Integrated metagenomics and molecular ecological network analysis of bacterial community composition during the phytoremediation of cadmium-contaminated soils by bioenergy crops

Zhaojin Chen a,b,*, Yuan Zheng a,b, Chuanyu Ding a,b, Xuemin Ren a,b, Jian Yuan a,b, Feng Sun a,b, Yuying Li a,b

a Key Laboratory of Ecological Security for Water Source Region of Mid-line Project of South-to-North Diversion Project of Henan Province, College of Agricultural Engineering, Nanyang Normal University, Nanyang 473061, People’s Republic of China
b Henan Collaborative Innovation Center of Water Security for Water Source Region of Mid-line Project of South-to-North Diversion Project, Nanyang 473061, People’s Republic of China

A R T I C L E   I N F O

Keywords:
Bacterial community structure
Cadmium
Phytoremediation
Bioenergy crops
MiSeq sequencing
Molecular ecological network analysis

A B S T R A C T

Two energy crops (maize and soybean) were used in the remediation of cadmium-contaminated soils. These crops were used because they are fast growing, have a large biomass and are good sources for bioenergy production. The total accumulation of cadmium in maize and soybean plants was 393.01 and 263.24 μg pot⁻¹, respectively. The rhizosphere bacterial community composition was studied by MiSeq sequencing. Phylogenetic analysis was performed using 16S rRNA gene sequences. The rhizosphere bacteria were divided into 33 major phylogenetic groups according to phyla. The dominant phylogenetic groups included Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes, and Bacteroidetes. Based on principal component analysis (PCA) and un-weighted pair group with arithmetic mean (UPGMA) analysis, we found that the bacterial community was influenced by cadmium addition and bioenergy cropping. Three molecular ecological networks were constructed for the unplanted, soybean- and maize-planted bacterial communities grown in 50 mg kg⁻¹ cadmium-contaminated soils. The results indicated that bioenergy cropping increased the complexity of the bacterial community network as evidenced by a higher total number of nodes, the average geodesic distance (GD), the modularity and a shorter geodesic distance. Proteobacteria and Acidobacteria were the keystone bacteria connecting different co-expressed operational taxonomic units (OTUs). The results showed that bioenergy cropping altered the topological roles of individual OTUs and keystone populations. This is the first study to reveal the effects of bioenergy cropping on microbial interactions in the phytoremediation of cadmium-contaminated soils by network reconstruction. This method can greatly enhance our understanding of the mechanisms of plant-microbe-metal interactions in metal-polluted ecosystems.

1. Introduction

Agriculture, mining and industry are major contributors to heavy metal soil contamination. Environmental degradation is prevalent in China, with approximately 20% of the arable land estimated to be contaminated with heavy metals (Teng et al., 2014; Zhao et al., 2015). This degradation is likely to have severe global economic and geopolitical consequences in the future. As a potential solution, phytoremediation is a sustainable and effective treatment for heavy metal-contaminated soils (Salt et al., 1995; Ali et al., 2013). Specifically, energy crop cultivation, as an alternative land-use strategy, is a significant component of biomass production and the ecological remediation of sites contaminated by heavy metals (Shi and Cai, 2009; Weyens et al., 2009; Gomes, 2012).

