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Long-term organic fertilization increased antibiotic resistome in phyllosphere of maize

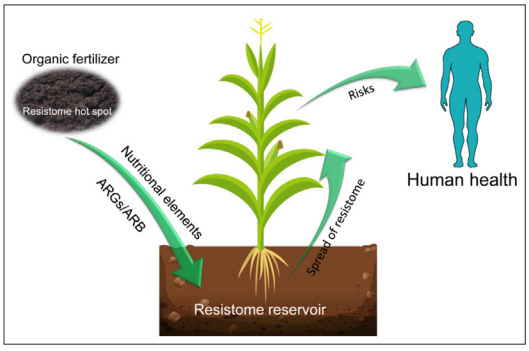
Qing-Lin Chen [a], Xin-Li An [a], Bang-Xiao Zheng [a,b], Yi-Bing Ma [c], Jian-Qiang Su [a,*]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] Ministry of Agriculture Key Laboratory of Plant Nutrition and Nutrient Cycling, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

HIGHLIGHTS

- Sewage sludge and manure amendment increased the occurrence and abundance of ARGs in phyllosphere.
- Bacterial community change was the major drives in shaping ARGs in the phyllosphere.
- Soil may serve as an antibiotic resistome reservoir for phyllosphere.

GRAPHICAL ABSTRACT



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ABSTRACT

Phyllosphere contains various microorganisms that may harbor diverse antibiotic resistance genes (ARGs). However, we know little about the composition of antibiotic resistome and the factors influencing the diversity and abundance of ARGs in the phyllosphere. In this study, 16S rRNA gene amplicon sequencing and high-throughput quantitative PCR approaches were employed to investigate the effects of long-term (over 10 years) organic fertilization on the phyllosphere bacterial communities and antibiotic resistome. Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes dominated in the phyllosphere bacterial communities. Long-term application of sewage sludge and chicken manure altered the phyllosphere bacterial community composition, with a remarkable decrease in bacterial alpha-diversity. A total of 124 unique ARGs were detected in the phyllosphere. The application of sewage sludge and chicken manure significantly increased the abundance of ARGs, with a maximum 2638-fold enrichment. Variation partitioning analysis (VPA) together with network analysis indicated that the profile of ARGs is strongly correlated with bacterial community compositions. These results improve the knowledge about the diversity of plant-associated antibiotic resistome and factors influencing the profile of ARGs in the phyllosphere.

1. Introduction

As the aerial parts of plants, phyllosphere is a ubiquitous and specific habitat for diverse microorganisms (Vorholt, 2012). It is estimated that on a global scale, the phyllosphere spans >10^8 km^2, which is >10 times

* Corresponding author at: Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China.
E-mail address: jqsu@iue.ac.cn (J.-Q. Su).

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of the vegetated area of earth (Lambin and Meyfroidt, 2011), harboring up to 10^{26} bacterial cells including epiphytes, as well as plant-pathogenic bacteria and even human pathogens (Dees et al., 2015; Delmotte et al., 2009; Lindow and Brandl, 2003). Previous studies have mainly focused on the bacterial community compositions and functions in phyllosphere (Bai et al., 2015; Kembel et al., 2014), demonstrating that bacteria associated with the leaves of plants generally fall into only a few phyla, i.e. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, and are often shared between different plant species (Ruiz-Perez et al., 2016; Ruppel et al., 2008). Nitrogen fixation genes, e.g., genes encoding dinitrogen reductase (*nifH*) and dinitrogenase (*nifD* and *nifK*) have also been detected in the phyllosphere metagenome (Knief et al., 2012). However, the composition of antibiotic resistome in the phyllosphere has gained less attention.

Although antibiotic resistance occurs naturally (D'Costa et al., 2011), the use of antibiotics is accelerating the emergence and dissemination of antibiotic resistance (Heuer et al., 2011; Yang et al., 2014). The increasing prevalence of ARGs is causing a threat to public health, due to the potential transmission of ARGs from environmental bacteria to human pathogens, giving rise to a global concern that ARGs should be considered as a new type of environmental contaminant (Forsberg et al., 2012; Martinez, 2008; Pruden et al., 2006).

Livestock farms and municipal wastewater treatment plants (WWTPs) appear to be hotspots of ARGs (Rizzo et al., 2013; Yang et al., 2014; Zhu et al., 2013). Antibiotics are commonly used in confined animal feeding operations worldwide to treat animal diseases and promote animal growth (Ji et al., 2012). Manure has been proved to be a rich reservoir of bacteria carrying ARGs and mobile genetic elements (MGEs) such as plasmids, transposons, and integrons (Sommer et al., 2009). Additionally, WWTPs receives a huge amount of domestic sewage containing antibiotic residues and antibiotic resistant bacteria, however, some of the ARGs cannot be effectively removed by wastewater treatment processes and are consequently discharged in the form of sewage sludge (Munir et al., 2011; Ju et al., 2016). Manure and sewage sludge application are common agricultural practices for recycling nutrients and cost-savings disposal of waste (Fahrenfeld et al., 2014; Joy et al., 2013. However, these practices are accelerating the occurrence and spread of ARGs in the environments (Bondarczuk et al., 2016; Kyselkova et al., 2015; Tien et al., 2017).

