly outnumber the cells of the plants themselves (Penuelas and Terradas 2014; Ansary *et al.* 2018). Bacteria are the most dominant inhabitants on leaf surfaces with a conservative estimate of 10^6–10^8 cells per cm^2 (Lindow and Brandl 2003; Penuelas and Terradas 2014). The persistently dominant bacterial phylum of phyllosphere microbiome is *Proteobacteria* (mainly including alpha and gamma classes), while *Actinobacteria*, *Bacteroidetes* and *Firmicutes* are also commonly found (Vorholt 2012; Bodenhausen, Horton and Bergelson 2013; Ren *et al.* 2014a).

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Additionally, relatively unknown phyla like TM7 and *Deinococcus-Thermus* are also found to be rich and dominant in the apple flower (Shade, McManus and Handelsman 2013). Furthermore, phyllosphere microbes have shown to play crucial roles in plant growth and health by protecting plants from diseases or being involved in nitrogen fixation and carbon cycles (Knief *et al.* 2012).

Rice (*Oryza sativa* L.) is one of the most important crops and a key model plant of grass family and monocotyledon (Hardke 2013), and its phyllosphere microbiome should not be overlooked. The rice culm (i.e. the jointed stem of rice) is also an important vegetative tissue for rice plants (Hardke 2013). However, about the microbiome on rice culms, most of the previous studies were focused on endophytes such as the bacterial genera of *Pseudomonus* and *Sphingomonas*, and the fungal genera *Fusarium*, *Penicillium* and *Pestalotiopsis* (Wang *et al.* 2016). Many of these endophytes like *Azoarus* sp. BH72, and *Azospillum* sp. B510 have the effects on nitrogen fixation and disease resistance (Ikeda *et al.* 2010; Hardoim *et al.* 2015). In addition, various species of phyllosphere microbiome have significant effects on the growth physiology of rice plants (Chi *et al.* 2005). Nevertheless, the microbiome on the surface of rice culms has poorly understood. Importantly, unlike other terrestrial plants that are sensitive to waterlogging or flooding (Jackson and Colmer 2005; Mommer and Visser 2005), basal culms or rice have to be flooded continually in order to achieve rice yield during the tillering stage (Hardke 2013). Therefore, rice culms provide a perfect material for investigating how water flooding shapes the microbiome on rice culms.

So far, both culture-dependent and -independent techniques are used to reveal the compositions of microbial communities in the phyllosphere (Hassani, Duran and Hacquard 2018), and the latter, such as 16S rRNA amplicon and metagenomic sequencing have recently become the mainstream research (Rastogi, Coaker and Leveau 2013). For example, Delmotte and his collaborators applied the culture-independent technique to disclose the diversity of the rice phyllosphere bacteria, which have difficulties in enriching using a specific medium (Delmotte *et al.* 2009; Andrews and Hirano 2012). Another study combined 16S rRNA amplicon with a metaproteogenomic approach revealed the main bacteria community comprised *Bacteroidetes*, *Firmicutes*, *Beta-* and *Gammaproteobacteria*, and emphasized the importance of one-carbon compound cycling in the rice phyllosphere (Knief *et al.* 2012).

However, most recent studies on phyllosphere microbiome have focused on the abundant taxa due to the dominant proportion of relative abundances (i.e. relative abundance >1%) (Shade, McManus and Handelsman 2013; Penuelas and Terradas 2014; Ren *et al.* 2014a; Ren, Zhu and Jia 2014b). On the contrary, less attention has been paid to the rare taxa in plant microbiome, which are defined as the fraction composed of low-abundant taxa (Sogin *et al.* 2006). Therefore, there are still remaining unsolved questions about the ecological importance and contribution of them (Kaminsky and Morales 2018). Actually, they may have a profound impact on shaping planetary processes, such as biochemical processes, community assembly and driving the functions of host-associated microbiomes (Jousset *et al.* 2017).

Therefore, in this study, the 16S rRNA gene amplicon sequencing technique was employed to analyze the microbial community inhabiting on the surfaces of non-flooded and flooded rice culms. Our aims of this study were (i) to reveal the compositions and structures of rice culm bacteria under non-flooded and flooded rice culms by taking abundant and rare taxa into account, (ii) to explore how water-flooding shapes the microbiome of rice culms. The results of this study would provide comprehensive insight into the microbiome on rice culms, both flooded and non-flooded culms, including abundant and rare taxa, which might offer the possibilities of utilizing these potential beneficial bacteria for improving crop health and sustainable productivity in agroecosystems.

MATERIALS AND METHODS

Sampling

Rice culms were collected from six different plots during rice tillering stage (6 m^2/plot, blue-clayed paddy soil, pH (1:2.5 in water) 7.08 ± 0.04, total carbon (TC) content 19.00 ± 0.60 g/kg, total nitrogen (TN) content 1.56 ± 0.05 g/kg) in Jiaxing Academy of Agricultural Sciences, Zhejiang Province, China (30°50′6.81″ N, 120°42′49.02″ E), on 15th August 2017. Rice cultivar was Xiushui-134. Five rice culms were randomly sampled in a plot site to form a composite sample, and three composite samples were collected from each plot sampled, that were three duplicates. After sampling, rice culms 2 cm above the water interface were collected as non-flooded (RSA: Rice culm Surface Above) samples and those 2 cm below the water interface were collected as flooded (RSB: Rice culm Surface Below) samples. Then, these subsampled rice culms were respectively sealed in sterile plastic bags, and transported to the laboratory in icy incubator, stored at −20°C before microbe extraction.

Surface rice culm microbes and DNA extraction

The microbial extraction on surface of rice culms was followed by (Zhu *et al.* 2017). In brief, around 3.0 g rice culms were transferred into a sterile 50 ml centrifuge tube under sterile condition for each sample. Into each tube, 45 ml autoclaved 1 × phosphate buffered saline (PBS, pH = 7.4) supplemented with 0.02% Tween20, then the tubes were shaken at 200 rpm, 30°C on a shaking incubator for 2 hr. After 2 hr, the solution, was filtrated with sterilized nylon nets (40 mesh), and then centrifuged at 7500 rpm for 30 min. After centrifugation, the pellets were used to extract DNAs with FastDNA™ Spin Kit for Soil (MP Biomedicals, Santa Ana, California, USA), following the manufacturer's instructions. The quality and concentration of DNA were checked by 1.0% agarose gel electrophoresis and spectrophotometric analysis using Nano-Drop ND-1000 (Nanodrop, Thermo Fisher Scientific, USA), and qualified DNA was stored at −20°C.