Important and complex symbioses exist between metal-tolerant and metal-accumulating plants and their rhizosphere microflora. Understanding these relationships might facilitate the development of phytoremediation technologies for removing heavy metals from contaminated soils (Rajkumar et al., 2012; Teng et al., 2015). Microorganisms coexist and interact within a complex system of networks, with both positive and negative feedback loops. Understanding these interactions is a central theme in microbial ecology (Faust et al., 2012;
Deng et al., 2016). For example, Sheng et al. (2012) examined the rhizosphere bacterial communities associated with copper-contaminated soils of the important biofuel crop maize (Zea mays) using denaturing gradient gel electrophoresis. Network models can be used for depicting plant-microbe relationships; however, the development of these models lags far behind the advances in metagenomic technologies (e.g., sequencing and microarrays) (Faust et al., 2012; Deng et al., 2016). Conventional analytical techniques have failed to rival network analyses in revealing pivotal information on the interactions among organisms (Bahram et al., 2014; Banerjee et al., 2016), keystone microbial taxa (Lu et al., 2013; Banerjee et al., 2016), and changes in environmental factors (Zhou et al., 2011; Jiang et al., 2015; Tu et al., 2015; Creamer et al., 2016). In a recent study by Deng et al. (2016), functional molecular ecological networks were constructed to study the effect of emulsiﬁed vegetable oil (EVO) amendment on groundwater microbial communities. The results showed that EVO injections triggered bacterial competition. Next-generation sequencing (NGS) technologies, including the 454 and MiSeq platforms, have the potential to revolutionize environmental microbiology because they allow the resolution of these complex networks and the association of microbial communities with their associated niche functions (Zhou et al., 2010, 2011; Hahn et al., 2016).

We chose maize and soybean in the current study due to their rapid growth rates, large biomass and bioenergy utility (Shi and Cai, 2009; Sheng et al., 2012; Van Slycken et al., 2013; Pandey et al., 2016). The rhizosphere bacterial community composition was studied by MiSeq sequencing. This study represents the ﬁrst network study of the impact of biofuel crops on microbial interactions in cadmium-contaminated soils, and it aims to enhance understanding of the mechanisms of plant-microbe interactions in these systems. The ﬁndings may contribute to the development of an effective bioenergy plant-microbe partnership for the phytoremediation of cadmium-contaminated soils.

2. Materials and methods

2.1. Pot experiments

Non-metal-contaminated soil samples were collected from cultivated soil in Nanyang, China (32°99′N and 112°47′E). The characteristics of the soil samples were as follows: yellow cinnamon soil; pH, 7.62; total P, 1.90 g kg⁻¹; available P, 18.27 mg kg⁻¹; total N, 1.31 g kg⁻¹; organic matter, 10.29 g kg⁻¹; cation exchange capacity, 13.2 cmol kg⁻¹. For the pot experiments, air-dried soil samples were thoroughly mixed and sieved (2 mm). The soils were treated with HClO₄ (4/1, v/v). The cadmium concentrations of root, stem and leaf tissues were analyzed by using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Optima 2100 DV, Perkin Elmer, USA).

Tolerance index (TI), bioconcentration factor (BCF) and translocation factor (TF) values were used to estimate the crop potential for phytoremediation use. TI was expressed as the ratio of lengths or weights of the roots and the above-ground tissues in heavy metal-contaminated soil to those in control soil as follows (Ait Ali et al., 2002):

\[
TI = \frac{\text{Growth parameters of the plants grown in cadmium − contaminated soil}}{\text{Growth parameters of the plants grown in control soil}} \times 100
\]

The TF of cadmium from the roots to the above-ground tissues was calculated as follows (Audet and Charest, 2007):

\[
\text{TF} = \frac{\text{cadmium accumulation in above − ground tissues}}{\text{cadmium accumulation in roots}}
\]

BCF was expressed as the ratio of the heavy metal concentration in plant tissues to that in soil. Two BCF measures (roots and above-ground tissues) were calculated as follows (Audet and Charest, 2007):

\[
\text{BCF} = \frac{\text{cadmium concentration in roots or above − ground tissues}}{\text{cadmium concentration in soil}}
\]

