Full Length Article

Responses of Ammonia-Oxidizing Archaea to Three Biofertilizers in Organic Soybean Fields

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Abstract

Ammonia-oxidizing archaea (AOA) are one of the main regulators of ammoxidation, which is the primary rate-limiting step of nitrification. However, the biofertilizers' effects on soil AOA are not presently well-understood. Therefore, soil samples treated with three biofertilizers *viz.,* nitrogen-fixing bacterium, phosphate-solubilizing bacterium and *Piriformospora indica*, both individually and mixed (MI) were collected, in order to explore the variation in the AOA composition and diversity using high-throughput sequencing. The results revealed that the available nitrogen content in soil treated with nitrogen-fixing bacterium and MI were significantly higher than that in soil treated with sterile water. In addition, the MI treatment significantly increased the diversity of AOA in soil. The composition of AOA was not different among the treatments at phylum, class, order, or family level. However, this was somewhat different at the genus and species level. *Nitrososphaera* was dominant at the genus level among the different soil samples. The structural similarity of AOA communities was higher in the three soil samples treated with *Piriformospora indica*, phosphate-solubilizing bacterium and sterile water, while the communities in soil treated with nitrogen-fixing bacterium and the mixture of nitrogen-fixing bacterium (MI) were different from that in sterile water. The partial least squares discriminant analysis revealed that the classification models for *P. indica*, sterile water and phosphate-solubilizing bacterium performed well. The correlation network analysis demonstrated that there was a positive correlation between *Candidatus*, *Nitrosotalea* and *Nitrosopumilus*. The phylogenetic analysis of the AOA community revealed that ammonia-oxidizing archaea mostly belonged to Thaumarchaeota, followed by Crenarchaeota. In addition, there were some unknown archaea. © 2021 Friends Science Publishers

Keywords: Ammonia-oxidizing archaea; Biofertilizers; Biodiversity; High-throughput sequencing; Organic soybean soil

Introduction

Nitrification is a crucial aerobic process in the nitrogen cycle on a global scale, which can reduce nitrate leaching and N_2O release in the soil (Gao *et al*. 2018a). It is known that the critical rate-limiting step of nitrification is ammonia oxidization through ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). For a long time, it has been considered that the former is the only participant and the main promoter for ammonia oxidation. The dominant position of AOB was removed when the first related archaea strain, *Nitrosopumilus martimus* SCM1, was isolated from seawater at the Seattle Aquarium in 2005. Since then, ammonia-oxidizing archaea have become a research hotspot, since these are new nitrogen-cycling microorganisms (Könneke *et al*. 2005). It has also been increasingly realized that ammonia-oxidizing archaea are important functional archaea, because these promote ammonia oxidation in the nitrogen cycle in natural environments (Hatzenpichler 2012).

Due to its unique physiology, the archaea group that oxidizes ammonia was proposed as a new phylum, Thaumarchaeota (Wu *et al*. 2019). It widely exists in soils, hot springs, marine sediments, fresh water and other environments, and is even more abundant and more sensitive to environmental changes, when compared to AOB, in soil (Zhou *et al*. 2016). AOA abundance and communities are often correlated to soil properties in agroecosystems. Some studies have shown that pH is the dominant factor that promotes the variation of AOA community (Hu *et al*. 2014; Li *et al*. 2015). Substrate availability, such as the amount of ammonium, is also a key driver for the richness of AOA species (Zhang *et al*. 2015; Norman and Barrett 2016). Soil properties, including soil moisture, temperature, and manure use, may also influence the activity of AOA (Liu *et al*. 2015a; Rudisill *et al*. 2016; Zhang *et al*. 2017). Some studies have also verified that AOA are crucial ammonia-oxidizing prokaryotes under various conditions on land, indicating that it can play more important roles in nitrification, when

compared to the bacterial counterparts (Prosser and Nicol 2012; Zhang *et al*. 2012; Liu *et al*. 2015b). Indeed, some debate remains on this opinion.

