Organic-Inorganic Fertilization Built Higher Stability of Soil And Root Microbial Networks Than Exclusive Mineral Or Organic Fertilization

Luhua Yang  
Ningbo Environment Observation and Research Station

Renhua Sun  
Chinese Academy of Agricultural Sciences Institute of Agricultural Resources and Regional Planning

Jungai Li  
Chinese Academy of Agricultural Sciences Institute of Agricultural Resources and Regional Planning

Limei Zhai  
Chinese Academy of Agricultural Sciences Institute of Agricultural Resources and Regional Planning

Huiling Cui  
Research Centre for Eco-Environmental Sciences Chinese Academy of Sciences

Bingqian Fan  
Chinese Academy of Agricultural Sciences Institute of Agricultural Resources and Regional Planning

Hongyuan Wang (wanghongyuan@caas.cn)  
Institute of agricultural resources and regional planning  https://orcid.org/0000-0002-7313-1509

Hongbin Liu  
Chinese Academy of Agricultural Sciences Institute of Agricultural Resources and Regional Planning

Research

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Abstract

Background

Root microbiome is critical for plant health and performance. Many studies have assessed the impact of agricultural management on soil microbiome. But a comprehensive understanding of how root microbiota is affected by soil types and fertilization is still lacking. It is clear yet whether the stability of root microbiome is affected by fertilization regimes, and whether in the same patterns as soil microbiome.

Methods

We conducted a long-term experiment and investigated the impact of soil type, plant type and fertilization regimes on soil and root bacterial communities using high-throughput sequencing and network analysis.

Results

Our results indicated that microbial network under combined organic-inorganic fertilization had higher stability than exclusive inorganic or organic fertilizer. In addition, fertilization exhibited stronger effects on root microbiome than on soil microbiome. While total nitrogen mainly contributes to the variance of root microbiome, pH and soil organic matter were responsible for the differences of soil microbiome. Bacteroidetes and Firmicutes appeared as important drivers in soil and root microbiome amended with organic fertilizer, whereas Actinobacteria was enriched in the soil microbiome under inorganic fertilizer.

Conclusions

Our results clearly indicated the responsive shifts of soil and root microbiome to different fertilization regimes, and gave hints for developing better fertilization practices and establishing healthy root associated microbiota.

Introduction

Root-associated microbiota play a key role in plant performance and productivity [1], often referred to as the “second genome” of plants [2]. By secreting root exudates, plants selectively recruit microbes in the rhizosphere, establishing resource-rich hotspots distinct from the bulk soil [3–5]. Though as important players in agro-ecosystems, few studies have assessed the impacts of agricultural practices on rhizosphere. Much of previous works focused exclusively on soil microbiome [6, 7], rarely considering both soil and root. How root microbiome is affected by agricultural practices, and whether to the same extent as soil microbiome, still remain elusive.

While management-induced shifts in bulk soil microbiomes affect environmental outcomes, plant-regulated rhizosphere communities are more directly relevant to yield outcomes. This knowledge can contribute to managing rhizosphere interactions that promote both plant productivity and agroecosystem
sustainability [8]. Clearly understanding the responsive shifts of soil and root microbiome to different fertilization regimes is vital for developing better fertilization practices and for further improving soil fertility and function.

To disentangle the complexities of soil and root microbiome, a system-level understanding of community function and structure is needed [9]. In the past decades, network-based approaches have been found useful in unravelling microbe-microbe associations in complex environments, ranging from human guts to oceans and soils [10–13]. By investigating the co-occurrence patterns, network analysis could offer new insights into potential interactions and reveal niche spaces shared by community members [14, 15]. However, previous studies of agricultural management mainly focused on network complexity [16], yet few have considered network stability, which is ecologically important.

Communities are considered more stable if more limited shifts occur in response to environmental perturbation; and are more likely to return to its previous state after the perturbation [17–19]. The gut microbiome, for instance, is often noted for its stability and the stability is considered critical for host health and well-being [20]. In natural systems, the stability of microbial networks decreased with increasing environmental stress [21]. Although presumably linked with community functions, such information about network stability in agro-ecosystems is lacking. The effect of agricultural management, especially fertilization regimes, on microbial networks is poorly understood.