Our knowledge of the diversity and abundance of ARGs in sewage sludge or manure amended soils is currently increasing rapidly (Fahrenfeld et al., 2014; Martinez, 2008). However, most of these studies have focus on the ARGs in soil, without considering the potential transportation of ARGs from soil to phyllosphere. By using high-throughput quantitative PCR (HT-qPCR) and Illumina sequencing of total bacterial 16S rRNA genes, this study aimed (1) to obtain a comprehensive profile of ARGs in the maize phyllosphere, (2) to evaluate the effects of long-term application of sewage sludge and manure on the abundance and diversity of ARGs, and (3) to explore the relationship between profiles of ARGs and bacterial community compositions.

2. Materials and methods

2.1. Sampling

The field experiment was conducted in a long-term experimental station of the Chinese Academy of Agricultural Sciences (CAAS), located in Dezhou of Shandong Province, China (37°20′ N, 116°38′ E). The experiment was initiated in 2006 with wheat-maize rotation, aiming to investigating the nitrogen (N) and phosphorus (P) input-output balances and soil P accumulation when biosolids (sewage sludge and chicken manure) were applied to a calcareous soil. In total, eight treatments including four sewage sludge treatments (0.5SS, 1SS, 2SS, 4SS), two urea treatments (0.5N, 1N,), one chicken manure treatment (CM) and one control treatment (CK) without addition of sewage sludge, chicken manure and urea (Table 1). Each treatment was conducted in triplicate and randomly distributed in the experimental field. The area for each plot is 40 m^2 (5 m × 8 m) and the fallow border is 1 m between each plot. All fertilizers were added to soil and fully mixed every year in October. The basic information including the ARGs content of the sewage sludge and chicken manure (obtained from a broiler farm) is provided in Table S1. The raw sewage sludge and chicken manure were air dried before application to soil. The planting density, tillage, irrigation, insect and weed control were performed according to the local agricultural practices. Such as, planting density is about 45 kg seeds/ha for maize. All plots were irrigated for two times in each seasons and use same amount of insecticide and herbicide to control insect and weed. Our previous study, conducted in the same plots, has demonstrated that the long term organic fertilization significantly increased the abundance and diversity of ARGs in soils (Chen et al., 2016).

Table 1
The detailed information of application rates of sewage sludge, chicken manure and chemical fertilizer.

Treatments	Sewage sludge t hm^{-2}	Chicken manure t hm^{-2}	Urea kg hm^{-2}	Superphosphate kg hm^{-2}	Potassium sulphate kg hm^{-2}
CK	0	0	0	600	240
0.5N	0	0	65.25	600	240
1N	0	0	130.50	600	240
0.5SS	4.5	0	65.25	600	240
1SS	9	0	65.25	600	240
2SS	18	0	65.25	600	240
4SS	36	0	65.25	600	240
CM	0	10	65.25	600	240

The air-dried raw sewage sludge was applied as the basic fertilizer incorporated into the soil prior to wheat sowing every year.

Leaves were sampled from maize (anthesis stage) in August 2015, according to reported methodologies (Ruiz-Perez et al., 2016) with some modifications. Briefly, leaves were collected from 24 plots, with 3 randomly selected plants in each plot. All samples were collected on the same day and immediately frozen on dry ice, transported to the laboratory within 24 h and stored at −80 °C before analysis.

2.2. DNA extraction

DNA was extracted according to (Xie et al., 2015), with some modifications. In brief, around 10 g frozen maize leaf (cut into pieces with sterilized scissors) was weighed into a conical flask (250 mL) containing 100 mL 0.01 M sterile phosphate buffered saline (PBS). The mixture was sonicated for 5 min and shaken at 180 rpm at 30 °C for 1 h. The washing solution was filtered with nylon gauze, and then further filtered with 0.22 μM cellulose membrane filter, which was subjected to DNA extraction using the FastDNA® Spin Kit for Soil (MP Biomedical, Santa Ana, CA) following the manufacturer's instruction. The quality of DNA was checked by spectrophotometric analysis using NanoDrop ND-1000 (Nanodrop ND-1000, Thermo Scientific, Waltham, MA). The concentration of DNA was determined using the QuantiFluor® dsDNA system (Promega, Madison, WI) according to the manufacturer's instruction with a microplate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA). The DNA was stored at −20 °C until use.

2.3. High-throughput quantitative PCR

High-throughput qPCR was performed using the Wafergen SmartChip Real-time PCR system. This platform can be used for large scale genotyping analysis, supporting 5184 nanoliter reaction wells. PCR mixtures (100 nL for each well) consisted of 1 X LightCycler 480 SYBR Green I Master (Roche Applied Sciences, Indianapolis, IN), 1 mg/mL BSA (bovine serum albumin) (New England Biolaboratories, Beverly, MA), 500 nM of each primer and a DNA template of 50 ng/μL. A total of 296 primer sets (Table S2) targeting 285 ARGs, 10 MGEs

marker genes including 8 transposases, 1 class1 integron, and 1 clinic integron, and 16S ribosomal RNA (rRNA) gene. After the initial enzyme activation at 95 °C for 10 min, 40 cycles of the following program were used for amplification: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s. The melting process was automatically generated by Wafergen software. The qPCR results were analyzed using SmartChip qPCR Software. Wells with multiple melting peaks as well as wells with amplification efficiency beyond the range (90%–110%) were discarded. Only samples with three replicates which had amplification were regarded as positive. Copy number was calculated according to (Eq. (1)). The normalized ARG copy number was transformed by Eq. (2). The average number of 16S rRNA gene per bacterium is currently estimated at 4.1 based on the Ribosomal RNA Operon Copy Number Database (rrnDB version 4.3.3) (Klappenbach et al., 2001; Stalder et al., 2014). The normalized copy numbers of ARGs were transformed to absolute copy numbers by normalizing to 16S rRNA gene copy numbers which were quantified separately from the Wafergen platform. A comparative C_T method was used to calculate the fold change of each ARG (FC value) in treatments compared to the control (Eq. (3)) (Schmittgen and Livak, 2008). The detection limit C_T (31) was taken as a replacement for the genes with no amplification.