Fertilization is an important agricultural practice that is often employed to improve soil nutrition and plant yield. However, it also intensively impacts the AOA community in soil (Liu *et al*. 2019). Some studies showed that long-term field fertilization can prominently change the community structure of AOB, rather than that of archaea, in paddy soil, and that the former were more susceptible to N, when compared to the latter, in a temperate steppe, indicating that AOB are more important (Wu *et al*. 2011; Chen *et al*. 2013). However, some studies revealed that the long-term application of milk vetch raised the abundance of AOA and AOB in red paddy soil. Furthermore, the long-term application of green manures has had larger effects on the AOA community, when compared to the AOB community, in red paddy soil (Aalto *et al*. 2018; Gao *et al*. 2018a, b). In addition, an affinity informs the environmental cooperation between them (Straka *et al*. 2019). These results suggest that AOA plays complex roles in different environments.

Biofertilizers are playing important roles in China's high-efficiency chemical fertilization policy. Nitrogen-fixing bacterium, phosphate-solubilizing bacterium and *Piriformospora indica* are the three kinds of important biofertilizeres, and these have received attention from researchers (Jayathilake *et al*. 2006; Aggani 2013). However, studies on AOA communities are still rare for soils treated with biofertilizeres. In order to determine the effects of these three biofertilizers on its communities, a study on the response of AOA to the three biofertilizers was performed in soils in the Swan Island organic field. These results would provide a theoretical basis for understanding the biodiversity in soils, and reveal the mechanism for accelerating the nitrogen cycle with different biofertilizers.

Materials and Methods

Site description and treatment

The test field was located on Swan Island in Shishou City, Hubei Province, China, where a cotton-soybean rotation was in place. As the old waterway of the Yangtze River, the location was unique due to its water erosion patterns and mud sediments. Five treatments were set for the experiment: (1) sterile H_2O, CK; (2) nitrogen-fixing bacteria, NF; (3) phosphate-solubilizing bacteria, PS; (4) *P. indica*, PI; (5) the mixture of the three strains. MI. NF and PS were provided by our research group, and PI was presented by Professor Kaiwen Ye from Taiwan University. The above bacterial agents were diluted with sterile H_2O before use. The effective number of viable bacteria in the diluted liquid bacteria agent was 5.0×10^8 mL^{-1}. The bacterial agents were sprayed at a distance of 3 cm from the seedlings at 25 days after sowing. Three replicates were performed for each treatment. The area of each replicate was 3×10 m^2 in the

field experiment, and 900 mL of bacterial solution was applied for each replicate. The distance between the blocks was 0.5 m. The soybean sowing quantity was 112 kg ha^{-1}. The experimental blocks were randomly arranged, and the methods of field management were based on local agricultural practices. The soil samples were collected after the soybean was harvested. These soil samples were collected in a plum blossom shape in each block. The 0–30 cm depth soils were collected after the removal of stones and crop residues, and placed in a -20°C soil refrigerator for conservation. The potted experiments were carried out at the Agricultural Environment and Ecological Engineering Laboratory in Yangtze University. The used pot was 25 cm in upper diameter, 20 cm in bottom diameter and 30 cm in height. The soil of the cultivated layer (0–30 cm) in test field was placed into the pot. The soil physical and chemical properties were as follows: organic matter content was 16.57 g kg^{-1}, total nitrogen amount was 1.16 g kg^{-1}, alkali-hydrolyzed nitrogen amount was 53.62 mg kg^{-1}, and soil pH was 7.65. The experimental treatment was the same as the field experiment, and the amount of bacterial agent was 10 mL per pot. When cotyledon was fully developed, two seedlings were preserved per pot. The field experiment and pot experiment were sown on April 20, 2019. The samples were taken at the maturity stage, and the seeds were tested for yield (Fehr *et al*. 1971). The number and dry weight of the nodule under the three biofertilizer treatments in the pot experiment were measured according to Yan and Han report (Yan and Han 2014).

Soil available nitrogen content

The available nitrogen (AN) content in soil from the field experiments was estimated using the Subbiah and Asija method (Subbiah and Asija 1956). A 2 g soil sample was placed in the outer chamber of a clean diffusion dish. The diffusion dish was gently rotated to make the soil sample distribution even. A 2 mL indicator solution of H_3BO_3 was placed into the inner chamber of the diffusion dish, and basic glycerin was smeared on the edge of the outer chamber and covered with ground glass. Then, 10 mL of NaOH solution (1 *M*) was added through a hole on one side of the ground glass, and the diffusion dish was covered tightly and gently rotated to cover all soil samples with lye. Afterwards, the diffusion dish was carefully placed in a constant temperature incubator (40 ± 1°C). After alkaline diffusion for 24 h, the NH_3 in the inner chamber was titrated with 0.025 *M* H_2SO_4 solution. The process was repeated for three times for each sample.