With these ideas in mind, we conducted a large-scale experiment and investigated the impact of soil type, plant type and fertilization regimes on soil and root bacterial communities using amplicon sequencing and network analysis. We specifically asked: (1) Do soil and root microbial communities differ in their responses to fertilization practices? (2) Which microbes are the indicator taxa for specific fertilization regimes? (3) How do fertilization practices impact network stability of soil and root microbial networks respectively?

**Materials And Methods**

**Sample collection**

Samples were collected from 9 sites in China (Fig. 1, Additional file 1). The sampling sites spanned from the Northeast Semi-humid Plain to the Huanghuaihai Semi-humid Plain, representing 3 typical soil types in China, namely black soil, cinnamon soil, and fluvo-aquic soil. The sampling sites were distributed in 3 provinces, i.e. Jilin (black soil), Beijing (cinnamon soil), and Henan (fluvo-aquic soil). In black soil (Northeast Semi-humid Plain), two plant types, spring maize (in open fields) and eggplants (in the greenhouse), were selected. All black soil sites were treated with inorganic N, P and K fertilizers (NPK). In fluvo-aquic soil (Huanghuaihai Semi-humid Plain), the fields were cropped with winter wheat (October to June) and summer maize (June to September) (i.e. wheat maize rotation). All fluvo-aquic soil sites were fertilized with mineral NPK. In cinnamon soil (Huanghuaihai Semi-humid Plain), spring maize, wheat maize rotation, as well as fallow fields were also sampled. For spring maize, five different kinds of fertilization were applied, including inorganic P and K fertilizers without N (PK), inorganic N, P, and K...
fertilizers (NPK), half substitution of the inorganic fertilizer by manure (1/2NPK + 1/2M), and equal substitution of the inorganic fertilizer with manure (M), respectively. For wheat maize rotation, five fertilization regimes were applied, namely no fertilization (CK), NPK, inorganic fertilization plus straw (NPK + S), inorganic fertilization plus 22.5 t ha$^{-1}$ manure (NPK + M), and inorganic fertilization plus 33.75 t ha$^{-1}$ manure (NPK + 1.5M). All the 9 sites were under consistent long-term treatment, from 4 years up to 28 years (Additional file 1).

Soil and root samples were collected two days after plants were harvested. Fields cropped with spring maize were sampled in the early September of 2018. Fields cropped with summer maize or eggplants, as well as the fallow fields were sampled in the later September of 2018. Each treatment in each site has three replicates. In total, 95 samples were collected, including 51 soil samples and 44 root samples (2 agro-climatic area, 3 soil types, 4 plant types, 8 fertilization treatments) (Additional file 2).

Five soil cores (at 0–20 cm depth) were collected in each plot between plant rows and were pooled as one replicate of bulk soil. Each replicate contained around 80 g of bulk soil. About 10 g of each well-mixed bulk soil replicate were put into sterile falcons on ice, transferred to the lab immediately and stored at -80°C until DNA extraction. The rest bulk soil was used for physiochemical analysis.

In each sampled subplot, five plants were selected. Plant roots were taken out from the soil and shackled to remove the loosely-attached bulk soil. The remaining soils that attached to plant roots including that need to be brushed off from plant rhizoplane were sampled and mixed as rhizosphere. The rhizosphere samples were treated in the same way as the bulk soil described above and were stored at -80°C until DNA extraction. The rest rhizosphere was used for physiochemical analysis.

Physiochemical analysis

The samples were sieved at 2 mm and kept at 4°C until analysis. Samples were analyzed for water content, pH, total nitrogen (TN), NO$_3$-N, NH$_4$-N and soil organic matter (SOM). The properties were determined according to the methods described in previous studies [22].