Illumina Sequencing of bacterial 16S rRNA gene amplicons

To investigate bacterial community structure and composition, the V3–V4 region of bacterial 16S rRNA gene was selected for amplification with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Castrillo *et al.* 2017). The reverse primer was tagged with a 12-bp unique barcode for each sample. The amplification was activated at 95°C for 3 min and then following the process: 95°C for 30 s, 55°C for 30 s, 72°C for 45 s with 27 cycles. The amplified products were sequenced using the Illumina Miseq2000 platform (i-Sanger, Beijing, China). All raw sequencing data are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP151699.

Bioinformatic analysis

The Illumina raw sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) following the online tutorials (Caporaso *et al.* 2010). In brief, paired reads were merged into a sequence according to the overlap relationship between paired-end reads, and the adaptor, primer sequences, chimeras, and low-quality reads simultaneously were removed. The filtered reads were clustered into the operational taxonomic units (OTUs) at the 97% similarity level using Usearch (version 7.0, http://drive5.com/upase/) (Edgar 2013). OTUs with only one read (singletons) were removed from the Illumina OTU table, and we randomly subsampled OTU table according to the lowest number of reads in all samples to get 33 636 reads per sample. The default method was picked up a representative sequence of the OTU in each cluster. Taxonomy assignment of the resulting OTU was carried out using RDP Classifier (version 2.2 http://sourceforge.net/projects/rdp-classifier/) with the SILVA 16S rRNA gene database (release128, http://www.arb-silva.de/) and aligned by PyNAST (Wang *et al.* 2007; Quast *et al.* 2013). Phylogeny tree was built by the Fast tree algorithm for downstream analyses (Zhu *et al.* 2018). The alpha-diversity of each sample was described by five different metrics: observed OTUs, Chao1, Shannon, Pielou's evenness and phylogenetic diversity. The microbial differences (beta-diversity) of different samples were compared by principal coordinate analysis (PCoA) based on a taxonomic metric (Bray–Curtis similarities), and a phylogenetic metrics (weighted Unifrac) (Aleklett *et al.* 2015).

Definitions of abundant and rare OTUs

As for individual sample, abundant OTUs were defined as having relative abundances >1% within a sample, while rare OTUs were defined as having relative abundances <0.01%, following the studies on bacteria (Galand *et al.* 2009) and prokaryotes (Logares *et al.* 2014). The definition of all OTUs was the OTUs pooled samples with the same condition. The group relative abundances were calculated as the average of relative abundances of OTUs across all samples, including zero values. The thresholds for abundant and rare OTUs were >0.1% and <0.001%, respectively (Logares *et al.* 2014).

Statistical analysis

Most of analyses were executed in R environment (version3.4.4) (R Development Core Team 2018). The previous mentioned five alpha-diversity indices were calculated by the package 'phyloseq' and statistically analyzed using Bonferroni corrected *t*-test (McMurdie and Holmes 2013). While the two different metrics were conducted by the package 'vegan', visualized by principal coordinate analysis (PCoA) and statistically analyzed using permutational multivariate analysis of variance (PERMANOVA) (Oksanen *et al.* 2018). Analysis of similarity (ANOMIS) and non-metric multidimensional scaling analysis (NMDS) was performed in 'vegan' (Oksanen *et al.* 2018). A hierarchical cluster analysis was performed by using Bray–Curtis similarity and a dendrogram inferred with the unweighted pair-group average algorithm. To determine the robustness of the clustering, data were subjected to bootstrapping with 1000 resampling and the analysis was rerun after removing the largest and smallest samples (Galand *et al.* 2009). Averages, standard deviations (SD), and Venn diagrams were conducted in Microsoft Excel 2016. Additionally, Microsoft Excel 2016 and OriginPro 9.1 were also used to generate other graphics.

RESULTS

The phyllosphere bacterial diversity on rice culms

Totally, there were 451 unique OTUs were detected and analyzed from 12 samples at a sequencing depth of 30 000. Initially, alpha diversity of bacteria on non-flooded (RSA) and flooded (RSB) rice culms was compared by both OTU richness (i.e. the observed OTUs, Chao1 index and phylogenetic diversity) and evenness (i.e. the Shannon index and Peilou's evenness). The results showed that all the indexes representing richness, including OTUs, Chao1 index and phylogenetic diversity, of RSB were significantly higher than those of RSA (Table 1, $P < 0.01$, $P < 0.001$ and $P < 0.01$, respectively); whereas no statistically significant difference between RSA and RSB in Shannon and Pielou's evenness (Table 1). These results suggested that flooding condition could broaden the diversity of phyllosphere bacteria on rice culms. The principal coordinate analysis (PCoA) showed that the phyllosphere bacteria on RSA and RSB were significantly separated along first two principal coordinates (PERMANOVA, $P = 0.0001$, ANOSIM $R = 0.809$, $P = 0.003$) (Fig. 1 and Fig. S1a, Supporting Information). This result demonstrated that flooding condition could be an important factor shaping phyllosphere bacteria on rice culms.

The abundant and rare community structure of bacteria on rice culms

From 12 samples (6 RSA and 6 RSB), each sample was normalized to 33 636 reads. The majority of these reads were abundant (>1%) reads, the proportion of which ranged from 86.30% to 92.92%, and the proportion of rare (<0.01%) reads only ranged from 0.13% to 0.62% (Fig. S2, Supplementary material). However, when considering the OTUs, the numbers of rare OTUs were much higher than those of abundant OTUs. The proportion of the number of abundant OTUs ranged from 4.96% to 8.74%, while rare OTUs ranged from 22.66% to 59.32% (Fig. S2, Supporting Information). These results showed that although the rare reads were much less than the abundant reads, the rare OTUs were much more than abundant OTUs. Interestingly, for the pooled samples with the same condition, the proportions of rare OTUs (<0.001%) in RSB (43.96%) were significantly higher than those in RSA (23.92%) (Table S1, Supporting Information). These results indicated that flooding condition could enrich the rare phyllosphere bacteria on rice culms.