Gene amplification and sequencing of AOA

The total DNA of 0.5 g of soil sample obtained from each field treatment was isolated using a MoBio PowerSoil^{TM} DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Three repeats were performed for each treatment. A

Nanodrop ND-2000 UV–VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 0.8% agarose gel electrophoresis were employed to determine the DNA quantity and quality, respectively. The high-quality DNA was conserved at -80°C in an icebox for further analysis. The highly variable V4 region of the bacterial 16S rRNA gene (about 250 bp) was used for sequencing, and the primer 520F and 802R were selected to use PCR amplification. The PCR conditions were based on those described by Francis *et al.* (2005). The paired-end sequencing of bacterial amplicons was conducted on the Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd. (Shanghai, China).

Quantification of bacterial abundance

The sequencing data were analyzed using QIIME (v1.8.0) pipeline (Caporaso *et al.* 2010). The raw reads were classified into the respective samples, according to the barcodes. After the low-quality sequences (sequence length of < 150 bp, sequence average Phred score of < 20, sequences with ambiguous bases and sequences with mononucleotide repeats of > 8 bp) were filtered out (Chen and Jiang 2014), the clean reads were obtained. The paired-end reads were assembled to use FLASH (Magoc and Salzberg 2011). Rarefaction curves were used to estimate the depth of the sequencing library of each soil type. After chimera detection, the sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity utilized UCLUST (Edgar 2010). A representative sequence was chose from each OTU using default parameters. The OTU classification was performed through BLAST, searching the representative sequences set against the Greengenes database for the best hit (DeSantis *et al.* 2006). Then, each OTU abundance in each sample was analyzed.

Bioinformatics analysis

The QIIME and R packages (v. 3.2.0) were primarily used to analyze the sequence data. The alpha diversity indices (Chao1 richness estimator, ACE metric, and Shannon diversity index) were analyzed. The OTU richness and evenness among the samples were compared using the ranked abundance curves. Principal component analysis (PCA) was conducted in terms of the genus-level compositional profiles. The differences in microbial structure among the samples were assessed using the R package "vegan". A Venn diagram was generated to visualize the shared and unique OTUs among samples using the R package "VennDiagram". The taxa abundances were compared among the samples using Metastats. Partial least squares discriminant analysis (PLS-DA) was also introduced to determine the microbiota changes among the samples using the R package "mixOmics" (Chen *et al.* 2011). The Spearman's rank correlations between the

predominant taxa were calculated in order to perform co-occurrence analysis. Correlations (|RHO| > 0.6 and $P < 0.01$) were visualized as co-occurrence networks using Cytoscape (Shannon *et al.* 2003). In addition, the microbial functions were analyzed using PICRUSt (Langille *et al.* 2013).

Statistical analysis

S.A.S. 8.1 was used to perform the one-way analysis of variance (ANOVA) and correlation analysis, and Duncan's test was used to evaluate the significance ($P < 0.05$) in the ANOVA.

Results

Effects of three biofertilizers on the content of available nitrogen

Compared to CK, the content of available nitrogen (AN) varied to some extent in soils treated with the three biofertilizers (Table 1). The highest AN content (106.98 mg kg^{-1} and 69.81 mg kg^{-1}) occurred in soils treated with nitrogen-fixing bacterium during the pot experiment and field experiment, respectively, while the AN content was the lowest (61.76 mg kg^{-1} and 52.84 mg kg^{-1}) in soils treated with phosphate-solubilizing bacterium during the pot experiment and field experiment, respectively. The content of AN in soils treated with the nitrogen-fixing bacterium significantly increased by 50.31% in the pot experiment and 29.49% in the field experiment, when compared to that in CK ($P < 0.05$). The AN content in soils treated with the solution of phosphate-solubilizing bacterium, *P. indica*, or the mixture of the three biofertilizers increased in the pot experiment and field experiment, when compared to CK, but the differences did not reach a significant level.