DNA extraction, PCR, library preparation, and sequencing

0.3 g from each soil/rhizosphere sample was used for DNA extraction using Griffiths’ protocol [23]. DNA was amplified using the PCR primer pair 515F and 806R targeting the variable region 4 of 16S rRNA gene [24]. PCR reactions were carried out in 30 µL reactions containing 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, UK), 0.2 µM of forward and reverse primers, about 10 ng template DNA and the remaining volume sterile distilled water. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, followed by 72 °C for 5 min. PCR products were checked on 2% agarose gel. Triplicate PCR products were then purified with GeneJET TM Gel Extraction Kit (Thermo Scientific, US) according to the manufacturer’s instructions. Libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, US) following the manufacturer’s recommendations. The library was
quality-assessed and quantified on the Qubit@ 2.0 Fluorometer (Thermo Scientific, US). All libraries were pooled into equal concentrations. The library was then sequenced on the Ion S5 TM XL platform (Thermo Scientific, US) and single-end reads were generated.

Bioinformatics

The raw sequences were quality-filtered and de-multiplexed using Cutadapt (v1.9.1) [25]. Barcode and primer sequences were thus truncated. Chimeric sequences were screened using UCHIME [26] against the Silva database (v138) [27] and removed. The clean reads were denoised using the unoise3 algorithm [28] implemented in usearch (v11). The denoised sequences, which are the correct biological sequences in the reads, are called "zOTUs" (zero-radius OTU), also referred to as ASVs (Amplicon Sequence Variants) by other authors in DADA2 [29] implemented in Qiime2 [30]. Taxonomy assignment was performed using the RDP training set v16 [31] with the SINTAX taxonomy prediction algorithm [32] implemented in usearch (v11). Reads which were assigned to chloroplast, mitochondria and archaea were filtered out.

Data analysis

All statistical analyses were conducted in R (v4.0.2). A workflow of the analysis steps presented below and the figures generated from each step is given in Fig. S1 (Additional file 3: Fig.S1).

Alpha diversity

Estimates of α-diversity (Shannon index) were calculated at each rarefaction level in usearch. We tested the effects of compartment, soil type, plant type and fertilization in overall samples and in subset samples. The normality of the dataset was checked using Shapiro-Wilk test and the homogeneity of variance across groups was computed using Levene's test. For the two-group comparison, the differences were tested using Student's t test if the dataset is normally distributed, or Wilcoxon test otherwise. For comparison of more than two groups, the differences were tested using one-way ANOVA if the samples have equal variance, or Kruskal-Wallis test otherwise. Tukey's Honest Significant Differences test was carried out for pair-wise comparison using the R package TukeyC [33] if applicable.

Beta diversity

We conducted a general analysis of β-diversity on the bacterial communities with all the samples together and then performed more specific hypothesis testing. For the general analysis, we normalized the filtered OTU sequence counts using the "trimmed means of M" (TMM) method with the BioConductor package edgeR [34] and expressed the normalized counts as relative abundance counts per million (CPM). We then carried out unconstrained principle coordinates analysis (PCoA) on Bray-Curtis dissimilarities to quantify the major variance components of β-diversity. For in-depth analysis, we performed constrained analysis of principal coordinates (CAP). All ordination analyses were performed using the R package phyloseq [35].

The community dissimilarity was tested with permutational analysis of variance (PERMANOVA) and permutational analysis of multivariate dispersions (BETADISP) using the functions adonis and betadisp,
respectively, in the vegan package [36] with $10^4$ permutations. Where applicable, pairwise differences between the groups were assessed with the function pairwise.perm.manova from the package RVAideMemoire [37]. Statistical significance of the CAP was assessed using the permutest function in the vegan package with $10^4$ permutations.

Microbiome network construction and analysis

OTUs with the relative abundance no less than 0.05% in at least one third samples were selected. Co-occurrence networks were constructed using Spearman rank correlations from R package psych [38]. Significant correlations ($r > 0.7$ and FDR adjusted $p < 0.001$) were visualized using Gephi [39] with the Fruchterman-Reingold layout.

We then quantified two network properties that have been associated with stability of ecological communities in perturbation studies: (1) how compartmentalized the network is, and (2) the number and strength of positive/negative correlations, via cohesion [40] and modularity [21] analyses, respectively.