Cluster analysis, based on either all OTUs, abundant OTUs or rare OTUs, showed that all the samples are classified into two groups: non-flooded (RSA) and flooded (RSB) (Fig. 2a–c). Especially, when the cluster analysis was based on rare OTUs, the classification between RSA and RSB showed more separation distance (Fig. 2a-c). This result further indicated that flooding condition is an important factor regulating phyllosphere bacteria on rice culms. The Venn diagram showed again that samples of RSB had more OTUs than that of RSA (Fig. 2d), and the abundant OTUs were similar between RSA and RSB (Fig. 2e), whereas RSB had much more rare OTUs than RSA (Fig. 2f). Meanwhile, nonmetric multidimensional scaling analysis (NMDS) also showed that the rare OTUs (ANOSIM $R = 0.983$, $P = 0.004$) better separated flooded samples (RSB) from non-flooded samples (RSA) than the abundant OTUs (ANOSIM R =

Table 1. Alpha-diversity indices of bacteria on non-flooded (RSA) and flooded (RSB) rice culms.

	Observed OTUs	Chao-1 Index	Phylogenetic diversity	Shannon index	Pielou's evenness
RSA[1]	144 ± 18	180.20 ± 30.33	16.72 ± 1.75	2.21 ± 0.27	0.45 ± 0.05
RSB[2]	196 ± 23**	312.65 ± 40.58***	21.91 ± 2.97**	2.32 ± 0.21	0.44 ± 0.04

[1]Non-flooded rice culms (n = 6)
[2]Flooded rice culms (n = 6).
**$P < 0.01$. ***$P < 0.001$ (t-test results for difference between RSA and RSB).

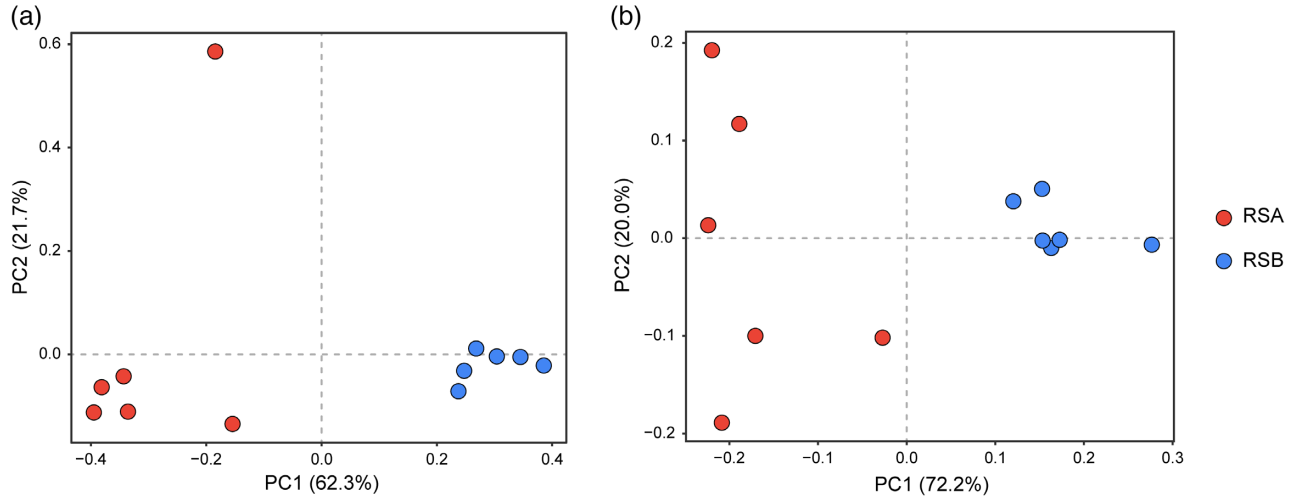

Figure 1. PCoA analysis of bacteria on non-flooded (RSA) and flooded (RSB) rice culms based on two different metrics (Bray–Curtis similarity **(A)** and weighted UniFrac **(B)**. P = 0.0001 by PERMANOVA test.

0.809, P = 0.001) did (Fig. S1b and c, and Fig. S3, Supporting Information). These results suggested that most of the rare OTUs were more adapted to flooded conditions. Additionally, ~57% of all OTUs, ~67% of abundant OTUs and ~93% of rare OTUs were found only in a single group (RSA or RSB) in Venn diagrams (Fig. 2d–f). The percentage of overlapped OTUs in abundant OTUs (32.27%) is much higher than that of overlap in rare OTUs (6.91%) (Fig. 2e, f).

Bacterial community compositions of rice culms

The comparison of the community composition on non-flooded and flooded rice culms showed that the bacterial community compositions of rice culms were significantly different between RSA and RSB (Fig. S4, Supporting Information). The taxonomic results showed that the relative abundance of *Gammaproteobacteria* was higher than *Firmicutes* in RSA, while the opposite pattern was shown in RSB. In RSA, the most abundant class was *Gammaproteobacteria* (*Proteobacteria* was divided into classes) accounting for 57% on average, followed by *Firmicutes* (17%), *Alphaproteobacteria* (8%) and *Cyanobacteria* (7%) (Fig. 3a). In RSB, the most abundant phylum was *Firmicutes* (54%), followed by *Gammaproteobacteria* (41%) (Fig. 3a). Besides, the relative abundance of *Actinobacteria* and *Cyanobacteria* was significantly reduced in RSB compared to RSA ($P < 0.01$ and $P < 0.05$, respectively), while the abundance of *Firmicutes* was significantly increased in RSB compared to RSA ($P < 0.001$).

When bacterial communities from abundant OTUs and rare OTUs were analyzed separately, *Gammaproteobacteria* and *Firmicutes* were the most dominant groups of abundant OTUs (Fig. 3b), and the results were similar to those of all OTUs (Fig. 3a). For both RSA and RSB samples, the number of taxonomic groups of rare OTUs at phylum level was much higher than that of abundant OTUs (Fig. 3c).

Since *Gammaproteobacteria* and *Firmicutes* were the most abundant taxa in RSA and RSB, respectively (Fig. 3a), further analysis at family and genera levels of these two phyla were performed. For *Gammaproteobacteria*, *Enterobacteriaceae* was the most predominant family (51.76%) in RSA, while *Aeromonadaceae* (17.37%) and *Enterobacteriaceae* (12.44%) were the most predominant family in RSB (Fig. 4a). In *Enterobacteriaceae*, both RSA and RSB were largely dominated by bacteria of the genera *Pantoea* and *Enterobacter*, accounting for 37.06% and 14.31%, respectively in RSA, 9.27% and 2.75%, respectively in RSB. (Fig. 4a). For *Firmicutes*, *Bacillaceae* was the most predominant family in RSA (16.36%) and RSB (52.63%). In *Bacillaceae*, both RSA and RSB were dominated by the genus of *Bacillus*, accounting for 16.35% in RSA and 52.59% in RSB. (Fig. 4b).