$$\text{Gene Copy Number} = 10^{(31-C_T)/(10/3)} \quad (1)$$

$$\text{Normalized ARG Copy Number} = (\text{ARG Copy Number}/\text{16S rRNA gene Copy Number}) \times 4.1 \quad (2)$$

$$\begin{aligned} \Delta C_T &= C_{T(ARG)} - C_{T(16S)} \\ \Delta\Delta C_T &= \Delta C_{T(Treatment)} - \Delta C_{T(Control)} \\ FC &= 2^{(-\Delta\Delta C_T)} \end{aligned} \quad (3)$$

Here C_T is the threshold value, ARG refers to one of the 295 antibiotic resistance gene assays, 16S refers to 16S rRNA gene, Treatment refers to the samples amended with sewage sludge or chicken manure, Control refers to the control samples without amendment.

2.4. Illumina sequencing of 16S rRNA genes, data processing and analysis

The V4-V5 region of bacterial 16S rRNA was amplified with primers F515 GTGCCAGCMGCCGCGG and R907 CCGTCAATTCMTTTRAGTTT (Zhou et al., 2011). In order to pool all samples, the reverse primer was tagged with unique barcodes for each sample. After the initial enzyme activation at 95 °C for 5 min, 30 cycles of the following program were used for amplification: 95 °C for 30s, 58 °C for 30 s and 72 °C for 30 s. Sequencing of these barcoded amplicons was performed using Illumina Hiseq2500 platform (Novogene, Beijing, China). Raw pair-end reads were assembled after filtering adaptor, low-quality reads, and barcodes to generate clean joined reads capturing the complete V4-V5 region of the 16S rRNA gene by Novogene. The generated high quality sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). The sequences were clustered into OTUs at the 97% similarity level using UCLUST clustering and taxonomy of each OTU were classified against the Ribosomal Database Project (RDP) database (Cole et al., 2009; Edgar, 2010). Chimeric sequences, chloroplast and mitochondrial OTUs and singleton OTUs were discarded from the final OTU data set by using the QIIME scripts: filter_otus_from_otu_table.py and filter_taxa_from_otu_table.py. Raw sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP111095.

2.5. Statistical analysis

Averages, and fold change values of ARGs were determined using Microsoft Excel 2007 (Microsoft Office 2010, Microsoft, USA). All statistical tests were considered significant at $P < 0.05$. Redundancy analysis (RDA) and Mantel test were used for correlation analysis between ARGs and bacterial communities. Variation partitioning analysis (VPA) was further conducted to determine the contributions of bacterial communities and MGEs to the variations of ARGs. Pearson correlation analysis was conducted to reveal the correlations between the abundance of MGEs and ARGs. Principal coordinate analysis was performed to evaluate the pattern of ARGs profiles among different samples based on the Bray-Curtis distance. VPA, mantel test and heatmap were conducted using R software with "vegan" and "pheatmap" packages (R Core Team, 2016). PCoA and RDA were performed using Canoco version 5.0 software. We constructed a correlation matrix by calculating all possible pair-wise Spearman's rank correlations between the ARGs and bacterial taxa to visualize the correlations in the network interface. A correlation between two items was considered statistically robust if the Spearman's correlation coefficient (ρ) was >0.8 and the *P*-value was <0.01 (Li et al., 2015). Cytoscape 3.3.0 (Shannon et al., 2003) was used to visualize the network graphs using circular layout algorithms, where edges were weighted according to the correlation coefficient and nodes (ARGs subtypes or bacterial taxa) size was weighted according to the relative abundance of ARGs or bacterial taxa.

3. Results

3.1. Assemblages of bacterial community in phyllosphere

A total of 1,992,059 high quality sequences were obtained from all 24 samples with sequences per sample ranging from 45,747 to 171,598. Sequence clustering yielded a total of 1840 operational taxonomic units (OTUs) (sequences binned at a 97% similarity cutoff), with OTUs per sample ranging from 324 to 833. Phylogenetic classification showed that these OTUs belonged to 13 phyla and 29 classes. Four dominant phyla (Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes) were identified, with Proteobacteria being the most abundant, ranging from 68.3% to 91.6%, and its abundance increased after application of chicken manure and sewage sludge (4SS) (Fig. 1). Alpha- and Gamma-Proteobacteria were the two dominant classes in all samples, ranging from 7.2% to 54.9% and from 22.5% to 86.3%, respectively (Fig. S1). Of the 1840 identified OTUs, 105 were shared by all samples (core community OTUs), which were dominated by *Sphingomonas*, *Pseudomonas*, *Chryseobacterium*, *Curtobacterium*, *Methylobacterium*, *Erwinia*, *Burkholderia*, and *Microbacterium*.