Modularity

Modules (groups of taxa whose abundances are more correlated/anti-correlated with each other than the rest of the community) were identified using the Clauset-Newman-Moore algorithm (greedy_modularity_communities) from the Python package networkx [41]. We then calculated modularity, a measure of whether connections tend to occur within or between modules, using the quality function in the Python package networkx [41]. We calculated one value of modularity for each microbial network under different fertilization regimes.

Large positive modularity values (i.e., close to 1) indicate that more connections occur within, rather than between, modules compared to random chance. Communities with high modularity tend to be more stable, as the impact of losing a taxon is restricted to its own module, thus preventing the effects of that taxon's extinction from propagating to affect the rest of the network.

Cohesion

Cohesion is an abundance-weighted, null model-corrected metric based on pairwise correlations across taxa [40]. We used the author-recommended ‘taxa shuffle’ null model with provided R code to calculate both positive and negative cohesions for each microbial community. The proportion of negative to positive cohesion was calculated as the absolute value of negative : positive cohesion.

By characterizing positive and negative co-occurrences separately, cohesion provides insights into associations among taxa caused by both positive and negative species interactions and/or by both similarity and differences in the niches of microbial taxa [42]. The ratio of absolute value of negative : positive cohesion indicates whether negative interaction or positive interaction dominant in the co-occurrence networks.

Identification of fertilization sensitive OTUs (fsOTUs)
Complementary approaches were adopted to identify the OTUs differ under varied fertilization regimes. We first carried out correlation based indicator species analysis with the R package *indicspecies* [43] to calculate the point-biserial correlation coefficient ($r$) of an OTU’s positive association to one or a combination of fertilization practice. The analysis was conducted with $10^4$ permutations and considered significant at $p < 0.05$. Additionally, we tested for differential OTUs among fertilization regimes using likelihood ratio tests (LRT) with the R package *edgeR* [34]. OTUs differed in abundance among fertilization regimes (false discovery rate (FDR) corrected $p$ value < 0.05) were identified. We then defined OTUs that were confirmed by both indicator species analysis and LRT as fertilization sensitive OTUs (fsOTUs).

**Bipartite networks**

The fsOTUs were visualized using bipartite networks. The networks were constructed using the Fruchterman-Reingold layout with $10^4$ permutations as implemented in the R package *igraph* [44].

**Identification of key drivers in networks**

The soil and root communities under each fertilization regime were combined to construct meta-networks. Four meta-networks were constructed consequently, in accordance with the fertilization regime NPK, M, NPK + M, and NPK + 1.5M.

We used a pair of parameters (i.e., within-module connectivity ($Z_i$) and connectivity among modules ($P_i$)) [45] to describe the topological roles of individual nodes (OTUs). The distribution of nodes in the networks was visualized using R package *ggplot2*. Nodes could be separated into four subcategories based on the values of $Z_i$ and $P_i$ [46]: (i) peripheral nodes ($Z_i \leq 2.5$, $P_i \leq 0.62$), which have only a few links that almost always connect to nodes in their modules; [47] highly linked connector nodes ($Z_i \leq 2.5$, $P_i > 0.62$), which have many modules; (iii) module hubs ($Z_i > 2.5$, $P_i \leq 0.62$), which are highly connected to many nodes in their respective modules (iv) network hubs ($Z_i > 2.5$, $P_i > 0.62$), which act as both module nodes and connection nodes.

We also used the method NetShift [48] to identify important microbial taxa which serve as “drivers” between two networks (https://web.rniapps.net/netshift). This method allows one to quantify the changes in association of a single taxon and compute the relative increase in importance of a node and thereby predict key microbial taxa.

**Results**

*The main drivers of soil and root microbiota*

We conducted bacterial community profiling of 51 soil and 44 root samples from 9 sites with 3 different soil types, 4 plant types and 8 fertilization regimes (Fig. 1). A total of 2 758 622 high-quality sequences was yielded (range 8 165 – 57 939; median 28 701; Additional file 2). In sum, we identified 18 097 bacterial zOTUs (zero radius OTU) across all samples.
The major phyla of the bacterial community were Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, and Firmicutes (Fig. S2). Soil and root, as different microbial habitats, were found inhabited by specific sets of microbes (Fig. 2a). Principal coordinate analysis (PCoA) indicated that microbial communities were clearly separated by soil types (Fig. 2b; Table S1). The discrete outlier in the bacterial communities was consistent with TN (Fig. S3b) and SOM (Fig. S3c). Soils supported higher species richness than roots (Fig. S4 and Table S2).