Changes in soybean yield and nodules under different biofertilizer treatments

The different biofertilizer treatments had certain effects on soybean yield and its constituent factors (Table 2). The application of MI, NF and PI could significantly increase the soy yield and its components ($P < 0.05$). Among these, MI had the most obvious effect on the increase in soybean yield under the pot or field experiment. Compared with CK, the soy yield increased by 15.00% in the pot experiment and increased by 8.57% in the field experiment under MI treatment. However, the effects of these different treatments on plant height were not significant. The root nodule number varied between 82–116 under the different biofertilizer treatments. The number of soybean root nodules under NF treatment increased most significantly ($P < 0.05$), followed by the MI treatment and PI treatment. The effects of the different biofertilizer treatments on root nodule weight were similar to the number of nodules ($P < 0.05$). The nodule weights/sizes were, as follows: NF > MI > PI >

Table 1: The content of AN in soils treated with three biofertilizers

Treatment	Pot experiment (mg kg^{-1})	Field experiment (mg kg^{-1})
CK	53.43 ± 0.11bc	53.91 ± 0.15bc
NF	106.98 ± 0.32a	69.81 ± 0.36a
PS	61.76 ± 0.52bc	52.84 ± 0.64bc
PI	66.52 ± 0.28bc	57.24 ± 0.45ab
MI	69.08 ± 0.13b	61.08 ± 0.22b

Note: 1) CK: sterile water; 2) NF: nitrogen-fixing bacterium; 3) PS: phosphate solubilizing bacterium; 4) PI: *Piriformospora indica*; 5) MI: a mixture of the three strains. Data were displayed in the form of mean ± SD (n = 3); t tests were used to detect the difference in the content of AN in soils treated with three biofertilizers

Table 2: Changes of soybean yield under biofertilizer treatment

Treatment	Pot experiment (per plant)					Field experiment
	Height (cm)	Pod number	Pod weight (g)	Pod Seed number	Yield (g)	Yield (kg/ha)
CK	46.37 ± 0.92a	7.99 ± 0.08c	1.87 ± 0.07b	28.26 ± 0.24c	5.33 ± 0.05b	2100 ± 10.53b
NF	46.58 ± 1.42a	11.46 ± 0.05a	2.12 ± 0.51a	36.30 ± 0.25a	6.08 ± 0.03a	2250 ± 16.88a
PS	47.16 ± 1.01a	8.02 ± 0.03b	1.91 ± 0.02ab	28.34 ± 0.17b	5.36 ± 0.07b	2130 ± 8.17b
PI	46.99 ± 0.77a	11.12 ± 0.73a	2.08 ± 0.09a	35.22 ± 0.33a	5.94 ± 0.09a	2220 ± 21.08a
MI	48.32 ± 0.65a	11.16 ± 0.27a	2.22 ± 0.01a	36.25 ± 0.47a	6.13 ± 0.03a	2280 ± 17.10a

Note: 1) CK: sterile water; 2) NF: nitrogen-fixing bacterium; 3) PS: phosphate solubilizing bacterium; 4) PI: *Piriformospora indica*; 5) MI: a mixture of the three strains. Data were displayed in the form of mean ± SD (n = 3); t tests were used to detect the difference in the relative indexes in soils treated with three biofertilizers

Table 3: Changes of the number and dry weight of soybean root nodule in the pot experiment

Treatment	Nodule number per plant	Dry weight of nodule per plant
CK	82.29d	188.16d
NF	116.11a	298.27a
PS	85.56d	188.91d
PI	94.78c	243.24c
MI	108.76b	281.67b

Note: 1) CK: sterile water; 2) NF: nitrogen-fixing bacterium; 3) PS: phosphate solubilizing bacterium; 4) PI: *Piriformospora indica*; 5) MI: a mixture of the three strains. Data were displayed in the form of mean ± SD (n = 3); t tests were used to detect the difference in the relative indexes in soils treated with three biofertilizers

PS > CK. Compared to CK, the dry weight of the root nodules in the NF treatment increased by 58.52%. In the MI treatment, the dry weight of the root nodules increased by 49.70%. In the PI treatment, the dry weight of the root nodules increased by 29.27% (Table 3).