2.3. DNA extraction, PCR, and MiSeq sequencing

To collect rhizosphere samples, plants were carefully excavated, and the soil loosely attached to the root was removed. Genomic DNA was extracted from 0.5 g of fresh rhizosphere soil using the Fast DNA SPIN for Soil Kit (MP Biochemicals, Solon, OH, USA) according to the manufacturer’s instructions. The V3-V4 region of the bacterial 16S rRNA gene was ampliﬁed (95 °C for 3 min; followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a ﬁnal extension at 72 °C for 10 min) using 338F (5′-ACTCTACGGGAGGCAGA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAAT-3′) with sample-identifying barcodes. The PCR assays were performed in 20 µl mixture containing 4 µl of 5 × FastPfu buffer, 2 µl of 2.5 mM dNTPs, 0.8 µl of each primer (5 µM), 0.4 µl of FastPfu Polymerase, 10 ng of template DNA, and Milli-Q water. PCR was performed in triplicate for each sample, and the products were puriﬁed using the AsyPrepDNA Gel Extraction Kit (Axygen, USA) and re-quantitated with QuantiFluor™ ST (Promega, USA). The sequencing was performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) with an Illumina MiSeq PE300 platform.

2.4. Pyrosequencing data

The sequence data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) Pipeline (Version 1.7.0 http://qiime.org/tutorials/tutorial.html). The operational taxonomic units (OTUs) at 97% similarity level were clustered using Usearch (version 7.1 http://drive5.com/uparse/). The OTU number of each sample was used to represent species richness. Rarefaction curves and Shannon-Wiener indices were generated, and the ACE, Shannon, and Chao1 estimators were calculated to compare the bacterial richness and diversity. Taxonomic classiﬁcation at the phylum and genus levels was performed using the Ribose2 Database Project (RDP) algorithm to classify the representative sequences of each OTU. A principle component analysis (PCA) was performed at a 3% dissimilarity level. The linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/lefse/), a statistical tool designed for the identiﬁcation of biomarkers from metagenome data, was used to identify potential statistically signiﬁcant taxa between different treatments (Segata et al., 2011).
2.5. Molecular ecological network analysis

Phylogenetic molecular ecological networks (pMENs) offer a robust statistical means of analyzing networks as they provide solutions for common issues encountered in the use of high-throughput metagenomic data, including noise reduction and automatic network definition (Zhou et al., 2010, 2011). The following steps were used for the ecological network construction using MENAP (http://129.15.40.240/ment/). 1) A relative abundance (RA) matrix and an OTU annotation structure. 2) The RA matrix was submitted for network construction. A cut-off value (similarity threshold, s) for the similarity matrix was automatically generated using default settings. 3) Calculations of "global network properties", "individual nodes' centrality", and "module separation and modularity" were performed. 4) The "output for Cytoscape visualization" procedure was performed in "greedy modularity optimization mode". The data network was then exported for visualization using Cytoscape software. 5) The "randomize the network structure and then calculate network procedure" was performed to calculate random network properties while maintaining the same number of nodes and links as the empirical networks and using the Maslov-Sneppen procedure (Lu et al., 2013; Yao et al., 2014).

2.6. Statistical analyses

The means of the treatments were compared by analysis of variance and the post-hoc Tukey's test (P < 0.05) in SPSS V. 19.0 for Windows.

3. Results

3.1. Bioenergy crops biomass, cadmium concentration and accumulation

At day 60, the biomass of energy crops was observed to decrease in the order maize > soybean. In both species, the root length and above-ground tissue length were significantly decreased (P < 0.05) in the treatment with cadmium addition (Table 1). Similarly, the biomass measures were significantly lower (P < 0.05) in the presence of cadmium with the exception of the root biomass in soybean. The decrease in the root biomass was lower than that observed for the above-ground tissue (stem + leaf) biomass, and cadmium addition elevated the root-to-above-ground tissue ratio in maize and soybean (Table 1). The TIs of the two energy crops are shown in Table S1 in the supporting materials. Maize and soybean exhibited high TIs for biomass and length, and these values were higher than 63.33 with the exception of the leaf biomass in soybean. The decrease in the two energy crops is strongly affected by the cadmium addition. Furthermore, samples from the same plant clustered together away from the unplanted group, suggesting that bacterial community composition was also affected by bioenergy cropping. This inference was supported by the UPGMA clustering analyses of the 16S rRNA gene fragments (Fig. 2). Based on the PCA and the calculated UPGMA results, the bacterial community composition was primarily affected by cadmium addition and bioenergy cropping.