Furthermore, analysis of the taxonomic variation between RSA and RSB at genus level showed that the abundance of 27 genera significantly increased in RSB compared with those in RSA, for example the relative abundances of *Bacillus*, *Aeromonas* and *Exiguobacterium* increased from 16.35% to 52.59%, 0.77% to 17.38%, and 0.23% to 1.08%, respectively. The abundance of 21 genera significantly decreased in RSB compared with those in

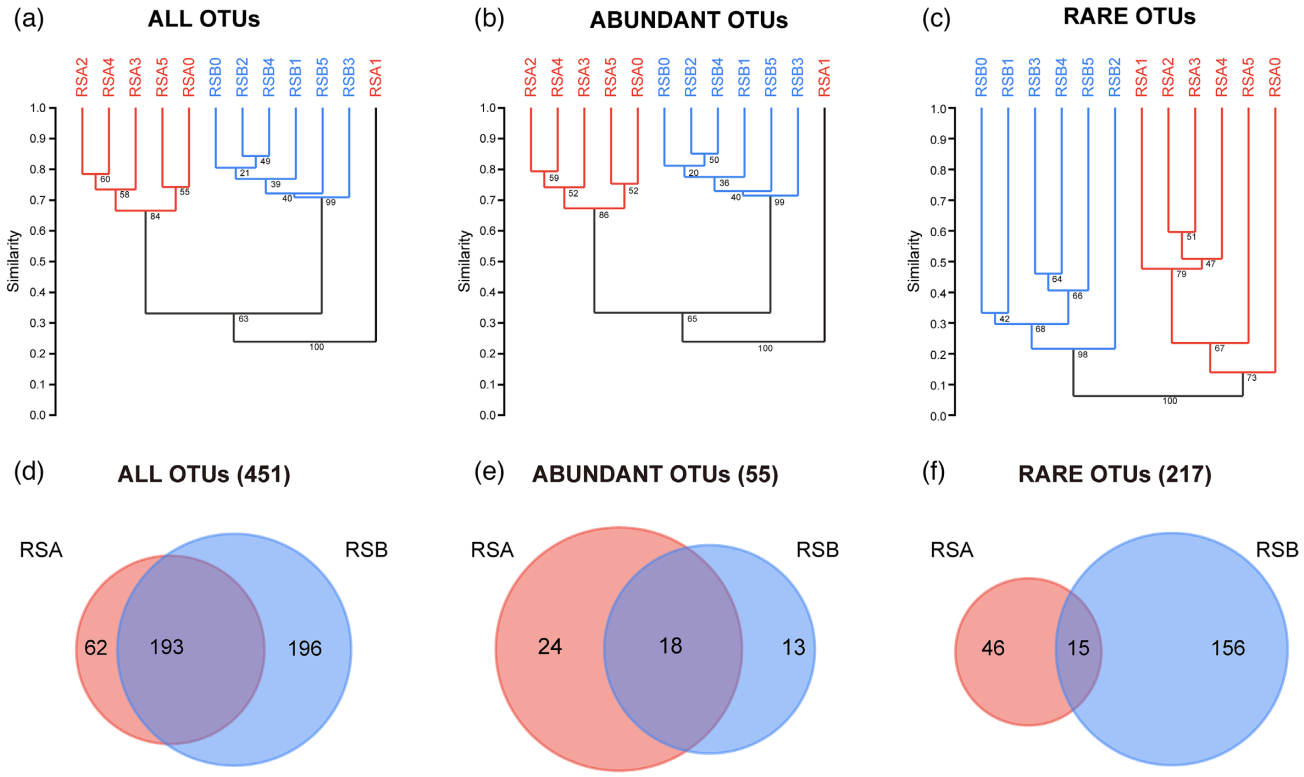

Figure 2. The structures of bacterial communities at OTU level on non-flooded (RSA) and flooded (RSB) rice culms. **(a–c)** Dendrograms represented the similarity between RSA and RSB, based on all OTUs, abundant OTUs only (>1%) and for rare OTUs only (<0.01%), respectively. Clustering was based on Bray–Curtis similarity. Colors highlight the constrained clusters. Bootstraps values (in percentages) were given at the nodes. **(d–f)** Venn diagrams indicated the distribution of OTUs (normalized Illumina dataset) in RSA and RSB. The distribution was shown for all OTUs in the group community (451), as well as for group abundant (>0.1%) (55) and rare (<0.001%) (217) OTUs.

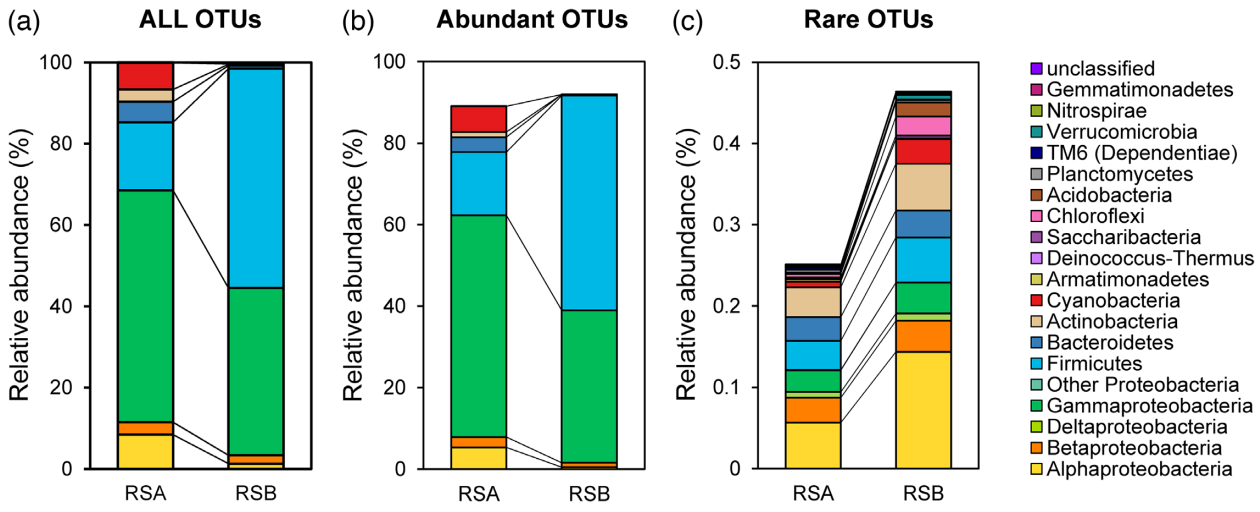

Figure 3. Bacterial compositions on non-flooded (RSA) and flooded (RSB) rice culms. **(a)** The average relative abundances of all OTUs at the phylum level (Proteobacteria divided into classes) on RSA and RSB. **(c)** and **(d)** only showed the relative abundances of the abundant and rare OTUs, respectively.