Analysis of the valid sequences of ammonia-oxidizing archaea under different treatments

The paired-end sequencing of DNA fragments from the community of ammonia-oxidizing archaea in soils treated with the three biofertilizers was conducted using the Illumina MiSeq platform. After removing the low-quality sequences, the sequence length of each sample was counted (Table 4). The statistics of length distribution of the sequences revealed that the length was distributed between 300 bp and 400 bp (Fig. 1A). The rarefaction curves revealed that the sequencing number exceeded 40,000, and the whole curve tended to be flat, indicating that the sampling was reasonable, and that the sequencing library of each soil sample reached saturation. Thus, this could truly reflect the ammonia-oxidizing archaea groups in the soil samples (Fig. 1B).

Analysis of OTUs and venn graph of ammonia-oxidizing archaea under different treatments

After removing the rare OTUs and OTUs with a abundance value lower than 0.001% of the total sequencing volume of all samples, the remaining OTUs were identified (Table 5).

The number of effective sequences obtained was within the range of 62,681–76,897, and the number of effective OTUs under the NF treatment was the highest under different taxon levels. According to the obtained OTU abundance matrix, the number of OTUs shared among the samples was calculated, and a Venn graph was drawn (Fig. 2A). The results revealed that the shared OUT number among all the treatments was 4,297. The unique OUT number in the CK, NF, PS, PI and MI treatments were 25, 11, 15, 20 and 57, respectively. The unique OTU number in the MI treatment was higher than that in the CK.

Diversity of ammonia-oxidizing archaea

In order to compare the ammonia-oxidizing archaea diversity in different soil samples under different biofertilizer treatments, a random resample was initially made for each sample in the matrix of the OTU abundance at the depth level of the minimum sequencing (90%) to revise the diversity differences generated by the sequencing depth. Then, the QIIME software was used to calculate the chao1, ACE and Shannon diversity indices for each sample (Table 6). There was no difference in the Shannon index in soils among the different bacterial fertilizer treatments and control. Furthermore, the MI treatment had higher Chao1 and ACE indices, when compared to the control, and there was a significant difference from the control. The other treatments had lower Chao1 and ACE index values, when

Table 4: Analysis of the sequencing amount of ammonia-oxidizing archaea in soils treated with different biofertilizers

Treatment	Sequencing amount
NF1	68287
NF2	66110
NF3	62720
PS1	57488
PS2	62677
PS3	67878
PI1	62737
PI2	61262
PI3	75889
MI1	76449
MI2	80373
MI3	73871
CK1	63934
CK2	69970
CK3	71124

Note: NF: Nitrogen-fixing bacterium; PS: phosphate solubilizing bacterium; PI: *Piriformospora indica*; MI: their mixture and CK: sterile water

Table 5: Analysis of OTU classification

Treatment	Sequence number	Phylum	Class	Order	Family	Genus	Species	Unclassified
NF	65705.67	6016.00	5991.33	6006.67	6006.00	3991.00	6033.00	0
PS	62681.00	5909.33	5887.67	5899.67	5898.33	3910.33	5925.67	0
PI	66629.33	5908.67	5869.33	5890.33	5888.33	3852.00	5924.00	0
MI	76897.67	5662.67	5630.67	5647.67	5647.00	3767.33	5678.67	0
CK	68342.67	5931.33	5908.33	5920.67	5919.33	3859.67	5947.67	0

Note: NF: Nitrogen-fixing bacterium; PS: phosphate solubilizing bacterium; PI: *Piriformospora indica*; MI: their mixtur and CK: sterile water
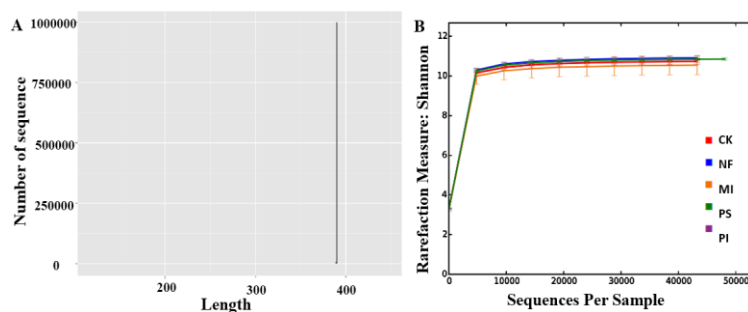
Fig. 1: Assessment of the sequencing data of ammonia-oxidizing archaea in soils treated with different biofertilizers. **A**. Distribution of sequence length, **B**. Rarefaction curves. NF: Nitrogen-fixing bacterium; PS: phosphate-solubilizing bacterium; PI: *Piriformospora indica*; MI: their mixture and CK: sterile water

compared to the control. The PS treatment and PI treatment had significant differences, when compared to the control. In conclusion, MI treatment can significantly increase the AOA diversity in soil.