PCoA analysis based on the Bray-Curtis distance revealed that the overall pattern of bacterial community in the phyllosphere were altered by soil amendments except for the treatments of 0.5N and 1SS (Fig. S2). The first two PCs explained 69.34% of the total variation. This shift in bacterial composition profiles was further demonstrated by Adonis test ($P < 0.001$). Bacterial alpha-diversity decreased after the application of sewage sludge and chicken manure by evaluation of observed OTUs (Fig. 1B).

3.2. Assemblages of ARGs in phyllosphere

A total of 124 ARGs out of 295 targeted genes were detected among all the phyllosphere samples. The detected number of ARGs ranged from 42 to 100. The application of sewage sludge and chicken manure significantly increased the occurrence of ARGs in the phyllosphere, in which treatment of 4SS harbored the most diverse ARGs followed by chicken manure treatment (Fig. 2).

In control, the normalized abundance of ARGs was 0.025 copies per cell and a significant increase in the abundance of ARGs was detected in almost all the treatments except for 0.5N (Fig. 2). When compared with chemical fertilizer treatment, sewage sludge or chicken manure significantly increased the abundance of ARGs, with the treatment of chicken manure being the highest among all the treatments (0.084 copies per cell). In the sewage sludge treatment, the abundance of ARGs was elevated with the increasing dose of sewage sludge.

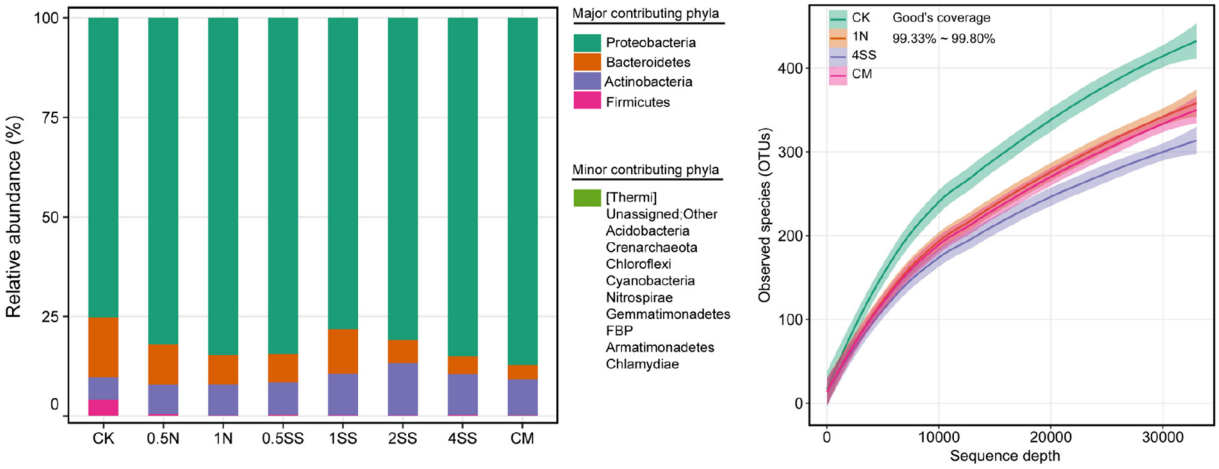
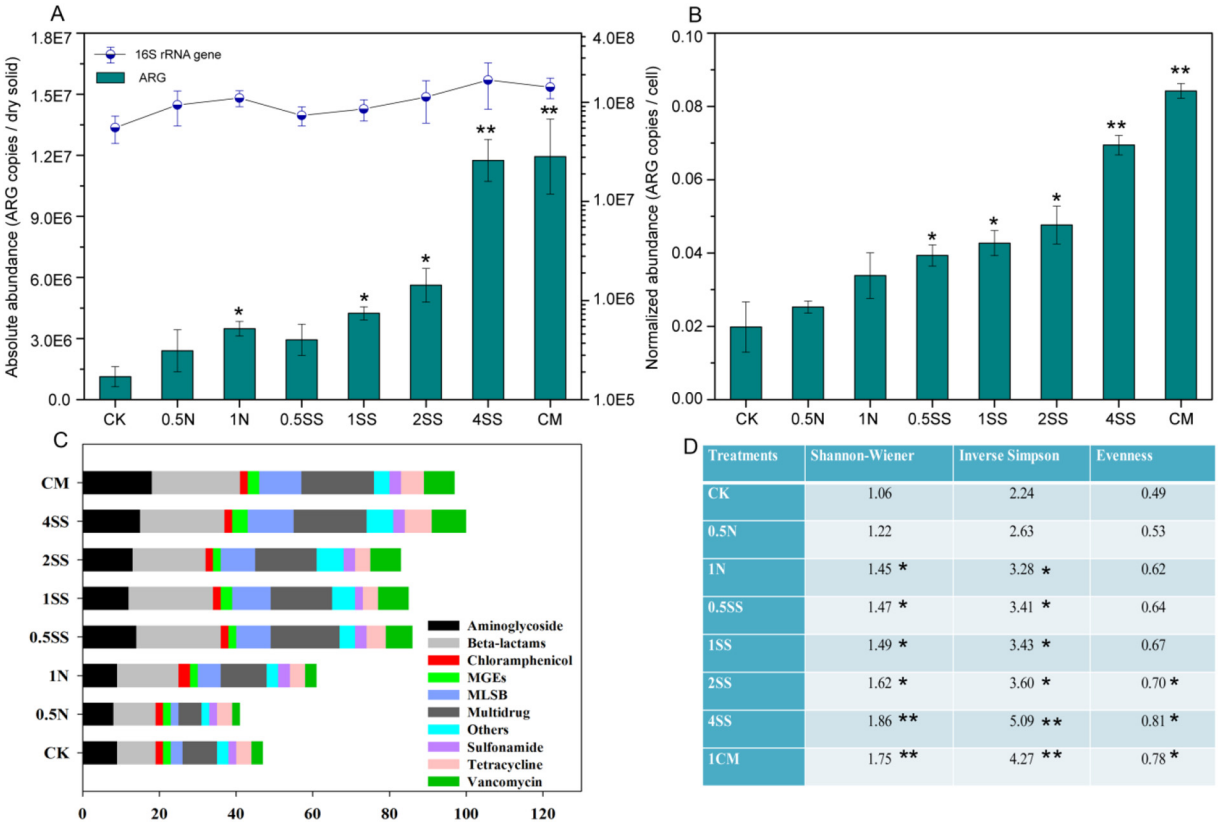