A total of 31,135 OTUs were found from the 18 samples and were affiliated with 33 phyla, 88 classes, 154 orders, 280 families and 471 genera. Furthermore, among these 31,135 OTUs, a large portion, representing 33.4–45.2% of the total 16s rDNA sequences, were taxonomically affiliated with Proteobacteria (Fig. 2). The OTUs were affiliated with Acidobacteria, Actinobacteria, Gemmatimonadetes, Bacteroidetes, Candidate division TM7, and Chloroflexi, accounting for 10.3–21.7%, 5.9–18.5%, 7.3–12.3%, 5.6–8.5%, 3.5–11.0% and 3.3–5.9% of the total reads, respectively (Fig. 2). Of the other OTUs that were affiliated with Elusimicrobia, SM2F11, BD1 5, Planctomycetes, Candidate division WS3, Deinococcus Thermus, Candidate division BRC1, MVP 21, Candidate division OD1, TM6, Chlamydiales, Candidate division WS6, BHI80 139, Candidate division OP3, Candidate division OP11, Fusobacteria and Lentisphaerae composed a small portion, i.e., a total of 0.9–3.3%, of the total sequence reads (Fig. 2).

Table 1

\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Treatments (mg kg\(^{-1}\)) & Sample number & Length (cm) & Dry weight (g) & \\
& & Root & Above-ground tissues & Root & Stem & Leaf & Root/above-ground tissues \\
\hline
0 & Unplanted & CK1-CK3 & nd & nd & 1.97 ± 0.30a & 1.16 ± 0.20b & 1.55 ± 0.34a & 0.73 ± 0.04a \\
& Maize-planted & M1-M3 & 27.03 ± 1.80a & 37.53 ± 3.56a & 1.16 ± 0.12c & 1.66 ± 0.35a & 1.60 ± 0.15a & 0.29 ± 0.04 cd \\
50 & Soybean-planted & S1-S3 & 17.08 ± 3.47b & 17.99 ± 1.53b & 1.47 ± 0.35b & 0.65 ± 0.16c & 1.12 ± 0.23b & 0.81 ± 0.03b \\
& Unplanted & CK4-CK6 & nd & nd & 0.59 ± 0.13 cd & 1.06 ± 0.22b & 0.88 ± 0.14b & 0.30 ± 0.03c \\
& Maize-planted & M4-M6 & 20.83 ± 1.96a & 32.18 ± 3.35a & 1.47 ± 0.35b & 0.65 ± 0.16c & 1.12 ± 0.23b & 0.81 ± 0.03b \\
& Soybean-planted & S4-S6 & 15.57 ± 2.78b & 14.29 ± 2.83e & 1.47 ± 0.35b & 0.65 ± 0.16c & 1.12 ± 0.23b & 0.81 ± 0.03b \\
\hline
\end{tabular}

\textit{nd}, not determinable. Means within the same column that followed by the same letter are not significantly different at \(P < 0.05\) based on an LSD test.

3.2. MiSeq sequencing and bacterial community composition

MiSeq pyrosequencing was used to research the rhizosphere bacterial community composition of the maize and soybean crops. In total, 590,204 available 16S rDNA sequences were obtained. The sequence information of the samples are presented in Table S2. Implementing a cutoff of 3%, the values of the OTUs, the ACE and Chao1 estimators, and the Simpson and Shannon-Weaver indices did not differ substantially among the unplanted and bioenergy cropping treatments (Table S2). The OTU, ACE and Chao1 estimator and Shannon-Weaver index values were lower in the unplanted treatment than in the bioenergy cropping treatments, whereas the Simpson index value was higher in the unplanted treatment. Bacterial diversity was higher in the bioenergy cropping treatments than in the unplanted treatment.