RSA, for example, the relative abundances of *Pantoea*, *Methylobacterium*, *Sphingomonas* decreased from 37.70% to 9.27%, 6.50% to 0.60% and 4.80% to 0.06%, respectively. (Fig. 5 and Table S2, Supporting Information).

DISCUSSION

Bacterial compositions with abiotic factors

Our results revealed that there are large populations of bacteria on non-flooded (RSA) and flooded (RSB) rice culms at the tiller-

(a)

Taxon at Family Level	RSA	RSB
Enterobacteriaceae	51.76%	12.44%
Pseudomonadaceae	3.24%	3.38%
Xanthomonadaceae	0.86%	0.21%
Aeromonadaceae	0.77%	17.37%
Moraxellaceae	0.28%	6.93%
Shewanellaceae	0.02%	0.05%
Others	0.08%	0.76%
Total of Gammaproteobacteria	57.02%	41.14%

Enterobacteriaceae at Genus Level	RSA	RSB
Pantoea	37.06%	9.27%
Enterobacter	14.31%	2.75%
Dickeya	0.21%	0.27%
Escherichia-Shigella	0.16%	0.09%
Plesiomonas	0.02%	0.06%
Rahnella	0.00%	0.00%
Morganella	0.00%	0.01%

(b)

Taxon at Family Level	RSA	RSB
Bacillaceae	16.36%	52.63%
Family XII_o__Bacillales	0.23%	1.08%
Staphylococcaceae	0.06%	0.01%
Planococcaceae	0.05%	0.07%
Enterococcaceae	0.02%	0.00%
Paenibacillaceae	0.01%	0.09%
Streptococcaceae	0.01%	0.01%
Others	0.01%	0.02%
Total of Firmicutes	16.75%	53.92%

Bacillaceae at Genus Level	RSA	RSB
Bacillus	16.35%	52.59%

Figure 4. Details of two main dominant taxa both on non-flooded (RSA) and flooded (RSB) rice culms. Average relative abundances of *Gammaproteobacteria* families (on the left) and *Enterobacteriaceae* genera (on the right) found in RSA and RSB **(a)**. Average relative abundances of *Firmicutes* families (on the left) and *Bacillaceae* genera (on the right) found in RSA and RSB **(b)**. Values were given as the percentage of sequences belonging to a certain taxon out of the total average bacterial community for RSA and RSB. The heatmap was colored from red (high abundance) to blue (low abundance).

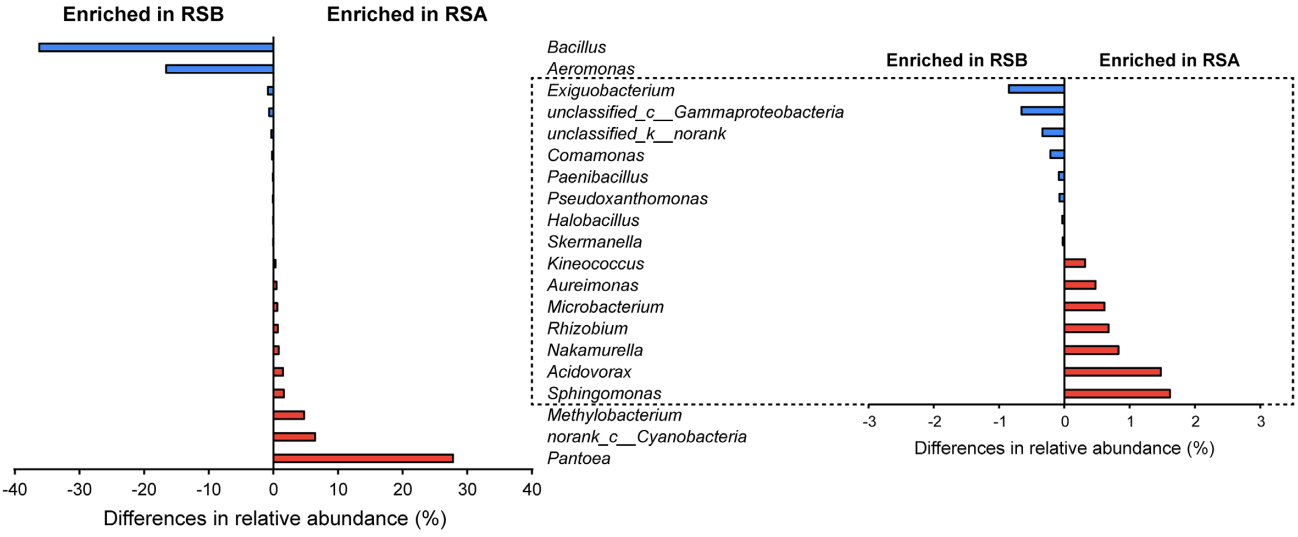

Figure 5. Taxonomic changes of the microbial community on surfaces of non-flooded (RSA) and flooded (RSB) rice culms at genus level (top 20). The figure on the right was an enlarged view of genera framed by the dotted line.