Fig. 1. Phylum distribution of the OTUs (A). Rarefaction curves showing the bacterial alpha-diversity (B).

The normalized (ARG copies per cell) and absolute (ARG copies per gram dry plant tissue) abundance of ARGs displayed a similar trend in the treatments. The absolute copy numbers of ARGs, ranging from 4.43×10^5 to 1.52×10^7 copies, was positively correlated with the 16S rRNA gene copy number ($P < 0.01$) (Fig. 2). ARGs were classed into nine subtypes, i.e. aminoglycoside, beta-lactams, chloramphenicol, Macrolide-Lincosamide-Streptogramin B (MLSB), multidrug, sulfonamide, tetracycline, vancomycin and others based on the type of antibiotics. Multidrug, beta-lactams and chloramphenicol resistance genes were the most dominant subtypes of ARGs. The application of sewage sludge and chicken manure significantly increased the abundance of genes conferring resistance to beta-lactams and chloramphenicol, especially for the treatments of 4SS and CM (Fig. S3). Antibiotic deactivation and efflux pump were the two dominant resistance mechanisms for antibiotic resistance (Fig. S4), and the application of sewage sludge and chicken manure increased efflux pump related ARGs.

The overall patterns of ARGs were shifted by different treatments, as indicated by Adonis test ($P < 0.001$). PCoA based on the Bray-Curtis distance indicated that control samples clustered together and were separated from the other samples, except for the treatment of 0.5N (Fig. S2). The treatments of 4SS, 2SS and CM were separated from the control along PC1 (explained 55.64% of the total variance), while the treatments of 0.5SS, and 1N were separated from the control along PC2 (explained 21.03% of the total variance), which indicated that the application of chicken manure and high doses of sewage sludge caused a major shift on ARGs.



Treatments	Shannon-Wiener	Inverse Simpson	Evenness
CK	1.06	2.24	0.49
0.5N	1.22	2.63	0.53
1N	1.45 *	3.28 *	0.62
0.5SS	1.47 *	3.41 *	0.64
1SS	1.49 *	3.43 *	0.67
2SS	1.62 *	3.60 *	0.70 *
4SS	1.86 **	5.09 **	0.81 *
1CM	1.75 **	4.27 **	0.78 *

Fig. 2. Characterization of ARGs in phyllosphere. A, the absolute abundance of ARGs, B, the normalized abundance of ARGs, C, the detected number of ARGs, D, the diversity index of ARGs in different treatments. **($P < 0.01$) and *($P < 0.05$) on the bar indicated that fertilization significantly increased the abundance and diversity of ARGs.

3.3. Enrichment of ARGs in phyllosphere

Among all of the detected 124 unique ARGs, a total of 62 unique ARGs were significantly enriched in at least one sample compared with the control. The heatmap of FC values (treatments versus the control) showed that the application of sewage sludge and chicken manure caused stronger enrichment of ARGs compared to the urea with a maximum enrichment of 2638-fold for *amp*C-06 was detected in CM treatment. In addition, other ARGs such as *bla*OXY, *aph*A1 (aka kanR), *aac*C were enriched >100 folds (Fig. 3).

3.4. Relationship between ARGs and bacterial communities

The ARG profiles were significantly correlated with bacterial community structures and compositions based on mantel test ($r = 0.52$, $P = 0.002$). RDA analysis indicated that the ARGs compositions were significantly correlated to the abundance of Actinobacteria, Proteobacteria and Firmicutes (Fig. 4). In addition, we observed that the ARG compositions were also positively correlated with the abundance of MGEs in the 4SS, 2SS and CM treatments. VPA analysis was further used to decipher the contribution of various factors to the shift of ARGs. Bacterial communities and MGEs accounted for 46.6% of total variation of ARGs profiles. Bacterial communities individually contributed 32.3% of the total variation, while MGEs only contributed 4.9% of the total variation, indicating that bacterial community changes were the major driver shifting the profile of ARGs in the phyllosphere (Fig. 5B).