For in-depth analysis, we employed canonical analysis of principal coordinates (CAP). Soil types could explain 13% of the variance in the soil microbiome and 15% of the root microbiome, both of which were confirmed by pairwise PERMANOVA test (Fig. S5; Table S3). A higher diversity of soil and root microbiome was found in cinnamon soil than in fluvo-aquic soil by the comparison of shannon index. Unexpectedly, no differences were observed in the α-diversity between black and cinnamon soil, though black soil is considered more fertile in general (Fig. S5; Table S4).

Soil and root microbiome were clearly separated by plant types, confirmed by both PCoA plots and PERMOVA tests (Fig. S6; Table S5). However, no statistical differences of α-diversity were found between different plant types (Fig. S7; Table S6).

We further investigated the fertilization impacts on soil and root bacterial communities. Clear differences of beta diversity among fertilization regimes were indicated by CAP and PERMANOVA tests (Fig. S8; Fig. 3). Notably, fertilization explained a higher proportion of variation in root microbiome (74%) than in soil microbiome (53%), indicating a stronger impact of fertilization exerted on root bacterial community.

The impact of fertilization on α-diversity was only observed in root microbiome but not in soil microbiome, regardless with crop types (Fig. S8; Table S7). Not surprisingly, the lowest α-diversity of root microbiome was observed under fertilization regime PK, where N is missing. Unexpectedly, root microbiome without fertilization and fertilized with NPK did not differ in the diversity. Interestingly, the addition of organic materials (straw and manure) significantly lowered the α-diversity. In particular, the addition of straw (NPK + S) showed the lowest α-diversity, much lower than the addition of manure (NPK + M and NPK + 1.5M).

Fertilization sensitive OTUs

To identify OTUs varied in abundance among different fertilization regimes, we employed indicator species analysis based on correlation. We further validated them using likelihood ratio tests implemented in edgeR. Finally, we defined the OTUs supported by both methods as fertilization sensitive OTUs (hereafter: fsOTUs) and summarized them in bipartite networks (Fig. 3; Fig. S9). The patterns were reminiscent of the effects seen in the beta diversity analyses. Each fertilization regime supports a specialized subset of soil and root bacteria. Particularly, we noted that Actinobacteria was enriched in the soil microbiome under inorganic NPK fertilizer. Instead, Acidobacteria and Bacteroidetes largely dominated the root microbiome, and were enriched with the addition of organic fertilizer. Firmicutes was enriched in under organic fertilization as well. Around 28% fsOTUs identified in root belonged to Acidobacteria, while only half of fsOTUs in soil (14%) were assigned to Acidobacteria. As approximation
for an “effect size” of fertilization on microbial communities, the fsOTUs accounted for 9.8% and 14.1% of the total soil and root bacterial OTUs, respectively.

*Network properties under different fertilization regimes*

We constructed co-occurrence networks of bacterial community under each fertilization regime. The overall community taxonomy changed by fertilization practices (Fig. 4). We further quantified the network stability via modularity, a reflection of how compartmentalized the network is, and cohesion, a metric quantifying the degree of community complexity, respectively.

In both soil and root microbiome, combined fertilization leads to the highest modularity, indicating higher community stability (Fig. 5). Organic fertilizer resulted in higher soil network modularity than inorganic fertilizer. However, the opposite trend was observed in the root microbiome, where bacterial networks showed higher modularity with inorganic fertilizer than organic fertilizer.

Similarly, both highest negative and positive cohesion metrics were observed with combined fertilization ($p < 0.01$, ANOVA), indicating higher network stability (Fig. 5). However, no statistical differences of cohesion were found between inorganic and organic fertilization. As to the soil microbiome, the differences among fertilization regimes were only found with negative cohesion but not positive cohesion ($p < 0.05$, ANOVA). As like in the root microbiome, combined fertilization showed higher negative cohesion than inorganic NPK or organic manure alone, but no differences were found between organic and inorganic fertilization.