Bacterial community structure was analyzed using PCA and UPGMA analysis to evaluate the specific effects of cadmium addition and bioenergy cropping. The first and second axes explained 37.45% and 19.06% of the variance, respectively. In total, the two axes explained 56.51% of the cumulative variance (Fig. 1). The 50 mg kg\(^{-1}\) cadmium samples formed a distinct cluster distant from the non-metal-contaminated samples in the PCA data space (Fig. 1), indicating that the rhizosphere bacterial community of the bioenergy crops was strongly affected by the cadmium addition. Furthermore, samples from the same plant clustered together away from the unplanted group, suggesting that bacterial community composition was also affected by bioenergy cropping. This inference was supported by the UPGMA clustering analyses of the 16S rRNA gene fragments (Fig. 2). Based on the PCA and the calculated UPGMA results, the bacterial community composition was primarily affected by cadmium addition and bioenergy cropping.
3.3. Effects of bioenergy cropping changes on the rhizosphere bacterial community in cadmium-contaminated soil

To investigate the effects of bioenergy cropping changes on the rhizosphere bacterial community, the LEfSe was used to identify differentially abundant taxons between bioenergy-planted samples and unplanted controls grown in 50 mg kg\(^{-1}\) cadmium-contaminated soils. The significant differences in community abundance among the samples are shown in the cladogram (Fig. 3). The adonis statistical test further revealed significant differences in the community composition between the unplanted controls and soybean-planted treatments \((P = 0.002)\) and between the unplanted controls and maize-planted treatments \((P = 0.003)\). The taxa with a higher bacterial abundance in the maize- and soybean-planted treatments mainly belonged to eight phyla (Actinobacteria, Proteobacteria, Bacteroidetes, Chloroflexi, Cyano bacteria, Fibrobacteres, Verrucomicrobia and Candidate_division_WS6) and nine phyla (Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Armatimonadetes, Verrucomicrobia, WCHB1_60 and Candidate_division_TM7), respectively, and the phyla Proteobacteria and Actinobacteria were more abundant in both the maize- and soybean-planted treatments. Thirteen genera presented significant differences in the maize-planted treatments: Ter riglobus, Blastococcus, Microbacterium, Arthrobacter, Aeromicrobium, Kribbella, Microlunatus and Patulibacter, more specific taxa within Actinobacteria were detected as biomarkers of maize-planted treatments. Twenty-six and twenty-one genus-level biomarkers for maize- and soybean-planted treatments were from Proteobacteria, mainly from the families Comamonadaceae, Sphingomonadaceae, Xanthomonadaceae, Rhodospirillaceae, Hyphomicrobiaceae, Burkholderiaceae and Rhizobiaceae, and the families Methylophilaceae, Phyllobacteriaceae, Rhizobiaceae, Burkholderiaceae, Alcaligenaceae, Bradyrhizobiaceae, Caulobacteraceae and Acidobacteriaceae, respectively.

3.4. The networks of bacterial communities and their responses to bioenergy (maize and soybean) cropping

Table 2

<table>
<thead>
<tr>
<th>Treatments (mg kg(^{-1}))</th>
<th>Cd concentration (mg kg(^{-1}))</th>
<th>BCF</th>
<th>Cd uptake (μg tissues(^{-1}))</th>
<th>TF</th>
<th>Total Cd uptake (μg pot(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>Stem</td>
<td>Leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize-planted</td>
<td>152.42 ± 14.68a</td>
<td>85.25 ± 3.30a</td>
<td>98.60 ± 13.38a</td>
<td>3.05 ± 0.29a</td>
<td>1.88 ± 0.19a</td>
</tr>
<tr>
<td>Soybean-planted</td>
<td>338.01 ± 49.98b</td>
<td>46.55 ± 8.10b</td>
<td>23.53 ± 0.42a</td>
<td>184.32 ± 22.53a</td>
<td>0.72 ± 0.09a</td>
</tr>
</tbody>
</table>
| Means within the same column that are followed by the same letter are not significantly different at \(P < 0.05\) based on an LSD test.