A network consisting of 46 nodes (ARG subtypes and bacterial taxa) and 98 edges was constructed to investigate the interactions between bacterial taxa and ARGs, in which six bacterial taxa were identified with close relationship with ARGs (Spearman's $\rho > 0.8$, P-value <0.01) (Fig. 5). For example, *Microbacteriaceae* was significantly correlated with chloramphenicol resistance gene *cmx*(A), beta-lactams resistance gene *cphA*-01 and multidrug resistance gene *ade*A, while *Sphingobacteriaceae* correlated with multidrug resistance genes *tol*C-02 and sulfonamide resistance gene *sul*I. Compared with the above families, *Pseudomonadaceae* and *Alcaligenaceae* had correlation with more diverse ARGs, including resistance genes for aminoglycoside (*aadA*-01), beta-lactams (*bla*-L1, *amp*C-01, *amp*C-05, *amp*C-06, *blaOX*A10-01, *cfx*A, *bla*TLA, *bla*-ACC-1), multidrug (*acr*A-05, *tol*C-02, *pmr*A, *mtr*C-02), MLSB (*msr*A-01), sulfonamide (*sul*I), tetracycline [*tet*(36)-01, *tet*(36)-02, *tet*(34)], vancomycin (*van*TE, *van*RA-02), and MGEs.

4. Discussion

4.1. Phyllosphere resistome

By using HT-qPCR, this study provides new insights into the antibiotic resistome in plant microbiome, as well as the factors driving the shift of antibiotic resistome in plant microbiome, which is critical for understanding the spread of ARGs in the environment and associated risks.

A total of 124 unique ARGs were detected in all phyllosphere samples encompassing the three major resistance mechanisms (efflux pumping, cellular protection, and antibiotic deactivation). It has been reported that diverse ARGs were detected in the phyllosphere of various vegetable plants (An et al., 2018; Fernandez-Alarcon et al., 2011; Ruimy et al., 2010; Wang et al., 2015). However, these studies covered limited

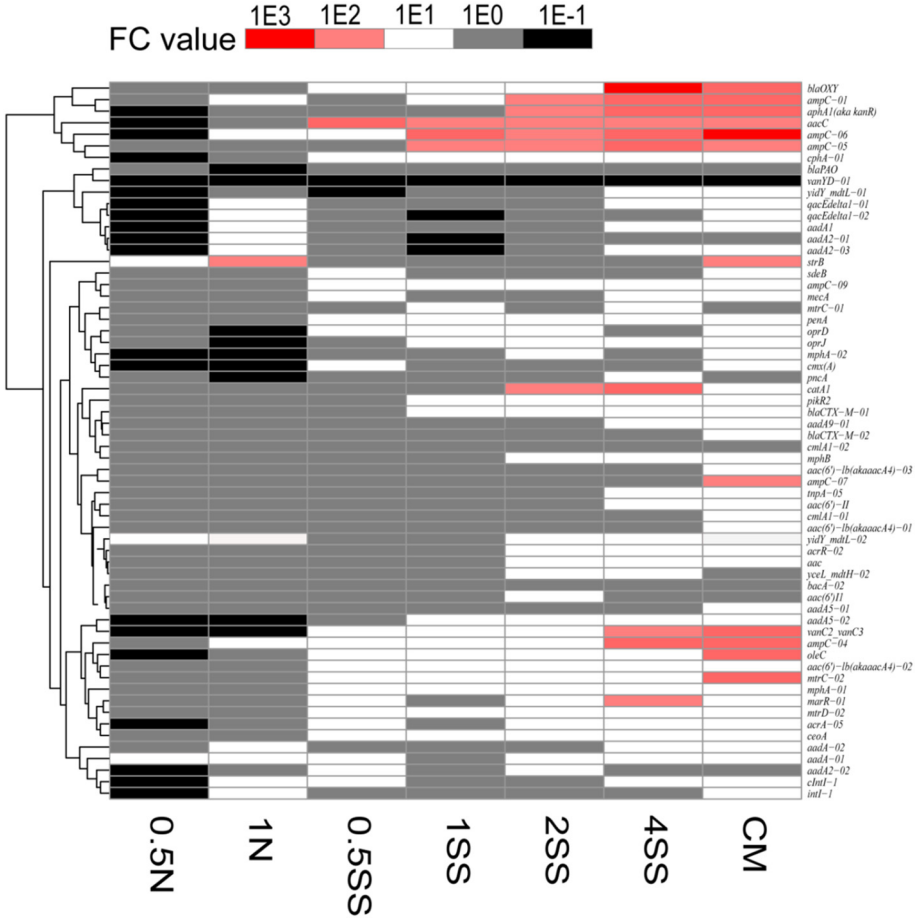


Fig. 3. Fold changes (FC values) showing the shift of resistance profiles of detected ARGs. The method of ΔΔCt comparison was used to calculate the FC values. When the FC value calculated by $2^{-(\Delta\Delta C_t+2s)} > 1$ or $2^{-(\Delta\Delta C_t-2s)} < 1$ (s is the standard deviation of the ΔΔCt value), it was considered as significantly enriched or decreased, respectively. The detection limit Ct (31) was taken as a replacement for the genes with no amplification.