We further carried out canonical correspondence analysis (CCA) to investigate the environmental variables corresponding with fertilization practices. SOM and pH were found significantly correlated with soil microbiome, while TN was found significantly correlated with root microbiome (Fig. S11).

*Key drivers in network shifting*

The soil and root microbiota under each fertilization regime were combined to construct meta-networks. Consequently, we obtained four meta-networks in accordance with the fertilization regime NPK, M, NPK + M, and NPK + 1.5M. On the basis of their within-module connectivity ($Z_i$) and among-module connectivity ($P_i$), we identified a series of module hubs (nodes highly connected to other members in a module) and connectors (nodes linking different modules), which could be regarded as keystone nodes that play key roles in shaping network structure (Fig. 6). The number of module hubs were highest under combined fertilization and lowest under inorganic fertilization, which is in line with the results of modularity analysis. Under inorganic fertilization, nearly one fourth of the connector OTUs with NPK were assigned to Acidobacteria, which was less abundant in connector OTUs of other networks. Instead, Firmicutes and *Candidatus_Saccharibacteria* became prominent as connectors under organic and combined fertilization, whereas they were absent in the connector OTUs with inorganic fertilizer.

We further explored the potential “driver microbes” in shaping microbial networks under different fertilization regimes using the newly-developed method “NetShift”. A taxon with an altered set of associations (identified by a high Neighbor shift (NESH) score), while being increasingly important for the
whole network (identified by a positive delta betweenness ($\Delta B$) score) is predicted as a “driver”. Accordingly, we selected top 30 taxa of highest NESH score with positive $\Delta B$ values (Fig. 7). In the shift from inorganic (NPK) to organic (M) fertilization, Bacteroidates and Verrumicrobiota stand out as the most prominent drivers, as both of their NESH and $\Delta B$ score are high. In comparison with pure organic fertilization (M), Bacteroidates, Acidobacteria, Firmicutes, BRC1, and Gammaproteobacteria (Pseudomonadales and Xanthomonadales) contributed as important members in driving network changes under combined fertilization (NPK + M). In shift of fertilization regime from NPK + M to NPK + 1.5M, *Turicibacter* and *Bacillus* from Firmicutes were identified as the key drivers with the highest NESH and $\Delta B$ score. Besides, increased number of Proteobacteria, particularly Rhizobiales, were found among the driver taxa.

**Discussion**

In this study, we characterized soil and root microbiome from 3 different soil types, 4 plant types and 8 fertilization regimes in a long-term field experiment. While soil types could largely determine microbial communities, fertilization practices were found as a primary factor in shaping soil and root microbiota under the same soil type. Our results suggested that combined organic-inorganic fertilization built higher stability of both soil and root microbial networks than exclusive inorganic or organic fertilization based on the analysis of network properties.

Network properties have been used to successfully predict the stability of microbial networks [11, 21, 42]. In particular, communities with greater modularity, reduced positive associations among taxa, and greater negative associations among taxa are more stable. Modularity could reflect biological processes such as shared ecological functions among taxa in a module [49–51], spatial compartmentalization [13], or similar niche requirement [52, 53]. High modularity could stabilize communities by restricting the impact of losing a taxon to its own module [54, 55]. Positive cohesion (positive relationships) represent high niche overlap and/or positive interactions between taxa, while negative cohesion (negative relationships) indicate divergent niches and/or negative interactions [40, 42]. It is argued that positive associations can create dependency and mutual downfall [17]. In contrast, negative co-occurrences/interactions could dampen positive feedbacks and thus improve stability [21]. In this study, we found higher modularity and connectivity (i.e. cohesion) as well as a dominance of negative correlations in microbial networks under combined fertilization, indicating that combined fertilization leads to microbial community with higher network stability.