ing stage (Fig. 3), indicating that rice culms provide an important microbial niche for bacteria under flooded and dry conditions. However, bacteria on rice culms were clearly clustered into two main groups: RSA and RSB (Fig. 1, Fig. 2, Fig. S1 and Fig. S3, Supporting Information), suggesting that water flooding is the driving factor shaping bacterial compositions on rice culms. In addition, the sharp decrease of the abundance of rare OTUs in RSA suggested that most of rare OTUs in RSB should be niche-specialized inhabitants and be sensitive to non-flooded condition. Besides, our results also indicated that bacteria of *Gammaproteobactereia*, *Actinobacteria* and *Cyanobacteria* might be sensitive to waterlogging or flooding, while those of *Firmicutes* might be adapted to that condition (Fig. 3a). Under flooded conditions, the diffusion of dissolved oxygen is about 10 000-fold slower than in air (Drew 1997). This anaerobic or partially anaerobic condition would inhibit the growth and propagation of aerobic bacteria. Our results showed that *Bacillus*, containing many facultative aerobes (Choudhary and Johri 2009), was the most dominant genus in RSB (52.65%), while much lower abundance in RSA (16.35%). In addition, water can function as a solvent of nutrients, relieve the transpiration rate and act as temperature buffer for phyllosphere bacteria (Aung, Jiang and He 2018). Therefore, water availability can significantly influence plant-associated microbial structures and communities (Lau

and Lennon 2012; Blazewicz, Schwartz and Firestone 2014; Aung, Jiang and He 2018). Previous studies have well demonstrated that microbes often aggregate near water sources on the leaf surfaces (Vorholt 2012; Xin *et al.* 2016). In our study, results showed that flooded samples (RSB) have significantly higher Chao1 index and phylogenetic diversity than non-flooded samples (RSA) (Table 1), confirming that water availability could increase the richness of phyllosphere bacteria on rice culms. On the contrary, bacteria on the non-flooded rice culms with lower richness might be attributed to a relatively hostile environment, such as the fluctuation of UV, temperature and wind velocity (Lindow and Brandl 2003; Vorholt 2012). Remarkably, in this study, we found some bacteria which are very sensitive to water flooding were identified, for example, the relative abundance of *Pantoea* sharply decreased from 37.06% in RSA to 9.27% in RSB (Fig. 4a), and *Bacillus* increased from 16.35% in RSA to 52.59% in RSB (Fig. 4b). These results propose potential bio-indicators for predicting bacteria which are from water flooded or non-flooded conditions.

In addition to water availability, microbial structures and communities of phyllosphere bacteria on rice culms could be influenced by other abiotic factors. In our study, *Gammaproteobacteria* and *Firmicutes* were the most dominated bacteria both in RSA and RSB (Fig. 3b), which is consistent with the results reported by Ren, Zhu and Jia. (2014b), whereas *Alphaproteobacteria* was found to be the most predominant taxon in some other studies (Delmotte *et al.* 2009; Knief *et al.* 2012; Vorholt 2012). These inconsistent research results may be caused by the distinct geographic climate types, which are one of the main influence factors regulating continental-scale airborne microbial compositions (Barberan *et al.* 2015). The two cities Jiaxing (in our study) and Yangzhou (Ren *et al.* 2014a) belong to the subtropical monsoon climate, while the experimental sites in Philippines (Knief *et al.* 2012) are under tropical rainforest climate. Therefore, airborne could be another important factor shaping the compositions of phyllosphere microbes under different continental-scales.

Relationships between plants and bacteria

Besides environmental conditions, the biotic factors also determine the microbial assemblies during all stages of plant growth (Lindow and Brandl 2003). In general, phyllosphere bacteria get benefits from plants and are also beneficial to plants, the active recruitment of microbes inhabit on plant is depend on the interaction between plants and bacteria (Carroll 1988). For example, *Methylobacterium* can make use of methanol produced by host plants (Gourion, Rossignol and Vorholt 2006), which in turn benefits host plants by producing auxins/cytokines to promote plant growth and induced systemic resistance against pathogens (Lidstrom and Chistoserdova 2002). In our study, *Methylobacterium* is an abundant genus in RSA (4.80%), which is consistent with many plant species like *Arabidopsis thaliana*, rice and wheat (Gourion, Rossignol and Vorholt 2006; Delmotte *et al.* 2009; Knief *et al.* 2010). However, under flooded condition, methylotrophic lifestyle is less essential for plant tissues (Knief *et al.* 2010; Knief *et al.* 2012), thus the relative abundance of *Methylobacterium* of RSB samples was significantly decreased (Table S2, Supporting Information). According to published studies, the majorities of abundant bacteria detected in this study are identified as plant beneficial bacteria, such as *Pantoea*, *Bacillus*, *Aeromonas*, *Methylobacterium* and *Rhizobium* (Turner, James and Poole 2013; Ren, Zhu and Jia 2014b). *Pantoea* (37.07% in RSA, 9.27% in RSB) and *Bacillus* (16.36% in RSA, 52.59% in RSB), which are predominantly abundant genera in our study (Fig. 4), have been reported to have abilities to promote plant nitrogen fixation, nutrient availability, enhance the productions of phytohormone and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, as well as increase plant tolerance to environmental stress (Lindow and Leveau 2002; Yang, Kloepper and Ryu 2009; Glick 2014; Ren, Zhu and Jia 2014b). Additionally, most species of *Pantoea*, containing large pantoea plasmids (LPP-1), have been shown to undergo extensive diversification exhibiting robust biological and ecological adaptation (De Maayer *et al.* 2012). Some members of *Bacillus* have been reported can protect crop plants against pests and disease as biocontrol agents (Kumar *et al.* 2012; Chumthong *et al.* 2016; Sha, Wang and Li 2016). Besides the abundant bacteria, some less abundant or even rare taxa detected in our study might also have important beneficial effects on host plants. *Stenotrophomonas* (0.09% in RSA, 0.05% in RSB) was detected in all samples, which has been reported to be important for the nitrogen and sulfur cycles (Ryan *et al.* 2009). *Exiguobacterium* (0.23% in RSA, 1.08% in RSB) and *Aeromonas* (0.77% in RSA, 17.38% in RSB) are common habitants on rice plants and might enhance the plant growth through enhancing nitrogen fixation and IAA production (Patten and Glick 2002; Venkatachalam *et al.* 2016; Kasana and Pandey 2018). In the future, further investigation about these phyllosphere bacteria would help to identify potential beneficial bacteria for improving crop health and sustainable productivity in agroecosystems.