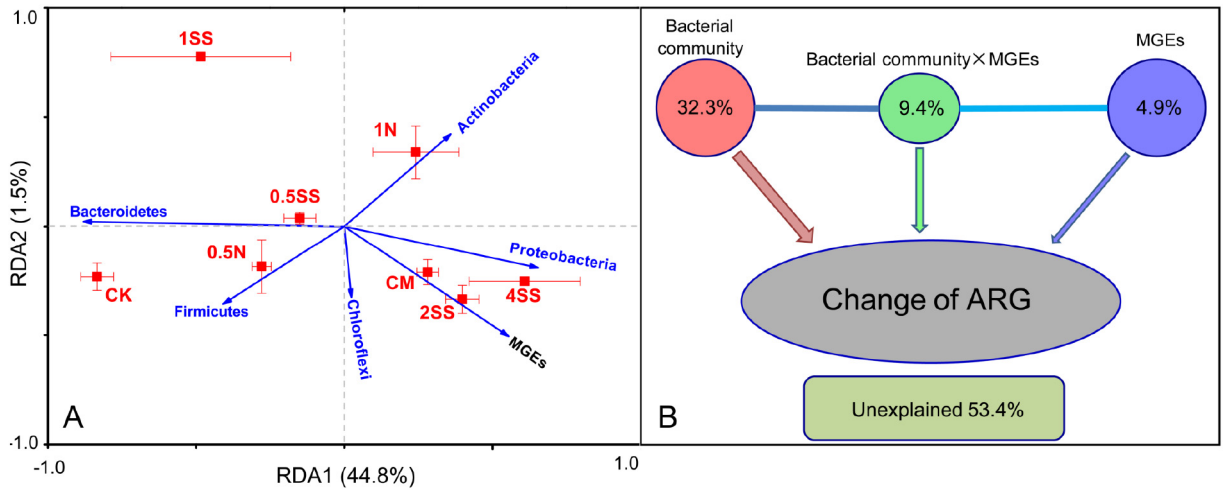


Fig. 4. Redundancy analysis (RDA) of the relationship between major microbial phyla (>1% in any sample) and ARGs (A), Variation partitioning analysis (VPA) differentiating effects of bacterial community and mobile genetic elements (MGEs) on the resistome (B).

number of ARGs by using conventional qPCR. Using HT-qPCR, 285 ARGs and 10 MGEs were targeted in the present study, providing a more comprehensive profile of ARGs in phyllosphere. In addition, we found that the abundance of MGEs was positively correlated with ARGs ($P < 0.01$) (Table S3), indicating that the MGEs may be a factor that increased the abundance of ARGs in phyllosphere. This inference is supported by previously published data that demonstrated the phyllosphere was conducive to conjugative plasmid transfer (Pontiroli et al., 2009), which could facilitate the dissemination of ARGs in the environment. Although, HT-qPCR is providing a way for rapid detection and quantification of many ARGs, the major limitation of PCR-based approach is that it depends on the availability of ARG sequences for primer design; novel ARGs in the environment cannot be detected (Su et al., 2017). With the advances in next generation sequencing and "omics" analysis, metagenomics survey is now widely adopted in characterizing antibiotic resistome in environment samples.

One of the major findings of this study is that the application of sewage sludge and chicken manure significantly increased the abundance and diversity of ARGs in the phyllosphere (Fig. 2). More than fifty unique ARGs were detected in the phyllosphere after the application of sewage sludge and manure (Fig. S5). Several studies have indicated that the foliage of crops carried antibiotic resistant bacteria in soil fertilized with struvite or biosolids including sewage sludge and animal manure slurries. For example, Marti et al. detected the emergence of ARGs on vegetables grown in freshly manured soil, which is consistent with our results (Marti et al., 2013; Marti et al., 2014). Our previous study also found the similar results that the application of struvite significantly increased the abundance and diversity of ARG in the phyllosphere of *Brassica*, and a fraction of the detected ARGs were shared exclusively between *Brassica* phyllosphere and struvite, indicating that struvite could be an important source of ARGs in phyllosphere (Chen et al., 2017). Therefore, development of waste treatment techniques (such as composting, anaerobic digestion, air-drying beds, and lime stabilization) is required to eliminate or reduce ARGs burdens from manures or sewage sludge (Burch et al., 2017). In addition, a period delay between application of organic fertilizer and crop harvest