A recent study provided evidence that naturally-occurring microbiome display properties characteristic of unstable communities when under persistent stress [21]. Networks with higher stability are more robust to environmental perturbations [56]. In this sense, our findings may indicate that the microbiota under combined fertilization is more resilient to environmental stresses. In addition, the success of pathogen invasion in the rhizosphere was reported to depend on the network structure of resident bacterial communities [57]. Therefore, the structure and stability of root community are highly important for plant health and fitness. Indeed, our previous results showed combined fertilization resulted higher crop yields.
than exclusive manure application than solely mineral fertilization [22]. The yield increase by combined fertilization was also confirmed in other long-term experiments [58], with enhanced soil nutrient availability, microbial biomass, enzymatic activities and soil nitrogen processes [59]. In a four-decade nutrient fertilization experiment, the application of combined inorganic fertilizers and cow manure led to the most resistant microbial community, which was associated with the lowest relative abundance of potential fungal plant pathogens after 35 years of nutrient fertilization [60]. In brief, our results are in line with the notion that host can benefit from increased microbiome stability.

Interestingly, the influence of fertilization is stronger on root microbiome, but less significant on soil microbiome, indicating compartment-specific responses of bacterial community. Our CAA analysis revealed that TN is the environmental factor responsible for the community variation of root microbiome, whereas pH and SOM explained the soil community differences. While pH is well-known for its decisive role in selecting bacterial community, it seems that C and N factors drive the soil and root microbiome respectively. Studies have shown that the quantity and quality of SOM following N enrichment were linked with soil microbial community and the associated enzyme activities [61]. Organic fertilizer is known to enhance SOM and improve soil fertility [62]. A recent study indicated that the reduced microbial stability in the active layer is associated with carbon loss under alpine permafrost degradation [63]. In line with previous reports, our results indicated that organic fertilization and combined organic-inorganic fertilization increased the microbial stability mainly by the increase of SOM. Root microbiome, however, are affected dynamically affected by both the surrounding edaphic conditions and the host plant. Compared with bulk soil, a higher demand of N is required by plants. The available N that can be assimilated by plants is strongly dependent on root-associated microbial guilds [64]. For instance, arbuscular mycorrhizal fungi were recently shown to be able to transfer N to plants, and this fungal symbiont-mediated N uptake was stimulated by carbon supplied from the host plant [65]. Growing evidence suggests that rhizosphere priming is an important strategy by which plants retrieve organic N [66].

Under mineral fertilization, Actinobacteria was found prominently enriched in soil microbiome. Many members of Actinobacteria were consistently identified as consumers of labile C substrates in stable-isotope probing (SIP) experiments [67]. The selective enrichment of Actinobacteria in the NPK fertilized soil indicated a community more reliant on simple C substrates, less suited to complex C decomposition and, by extension, nutrient mineralization. In the shift from inorganic fertilization to organic fertilization, Bacteroidates and Verrumicrobiota were among the most important drivers in community change. Bacteroidetes are primary degraders of complex carbohydrate-based biomass [68, 69]. Members of Verrucomicrobia were identified by SIP experiments as consumers of more complex C compounds, such as cellulose [70]. Cultured members of these phyla have genomic pathways for the breakdown of complex, plant-derived polysaccharides. Considered together, they may play an important role in decomposing complex organic material, and thereby contribute to the community shift from inorganic to organic fertilization. With extra manure applied (i.e. NPK + 1.5M), a dominance of Firmicutes was observed as the potential drivers. Firmicutes are likely to increase in nutrient-rich conditions [71]. For instance, the increase in Firmicutes in gut microbiota was often correlated with obesity [72]. In our case,
the large dominance of Firmicutes under the fertilization regime NPK + M might be an indication of over-fertilization. Fertilization regimes that relies exclusively based on inorganic inputs may result in root selection of microbial communities more dependent on easily accessed C and disrupt the plant’s ability to select for a prokaryotic community that mineralizes nutrients from existing organic matter.

**Conclusions**

Overall, we found that fertilization regimes had strong impacts on soil and root microbiome. Network analysis with modularity and cohesion indicated that microbial network under combined fertilization had higher stability than inorganic or organic fertilizer alone. In addition, the response of root microbiome to fertilization is stronger than soil microbiome and exhibited different patterns. While TN contributes mostly to the variance of root microbiome, pH and SOM could largely explain the differences in soil microbiome. Bacteroidetes and Firmicutes appeared as important drivers in soil and root microbiome amended with organic fertilizer, while Actinobacteria was enriched in the soil microbiome under inorganic NPK fertilizer alone. Our study imply that combined organic-inorganic fertilization might be a sound practice better than exclusive mineral or organic fertilization. However, the risk of over-fertilization still need to be taken care of.