Taxonomic composition analysis of ammonia-oxidizing archaea

According to the OTU identification, the specific composition of ammonia-oxidizing archaea from each soil sample can be obtained at different classification levels. However, the composition is not different at the phylum, class, order and family level, while there were some differences at the genus and species level (Table 7). Furthermore, *Nitrososphaera* was dominant at the genus level in the different soil samples, and *Nitrosopumilus*, *Candidatus Nitrosotalea* and *Candidatus Nitrosoarchaeum*

were relatively disadvantaged. In addition, other genera were identified (Fig. 2B). The heat map analysis also revealed that the abundance of *Nitrososphaera* in samples treated with NF, PI and MI increased, when compared to that in CK (Fig. 3A).
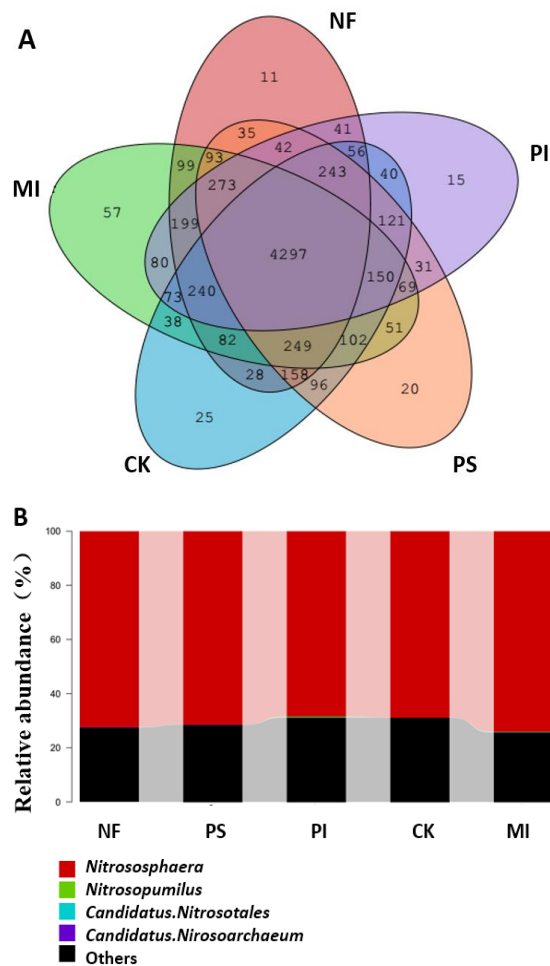
Analysis of the AOA communities under different treatments

Principal component analysis (PCA) was used to o assess differences in the AOA composition in soil under different treatments. The results showed the significant clustering of AOA communities (Fig. 3B). PC1 and PC2 explained 99.48 and 0.45% of the total changes, respectively. NF, PS, PI and MI were obviously distinguished from CK, suggesting that there were significant influences from the different treatments on soil AOA. Furthermore, the community

Table 6: Diversity of ammonia-oxidizing archaea communities in soil treated with different microbial fertilizers

Treatment	Shannon	Chao1	ACE
CK	10.74 ± 0.04a	4930.92 ± 68.66b	5041.49 ± 63.19b
NF	10.92 ± 0.07a	4782.62 ± 37.17bc	4818.54 ± 17.5bc
OP	10.85 ± 0.04a	4688.98 ± 56.54cd	4711.59 ± 78.49cd
PI	10.75 ± 0.11a	4532.53 ± 108.77cd	4504.38 ± 97.6d
MI	10.89 ± 0.04a	5350.93 ± 52.41a	5336.21 ± 74.83a