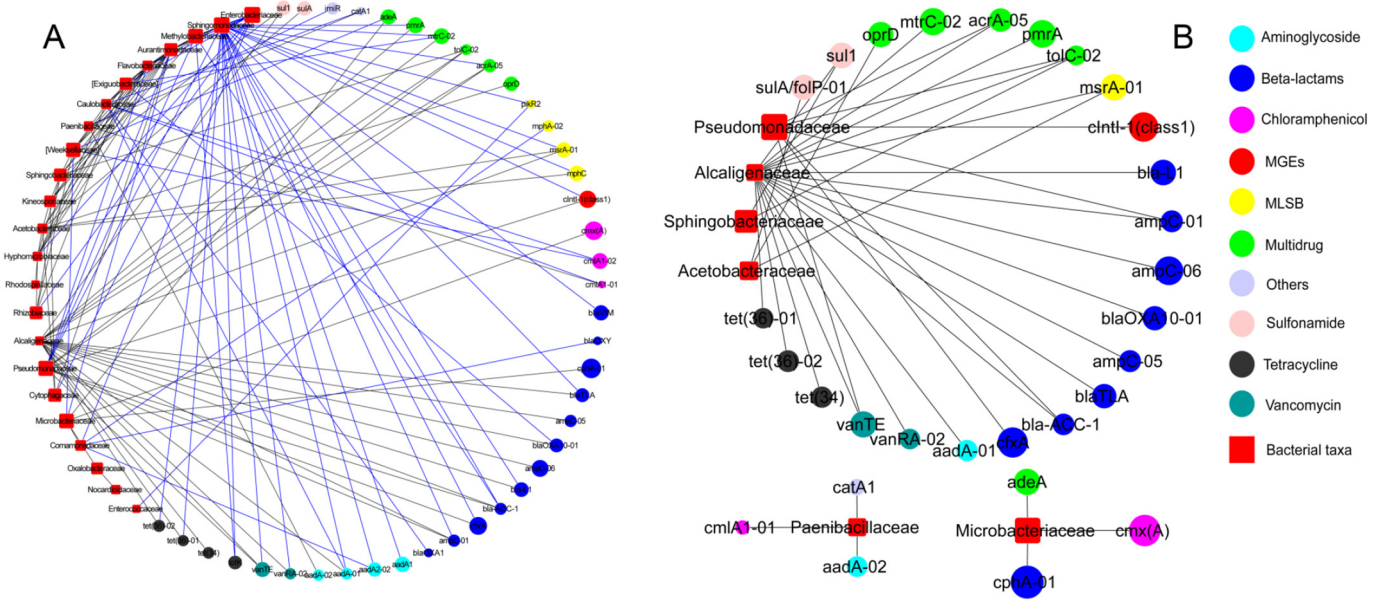


Fig. 5. The Network analysis revealing the co-occurrence patterns between ARG subtypes and microbial taxa. The connection represents a strong (Spearman's correlation coefficient $\rho > 0.8$) and significant (P-value < 0.01) correlation (A). Simplified network revealed the possible potential hosts for ARGs (B). The edges were weighted according to the correlation coefficient and the node sizes were weighted according to the relative abundance of ARGs or microbial taxa. The blue and black edge indicated a negative and positive correlation, respectively.

may reduce the potential risks of antibiotic resistance (Rahube et al., 2014).

ARGs that residing on MGEs in the phyllosphere might pose higher risks to human health via direct contact, particularly consumption of fresh leafy vegetables. In addition, a fraction of plant-associated bacteria were affiliated to *Pseudomonas*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Burkholderia*, some strains of these genera are pathogenic and of clinical importance because they possess diverse ARGs and the ability to form biofilms (Booth et al., 2003; Perreten et al., 2005; Strauss et al., 2015; Zhang et al., 2013). With the increasing demands for organically produced food by humans, the usage of animal manures and sewage sludge will likely increase in the future (Jechalke et al., 2013). The potential horizontal gene transfer of ARGs and the presence of human pathogens in phyllosphere raises the risk of transmission of ARGs from organic fertilizers to pathogenic bacteria (Brandl, 2006).

4.2. Relationship between resistome and microbiome in phyllosphere

In recent years, the phyllosphere associated microbiome of plants have been extensively studied by culture-independent analyses and revealing that the co-occurrence of four main bacterial phyla i.e. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, which is consistent with our observation. The relative incomplex taxonomically structure of bacterial communities in phyllosphere could be attributed to the effects of environmental factors, such as elevated and fluctuating salinity, periodic desiccation, high temperatures, high incidence of UV radiation (Lambais et al., 2006; Ruiz-Perez et al., 2016), organic carbon and moisture provided by leaves (Qvit-Raz et al., 2008).

In the present study, we found that bacterial community compositions were significant correlated with ARG profiles. VPA analysis further demonstrated that changes in bacterial community driving the shifts of ARGs in phyllosphere, which is supported by prior studies conducted in soils and during sewage sludge composting (Chen et al., 2016; Su et al., 2015). The phyllosphere of plants are able to recruit bacteria from broad origins, including soil and air (Bulgarelli et al., 2013; Vorholt, 2012) owing to the open nature of leaves. Therefore, land application of organic fertilizers could influence the phyllosphere microbiome via soil-plant system or directly recruit the manure- and sewage sludge-borne bacteria via air.

An interesting finding is that the profile of phyllosphere microbiome was shifted by soil amendments with different fertilizers, and sewage sludge application decreased its alpha-diversity. It was suggested that there was potential cross-talk between above - and below-ground parts of the plant, in which bacteria in plant rhizosphere and phyllosphere were interconnected (Bodenhausen et al., 2013; Ruiz-Perez et al., 2016). Our previous study has demonstrated that the long-term application of sewage sludge and chicken manure significantly changed the bacterial community compositions and structures in soil from the same site, and further increased the abundance and diversity of ARGs in soil (Chen et al., 2016). Based on the comparison of results between the present study and our previous study, we observed that the abundance of ARG in the eight treatments displayed a similar trend between phyllosphere and soil. In addition, 24 ARGs and class 1 integron-integrase gene were shared between soil and phyllosphere and the abundance of the shared ARGs occupied over 60% of the total abundance of ARGs in phyllosphere (Fig. S6). These results imply that soil resistome could serve as reservoir for above-ground parts of plants, and shifts in soil microbial compositions could affect the plant-associated microbiota and eventually shape the ARG compositions in phyllosphere.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.07.260.

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