**Declarations**

**Availability of data and materials**

The clean reads were deposited under the accession number PRJCA004095 in the GSA database ([https://bigd.big.ac.cn/gsa/](https://bigd.big.ac.cn/gsa/)). The scripts were deposited at [https://github.com/yysmile2014/Fertilization](https://github.com/yysmile2014/Fertilization).

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**Author information**

Luhua Yang and Renhua Sun contributed equally to this work.

**Affiliations**
Contributions

HW, HL and LZ conceived the project. JL and BF collected samples and completed the laboratory work. LY and RS completed downstream computational analysis. LY, RS and HW interpreted the results with contribution from HC. All authors contributed to the preparation of the manuscript and approved its final version.

Corresponding authors

Correspondence to Hongyuan Wang or Hongbin Liu

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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Figures
Figure 1

Geographical distributions of the 9 sampling sites in China. The green shaded area represents croplands. The samples were collected in 2 typical agro-climatic area with 3 typical soils in China, namely black soil, cinnamon soil, and fluvo-aquic soil, represented by different colors. The sampling sites were distributed in 3 provinces, Jilin (a), Beijing (b), and Henan (c), which were illustrated in detail in the right panels. The plant types were indicated by different shapes. Fertilization were indicated with I and II in Beijing, and other sites were under NPK treatment.
Figure 2

The main drivers of soil and root microbiome. (a) The plot displayed the abundance patterns of bacteria in soil and root microbiomes. X-axis reports average OTU abundance (as counts per million, CPM), and Y-axis log2-fold change (root relative to soil). Root and soil-specific OTUs were colored in green and brown respectively. Non-differentially abundant OTUs were colored in gray (likelihood ratio test, p < 0.05, FDR corrected). (b) Unconstrained PCoA ordinations of bacteria. Percentage of variation given on each axis refers to the explained fraction of total variation in the community. Symbols refer to the different fertilization treatments. Figure (a) are colored by soil types and compartments.
Figure 3

Co-occurrence networks of soil and root OTUs under each fertilization regime. (a) spring maize (b) summer maize (wheat maize rotation). Each node (circle) represents an OTU. The color of nodes indicate the assigned phyla. The size of the node were proportional to the relative abundance (25:1).
Figure 4

Fertilization induced differences of soil/root microbiome and fertilization sensitive OTUs. (a) CAP analyses were constrained by the factor “fertilization”. The explained fraction of the total variance is indicated above the plots. Percentage of variation given on each axis refers to the explained fraction of total variation. (b) Defining fertilization sensitive bacteria OTUs in soil and root under different fertilization treatments. Bipartite networks display fertilization system specific OTUs in the soil and root.
bacterial communities determined by both methods in the Venn diagram. Circles represent soil bacteria OTUs and triangles represent root bacteria OTUs. OTUs are colored according to their Phylum assignment.

Figure 5

The modularity, absolute value of cohesion and positive cohesion of microbial networks under different fertilization regimes.
Figure 6

The zi-pi plot of the microbial networks under each fertilization regime. Each node represents a OTU. Zi represent within-module connectivity and Pi represent among-module connectivity. Nodes were separated into four subcategories based on the values of Zi and Pi: peripheral nodes (Zi ≤ 2.5, Pi ≤ 0.62), connector nodes (Zi ≤ 2.5, Pi > 0.62), module hubs (Zi > 2.5, Pi ≤ 0.62), and network hubs.
Figure 7

The top 30 taxa of “driver microbes” in microbial networks during the shift of fertilization regime. Each dot represents a taxa in the microbial networks. The X axis denotes the delta between score (∆B), implying the changes of importance of each taxa in the network in comparison with the former network. The size of the dot corresponds to the NESH score, indicating the changes of node associations. Taxa with high NESH score and positive delta between value were predicted as “driver microbes”.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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