To identify the effects of bioenergy (maize and soybean) cropping
on microbial interactions in the phytoremediation of cadmium-contaminated soils, three molecular ecological networks were constructed for the unplanted, soybean-planted and maize-planted bacterial communities (Fig. 4). As shown in Table 3, networks with 435, 485 and 502 nodes were constructed from the unplanted, soybean-planted and maize-planted samples, respectively. The network connectivity yielded $R^2$ values of 0.897, 0.831 and 0.818 for the unplanted, soybean-planted and maize-planted samples, respectively. These values conform to the power law, which suggests that these networks displayed scale-free behavior. The average geodesic distance (GD) values for the unplanted, soybean and maize networks were 4.638, 4.122 and 2.866, respectively, which are comparable to those of networks that display small-world behavior. The average clustering coefficient (avgCC) values ranged from 0.238 to 0.337, which are higher than those of random networks (0.008–0.024). The networks constructed from the unplanted, soybean-planted and maize-planted samples yielded modularity values of 0.739, 0.814 and 0.806, respectively. These values are higher than the suggested threshold value of 0.4 for a modular structure, indicating that the three networks were modular (Newman, 2006). Although the soybean-planted and maize-planted networks contained fewer total links and had lower average connectivity (avgK) values relative to the unplanted network, these networks were more complex than the unplanted network with respect to the number of original OTUs, the total number of nodes, average GD and modularity. In addition, the number of nodes in the soybean-planted and maize-planted networks (485 and 502 nodes, respectively) was greater than that in the control network (435 nodes), and the bioenergy cropping network was denser, with a lower average GD. All of these key topological properties suggest that bioenergy cropping altered the co-occurrence patterns of the bacterial communities (Fig. 4 and Fig. S3).

Implementing the threshold values of $Z_i = 2.5$ and $P_i = 0.62$, all of the nodes were divided into four categories. The majority of the nodes (>99%) from the unplanted, soybean-planted and maize-planted networks fell into peripherals (specialists, $Z_i < 2.5$ and $P_i < 0.62$, which indicate nodes with few links that are predominantly to other nodes within their modules) (Fig. 5). Five, four and two nodes belonging to Proteobacteria (Seven nodes, including Devisia sp., Aquicella sp., Cell-vibrio sp., Nocardioides sp., Rhizomicrobiurn and GR-WP33-30 no rank), Acidobacteria (three nodes, including Blastocatella, Nocardioides and no rank), and Bacteroidetes (one node, Flavisolibacter sp.) from the unplanted, soybean-planted and maize-planted networks fell into module hubs (generalists, $Z_i ≥ 2.5$ and $P_i < 0.62$, which are predominantly linked to many species in their own modules) (Table S3). Six nodes fell into connectors (generalists, $Z_i < 2.5$ and $P_i ≥ 0.62$, which are predominantly linked to several modules). One of these six nodes belonged to Ramlibacter sp. (Proteobacteria, OTU2045) from the unplanted network, four belonged to Blastocatella sp. (Acidobacteria, OTU729), Chitinophaga sp. (Bacteroidetes, OTU711), uncultured Erythrobacteraceae (Proteobacteria, OTU2040) and Subgroup 6 No Rank (Acidobacteria, OTU2040) from the soybean-planted network, and one belonged to Gemmatimonas sp. (Gemmatimonadetes, OTU519) from the maize-planted network. Network connectivity is a central factor dictating the nature of a network, and the degree of connectivity can provide an indication of how generalist or specialist an organism is. Generalists will bridge different nodes within their own modules and/or among different modules, whereas specialists will link to only a few nodes. Thus, generalists are considered to be key organisms in the community (Zhou et al., 2011; Deng et al., 2012). Our results suggest that there was no node in the network hub (supergeneralists, $Z_i ≥ 2.5$ and $P_i ≥ 0.62$) category that existed as both a module hub and a connector. Expectedly, more connectors (generalists) existed in the...
bioenergy cropping networks than in the unplanted network, and more module hubs (generalists) were present in the soybean-planted network than in the unplanted network.