Note: 1) CK: sterile water; 2) NF: nitrogen-fixing bacterium; 3) PS: phosphate solubilizing bacterium; 4) PI: *Piriformospora indica*; 5) MI: a mixture of the three strains. Data were displayed in the form of mean ± SD (n = 3); t tests were used to detect the difference in the relative indexes in soils treated with three biofertilizers
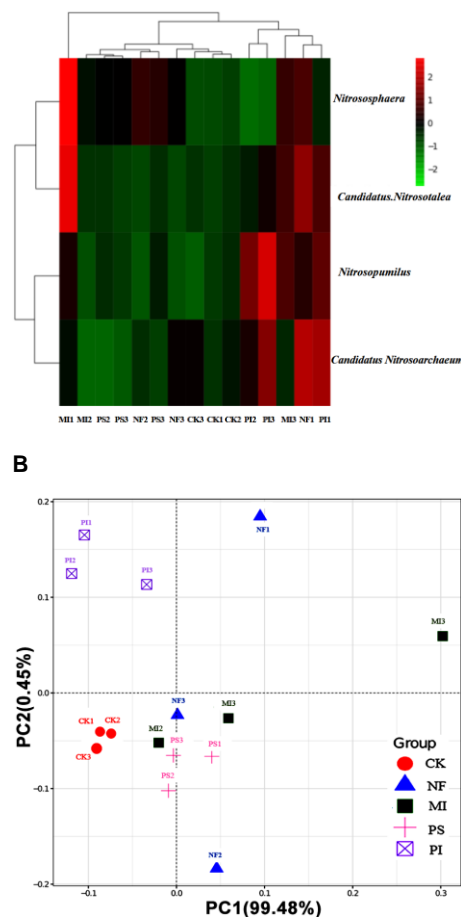
Table 7: Statistical table of microbial groups at each classification level

Sample	Phylum	Class	Order	Family	Genus	Species
NF	2	2	3	3	5	13
NF	2	2	3	3	4	10
NF	2	2	3	3	4	10
PS	2	2	3	3	5	11
PS	2	2	3	3	5	12
PS	2	2	3	3	5	12
PI	2	2	3	3	5	12
PI	2	2	3	3	5	13
PI	2	2	3	3	5	13
MI	3	2	3	3	5	17
MI	2	2	3	3	5	10
MI	2	2	3	3	5	12
CK1	2	2	3	3	5	11
CK2	2	2	3	3	5	11
CK3	2	2	3	3	5	12

Note: NF: Nitrogen-fixing bacterium; PS: phosphate solubilizing bacterium; PI: *Piriformospora indica*; MI: their mixture and CK: sterile water

Fig. 2: Analysis of ammonia-oxidizing archaea groups in soils treated with different biofertilizers. **A**. Venn graph of ammonia-oxidizing archaea groups. **B**. The relative abundance of ammonia-oxidizing archaea at the genus level. NF: Nitrogen-fixing bacterium; PS: phosphate-solubilizing bacterium; PI: *P. indica*; MI: their mixture and CK: sterile water

difference between PI and CK was the largest, while that between PS and CK was the smallest. The community differences for MI or PS were relatively small, when compared to CK. a PLS-DA discrimination model was established according to the species abundance matrix and sample grouping data for AOA using the R software. The

**Fig. 3:** The relative abundance of ammonia-oxidizing archaea in the soil at the genus level. **A**. Heat map of the genus level. **B**. PCA analysis of the structural similarity. NF: Nitrogen-fixing bacterium; PS: phosphate-solubilizing bacterium; PI: *Piriformospora indica*; MI: their mixture and CK: sterile water

results revealed that the classification models for PI, CK and PS performed well, while those for NF and MI did not