Ecological strategies of microbes on rice culms

Bacteria have to withstand various environmental pressures in the more open habitat (i.e. culms) compared to ground compartments (i.e. roots) (Vorholt 2012), as well as interact with other microorganism and host plants before they can inhabit on plant culms. Phyllosphere bacteria implement several ecological strategies to inhabit on rice culms. Their survival and growth support tolerance strategies of phyllosphere bacteria, which requires the ability to tolerate direct exposure to stresses on epiphytic sites (Beattie and Lindow 1999). Some phyllosphere bacteria, such as *Methylobacterium*, *Sphingomonas* and *Pseudomonas*, can protect against UV radiation by possessing pigmentation (Nogueira, Luisa Botelho and Tenreiro 1998; Lindow and Brandl 2003). Therefore, these bacteria were more abundant in RSA than RSB (Table S2, Supporting Information). One other paramount adaptive strategy for phyllosphere bacteria is so-called the avoidance, which requires bacteria seek shelters like endophytic sites that are protected from environmental stresses (Beattie and Lindow 1999). In our study, over 70% of bacteria on rice culms have close affinity with endophytes, such as *Pantoea*, *Enterobacter*, *Bacillus*, *Aeromonas*, *Exiguobacterium* and *Rhizobium* which were reported by Hardoim *et al.* (2015) (Hardoim *et al.* 2015,,). This close affinity to endophytes suggests that these detected phyllosphere bacteria are latent endophytes. It has been demonstrated that the horizontal transmission of phyllosphere bacteria, from leaves and stems via stomata or wounds caused by surrounding factors (e.g. wind, rain and insects), might be the major source of plant endophytes (Bright and Bulgheresi 2010; Kumar *et al.* 2012; Frank, Guzmán and Shay 2017). For example, *Pantoea* sp. is known as aggressive endophytic colonizer (Verma *et al.* 2004). In our study, *Pantoea* was the most dominant genus in RSA samples (37.07%), while sharply decreased to 9.27% in RSB samples (Fig. 4a) It is possible that several members of *Pantoea* chose avoidance strategy to enter rice culms via stomata when flooded, and became endophytes to occupy new niches. In addition, the variation of the relative abundance of *Enterobacter* (14.31% in RSA, 2.74% in RSB),

Bacillus (16.36% in RSA, 52.59% in RSB) and *Aeromonas* (0.77%% in RSA, 17.38%% in RSB) (Fig. 5 and Table S2, Supporting Information) could also be explained by potential horizontal transmission from endophytes, since these species have been identified as plant endophytes (Mano and Morisaki 2008; Sun *et al.* 2008). In the future, the interesting horizontal transmission between phyllosphere microbes and endophytes is worthy further investigation.

CONCLUSION

The present study provides new information about the structure and composition of microbiome on rice culms under flooded and non-flooded conditions. We found that bacterial communities on flooded and non-flooded rice culms were significantly different. In both RSA and RSB, *Gammaproteobacteria* and *Firmicutes* are the most dominant taxa, but the response of them to water flooding was different. The members of *Gammaproteobacteria*, especially *Pantoea* might be sensitive to waterlogging or flooding, while those of *Firmicutes* like *Bacillus* might adapt to that condition. Water flooding increased the bacterial richness on rice culms, and especially enriched the relative abundance of rare bacteria. In addition, we found that the rare reads and OTUs are more sensitive to environment change, which should be important feature for characterizing phyllosphere microbiome. The results of this study suggested that bacteria inhabiting on the surfaces of rice culms was regulated by both abiotic factors, such as flooding and biotic factors, including the interactions between plant and microbes.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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REFERENCE

Aleklett K, Leff JW, Fierer N *et al.* Wild plant species growing closely connected in a subalpine meadow host distinct root-associated bacterial communities. *PeerJ* 2015;**3**:e804.

Andrews JH, Hirano SS. *Microbial ecology of leaves*. Springer Science & Business Media, 2012.

Ansary WR, Prince FRK, Haque E *et al.* Endophytic *Bacillus* spp. from medicinal plants inhibit mycelial growth of Sclerotinia sclerotiorum and promote plant growth. *Z Naturforsch C* 2018;**73**:247–56.

Aung K, Jiang Y, He SY. The role of water in plant-microbe interactions. *Plant J* 2018;**93**:771–80.

Barberan A, Ladau J, Leff JW *et al.* Continental-scale distributions of dust-associated bacteria and fungi. *Proc Natl Acad Sci U S A* 2015;**112**:5756–61.

Beattie GA, Lindow SE. Bacterial Colonization of Leaves: a Spectrum of Strategies. *Phytopathology* 1999;**89**:353–9.

Blazewicz SJ, Schwartz E, Firestone MK. Growth and death of bacteria and fungi underlie rainfall-induced carbon dioxide pulses from seasonally dried soil. *Ecology* 2014;**95**:1162–72.

Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS One* 2013;**8**:e56329.

Bright M, Bulgheresi S. A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* 2010;**8**:218–30.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;**7**:335–6.

Carroll G. Fungal Endophytes in Stems and Leaves: from Latent Pathogen to Mutualistic Symbiont. *Ecology* 1988;**69**:2–9.

Castrillo G, Teixeira PJPL, Paredes SH *et al.* Root microbiota drive direct integration of phosphate stress and immunity. *Nature* 2017;**543**:513–8.

Chi F, Shen SH, Cheng HP *et al.* Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl Environ Microbiol* 2005;**71**:7271–8.

Choudhary DK, Johri BN. Interactions of *Bacillus* spp. and plants–with special reference to induced systemic resistance (ISR). *Microbiol Res* 2009;**164**:493–513.

Chumthong A, Wiwattanapatapee R, Viernstein H *et al.* Spray-dried powder of *Bacillus* megateriumfor control of rice sheath blight disease: formulation protocol and efficacy testing in laboratory and greenhouse. *Cereal Res Commun* 2016;**44**:131–40.

De Maayer P, Chan WY, Blom J *et al.* The large universal *Pantoea* plasmid LPP-1 plays a major role in biological and ecological diversification. *BMC Genomics* 2012;**13**:625.

Delmotte N, Knief C, Chaffron S *et al.* Community proteogenomics reveals insights into the physiology of phyllosphere bacteria, *Proc Natl Acad Sci U S A* 2009;**106**:16428–33.

Drew MC. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia, *Annu Rev Plant Physiol Plant Mol Biol* 1997;**48**:223–50.

Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;**10**:996–8.

Frank AC, Guzmán JPS, Shay JE. Transmission of Bacterial Endophytes. *Microorganisms* 2017;**5**.

Galand PE, Casamayor EO, Kirchman DL *et al.* Ecology of the rare microbial biosphere of the Arctic Ocean. *Proc Natl Acad Sci U S A* 2009;**106**:22427–32.

Glick BR. Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol Res* 2014;**169**:30–39.

Gourion B, Rossignol M, Vorholt JA. A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc Natl Acad Sci U S A* 2006;**103**:13186–91.