4. Discussion

4.1. Phytoremediation of cadmium-contaminated soils by bioenergy crops

Maize and soybean plants were used in the current study because these crops are fast growing, have a large biomass and are good sources for bioenergy production (Shi and Cai, 2009; Sheng et al., 2012; Van Slycken et al., 2013; Pandey et al., 2016). Many indicators, such as root, stem, and leaf biomass and plant growth parameters, can be used to evaluate heavy metal toxicity in plants (Salt et al., 1995; Shi and Cai, 2009). The cadmium sensitivities of the different parameters evaluated in the present study were found to be species-specific. The analyses of plant growth parameters (Table 1) and TI (Table S1) indicated that maize and soybean were tolerant to cadmium. The biomass of above-ground tissues and the heavy metal concentration in above-ground tissues determined phytoremediation efficiency (Ali et al., 2013). The ability to translocate the metal to the above-ground tissues is important for phytoremediation. In the current study, cadmium that was accumulated by the maize and soybean was mainly accumulated in the roots, indicating that these plants had a low ability to translocate cadmium to the above-ground tissues, and their TF values were less than 1 (Table 2). Among the two crops, the cadmium concentrations in the leaf and stem of maize were 98.60 and 85.25 mg kg\(^{-1}\), and the TF value of maize was 0.77. These results suggest that this crop has a greater ability than soybean to translocate cadmium to above-ground tissues. An excellent indicator of the metal accumulation capacity of a plant is its BCF value. With the exception of the above-ground tissue BCF of soybean, the crops included in this study obtained root BCF and above-ground tissues BCF values greater than 1, indicating that they have a high capacity for cadmium phytoremediation.

Table 3

Topological properties of the co-occurrence networks of the bacterial communities in the unplanted, soybean-planted and maize-planted samples and their corresponding random networks.

<table>
<thead>
<tr>
<th>Treatments (mg kg(^{-1}))</th>
<th>Experimental networks</th>
<th>Random networks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of original OTUs</td>
<td>Total nodes</td>
</tr>
<tr>
<td>Unplanted</td>
<td>494</td>
<td>435</td>
</tr>
<tr>
<td>Soybean-planted</td>
<td>620</td>
<td>485</td>
</tr>
<tr>
<td>Maize-planted</td>
<td>625</td>
<td>502</td>
</tr>
</tbody>
</table>

Fig. 4. An overview of the bacterial networks in the unplanted (A), soybean-planted (B) and maize-planted (C) soil samples. Modules (groups of OTUs) with more than five OTUs are shown. The colored circles indicate those OTUs affiliated with particular phyla (color code on the right). The size of the nodes represents the relative abundance of the OTUs. A blue edge indicates a negative interaction between two individual nodes, whereas a red edge indicates a positive interaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
fertilization. They further found that this or maize-planted (blue triangles) network. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. MiSeq-pyrosequencing reveals rhizosphere bacterial community composition