Moldenbauer KEWC, Slaton N. Rice growth and development. *Rice production handbook 192* 2001; **192**: 7–14.

Hardoim PR, van Overbeek LS, Berg G *et al.* The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev* 2015;**79**:293–320.

Hassani MA, Duran P, Hacquard S. Microbial interactions within the plant holobiont. *Microbiome* 2018;**6**:58.

Ikeda S, Okubo T, Anda M *et al.* Community- and genome-based views of plant-associated bacteria: plant-bacterial interactions in soybean and rice. *Plant Cell Physiol* 2010;**51**:1398–410.

Jackson MB, Colmer TD. Response and adaptation by plants to flooding stress. *Ann Bot-London* 2005;**96**:501–5.

Jousset A, Bienhold C, Chatzinotas A *et al.* Where less may be more: how the rare biosphere pulls ecosystems strings. *ISME J* 2017;**11**:853–62.

Kaminsky R, Morales SE. Conditionally rare taxa contribute but do not account for changes in soil prokaryotic community structure. *Front Microbiol* 2018;**9**. DOI: 10.3389/fmicb.2018.00809.

Kasana RC, Pandey CB. *Exiguobacterium*: an overview of a versatile genus with potential in industry and agriculture. *Crit Rev Biotechnol* 2018;**38**:141–56.

Knief C, Ramette A, Frances L *et al*. Site and plant species are important determinants of the Methylobacterium community composition in the plant phyllosphere. *ISME J* 2010;**4**:719–28.

Knief C, Delmotte N, Chaffron S *et al*. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J* 2012;**6**:1378–90.

Kumar AS, Lakshmanan V, Caplan JL *et al*. Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. *Plant J* 2012;**72**:694–706.

Lau JA, Lennon JT. Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proc Natl Acad Sci U S A* 2012;**109**:14058–62.

Lidstrom ME, Chistoserdova L. Plants in the pink: cytokinin production by *Methylobacterium*. *J Bacteriol* 2002;**184**:1818.

Lindow SE, Leveau JH. Phyllosphere microbiology. *Curr Opin Biotechnol* 2002;**13**:238–43.

Lindow SE, Brandl MT. Microbiology of the phyllosphere. *Appl Environ Microbiol* 2003;**69**:1875–83.

Logares R, Audic S, Bass D *et al*. Patterns of rare and abundant marine microbial eukaryotes. *Curr Biol* 2014;**24**:813–21.

Mano H, Morisaki H. Endophytic bacteria in the rice plant. *Microbes Environ* 2008;**23**:109–17.

McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;**8**:e61217.

Mommer L, Visser EJ. Underwater photosynthesis in flooded terrestrial plants: a matter of leaf plasticity. *Ann Bot-London* 2005;**96**:581–9.

Muller DB, Vogel C, Bai Y *et al*. The plant microbiota: systems-level insights and perspectives. *Annu Rev Genet* 2016;**50**:211–34.

Nogueira F, Luisa Botelho M, Tenreiro R. Radioresistance studies in *Methylobacterium* spp. *Radiat Phys Chem* 1998;**52**:15–19.

Oksanen J, Blanchet FG, Friendly M *et al*. vegan: Community Ecology Package. 2018.

Patten CL, Glick BR. Role of pseudomonas putida indoleacetic acid in development of the host plant root system. *Appl Environ Microb* 2002;**68**:3795–801.

Penuelas J, Terradas J. The foliar microbiome. *Trends Plant Sci* 2014;**19**:278–80.

Quast C, Pruesse E, Yilmaz P *et al*. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;**41**:D590–6.

R Core Team. 2018. *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Rastogi G, Coaker GL, Leveau JH. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. *FEMS Microbiol Lett* 2013;**348**:1–10.

Ren GD, Zhang HY, Lin XG *et al*. Response of phyllosphere bacterial communities to elevated CO_2 during rice growing season. *Appl Microbiol Biotechnol* 2014a;**98**:9459–71.

Ren GD, Zhu JG, Jia ZJ. Contrasting response patterns of rice phyllosphere bacterial taxa to elevated CO_2. *Pedosphere* 2014b;**24**:544–52.

Ryan RP, Monchy S, Cardinale M *et al*. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol* 2009;**7**:514–25.

Sha Y, Wang Q, Li Y. Suppression of Magnaporthe oryzae and interaction between *Bacillus subtilis* and rice plants in the control of rice blast. *Springerplus* 2016;**5**:1238.

Shade A, McManus PS, Handelsman J. Unexpected diversity during community succession in the apple flower microbiome. *MBio* 2013;**4**, e00602–12, doi: 10.1128/mBio.00602-12..

Sogin ML, Morrison HG, Huber JA *et al*. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc Natl Acad Sci U S A* 2006;**103**:12115–20.

Sun L, Qiu FB, Zhang XX *et al*. Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microb Ecol* 2008;**55**:415–24.

Turner TR, James EK, Poole PS. The plant microbiome. *Genome Biol* 2013;**14**:209.

Venkatachalam S, Ranjan K, Prasanna R *et al*. Diversity and functional traits of culturable microbiome members, including cyanobacteria in the rice phyllosphere. *Plant Biol* 2016;**18**:627–37.

Verma SC, Singh A, Chowdhury SP *et al*. Endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp. using green fluorescent protein reporter. *Biotechnol Lett* 2004;**26**:425–9.

Vorholt JA. Microbial life in the phyllosphere. *Nat Rev Microbiol* 2012;**10**:828–40.

Wang Q, Garrity GM, Tiedje JM *et al*. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**:5261–7.

Wang WF, Zhai YY, Cao LX *et al*. Endophytic bacterial and fungal microbiota in sprouts, roots and stems of rice (*Oryza sativa* L.). *Microbiol Res* 2016;**188**:1–8.

Xin XF, Nomura K, Aung K *et al*. Bacteria establish an aqueous living space in plants crucial for virulence. *Nature* 2016;**539**:524–9.

Yang J, Kloepper JW, Ryu CM. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci* 2009;**14**:1–4.

Zhu BK, Chen QL, Chen SC *et al*. Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced. *Environ Int* 2017;**98**:152–9.

Zhu D, An XL, Chen QL *et al*. Antibiotics disturb the microbiome and increase the incidence of resistance genes in the gut of a common soil Collembolan. *Environ Sci Technol* 2018;**52**:3081–90.