As approximately 99% of microorganisms have never been recovered by standard cultivation techniques, cultivation-independent techniques are necessary for bacterial community composition analysis. For example, using a DGGE analysis of rapeseed rhizosphere bacterial communities in the remediation of cadmium-contaminated soil, Chen et al. (2013) identified seven bacterial phyla. In the current study, the bacterial community phylotypes were analyzed based on MiSeq sequencing data, and the taxonomically classified OTUs were found to be affiliated with 33 phyla, 154 orders, and 471 genera. Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes, and Bacteroidetes were the dominant bacterial phyla in the soil samples. Compared with other molecular methods, such as DGGE, high-throughput sequencing analysis provides much more accurate estimates and information regarding microbial diversity (Zhang et al., 2016). In addition, it can recognize keystone organisms (Banerjee et al., 2016), which can be used to reveal the composition and function of the rhizosphere bacterial community associated with bioenergy crops used for the remediation of heavy metal-contaminated soil. For example, Feng et al. (2015) used pyrosequencing assays and found that *Bacillus asahii* had the strongest influence on the response of bacterial communities to organic manure fertilization. They further found that *Bacillus asahii* was crucial in the development of crop growth and soil fertility. The PCA and the calculated UPGMA results of the present study indicate that the rhizosphere bacterial community was influenced by cadmium addition and bioenergy cropping. The relative abundances of 696 and 608 OTUs increased in response to the planting of soybean and maize, respectively, in cadmium-contaminated soils. The associated phyla included Proteobacteria, Acidobacteria, Gemmatimonadetes, Bacteroidetes and others, and some important genera of plant growth-promoting bacteria were also associated with these OTUs, including *Bacillus, Pseudomonas, Burkholderia, Enterobacter* and others (Rajkumar et al., 2012; Chauhan et al., 2015; Ullah et al., 2015). For example, OTU860 is assigned to the genus *Sphingomonas* and was a dominant and highly changed bacterial species. Chen et al. (2014) isolated the plant-growth-promoting endophyte *Sphingomonas* SaMR12, which increased *Sedum alfredii* biomass and zinc extraction from contaminated soil. Such changes in bacterial abundance, such as that of *Sphingomonas* (OTU860), might play crucial roles in bioenergy crop growth, the tolerance of bioenergy crops to heavy metals, and the phytoremediation of heavy metal-contaminated soils.

4.3. Bacterial networks and their responses to bioenergy cropping

Bioenergy cropping and heavy metal pollution are important factors influencing the soil microbial community, and many studies have quantified the effects of bioenergy cropping on the diversity and function of soil organisms (Ellis et al., 2003; Mao et al., 2013). In the current study, the OTU values, the ACE and Chao1 indicator values and the Shannon-Weaver index values were lower in the unplanted treatment than the bioenergy crop treatments, whereas the Simpson index was higher in the unplanted treatment. Comparative analysis indicated that bioenergy cropping affected the bacterial community. Furthermore, the network analysis of taxonomic co-occurrence patterns offers new insights into keystone populations as well as into significant module memberships in biotic communities and their responses to habitat conditions. For example, Zhou et al. (2011) demonstrated the role of Actinobacteria as a keystone bacteria taxon, the abundance of which was found to be significantly correlated with soil geochemical variables. In this study, five, four and two nodes belonging to the unplanted, soybean-planted and maize-planted networks, respectively, fell into module hubs (generalists) (Table S3). In addition, one, four and one nodes belonging to the unplanted, soybean-planted and maize-planted networks, respectively, were categorized as connectors (generalists), which were highly connected to several modules. Proteobacteria and Acidobacteria were the keystone bacteria taxa connecting different co-expressed OTUs. The results indicate that bioenergy cropping alters the topological roles of individual OTUs and keystone populations and that different species may become keystone populations. To our knowledge, this is the first study to use time network analysis based on MiSeq sequencing to investigate microbial interactions in unplanted soils and soils planted with bioenergy (maize and soybean) crops for the phytoremediation of cadmium-contaminated soils.

5. Conclusions

Two energy crops were used to remediate cadmium-contaminated soils, and the rhizosphere bacterial community composition was studied by MiSeq sequencing. The PCA and the calculated UPGMA results indicated that the bacterial community was influenced by cadmium addition and bioenergy cropping. The molecular ecological network analysis constructed based on MiSeq sequencing indicated that the bioenergy cropping systems increased the complexity of the bacterial community network and altered the topological roles of individual OTUs and keystone populations. Through the reconstruction of networks, this study revealed the effects of bioenergy cropping on microbial interactions in the phytoremediation of cadmium-contaminated soils. This study represents an important step towards an integrated ecosystem-based understanding of the influence of heavy metals on plant-microbe interactions.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant no. 41601332), the Key Research Project of Colleges and Universities of Henan Province Education Department (Grant no. 16A210012) and the Key Scientific and Technological Project of Henan Province (Grant no. 172102110259).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.07.019.
References