B.
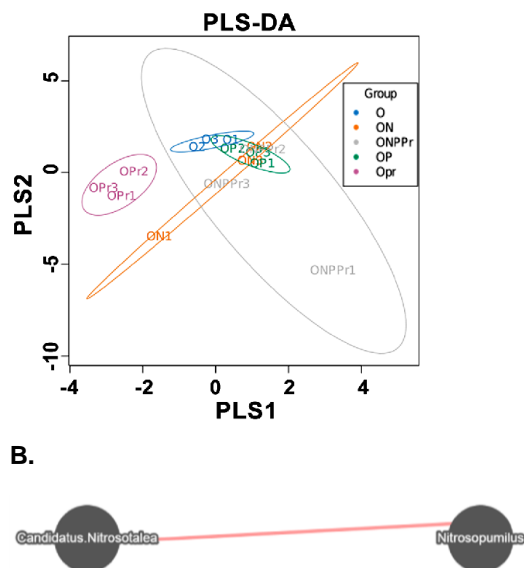
Fig. 4: Comparative analysis of the AOA colony and screening of their key species. **A**. PLS-DA discriminant analysis. **B**. The nodes represent the dominant genera and are marked with different colors. The connections between the nodes indicate the correlation between the two genera; a red line indicates a positive correlation, and a green line indicates a negative correlation. The more connections there are through a node, the more associations the genus has with other members of the community. NF (ON): Nitrogen-fixing bacterium; PS (OP): phosphate-solubilizing bacterium; PI (OPr): *Piriformospora indica*; MI: their mixture (ONPPr) and CK (O): sterile water
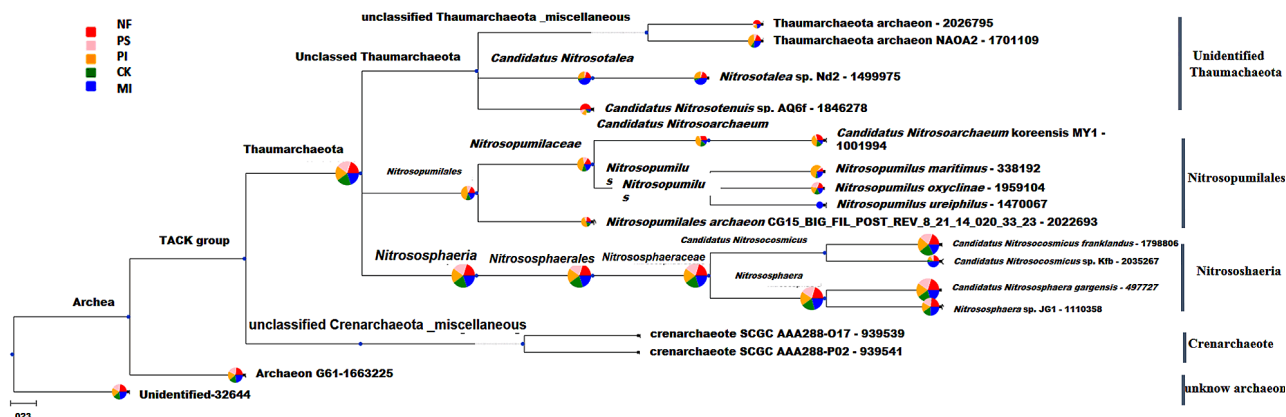
Fig. 5: Map of the evolution and abundance of AOA species. The pie chart of each branch node of the classification showed the abundance of this taxon in each sample. The larger the sector area is, the higher the abundance in the corresponding sample.

perform well (Fig. 4A). Among these, the classification model for PI was the best, and was quite different from CK, suggesting that the community difference between PI and CK is significant.

Analysis of the correlation network among the dominant species of AOA

In terms of the abundance distribution of OTUs or taxa in the different soil samples, AOA groups that were negatively or positively correlated with each other can be found, and an association network of the dominant AOA groups can be built to analyze the ecological significance of the correlation

(Fig. 4B). In the present study, with the use of the Mothur software, the Spearman's correlation coefficient was calculated between the dominant genera, in which the abundance was within the top 50. The correlation network was constructed for the dominant genus with an abundance of rho of > 0.6 and a P-value of < 0.01, and the correlation network was imported into the Cytoscape software for visualization. The results revealed a positive correlation between *Candidatus, Nitrosotalea* and *Nitrosopumilus*, which indicating that there was a cooperative relationship between these. However, the correlation networks between the other dominant genera were relatively low.

Phylogenetic analysis of AOA communities

In the process of OTU identification, the phylogenetic tree that represented the sequence of OTUs was constructed using the Fast Tree tool. Using the MEGAN software, the OUT abundance and composition data in each soil sample were mapped to the classification hierarchy tree provided by the NCBI Taxonomy. The result revealed that ammonia-oxidizing archaea mostly belonged to Thaumarchaeota (including *Nitrosopumilales*, *Nitrososphaeria* and unidentified Thaumarchaeota), followed by Crenarchaeota. In addition, there were some unknown archaea (Fig. 5).

Discussion

Nitrification is an important part of the process of the nitrogen cycle, and is closely correlated to nitrogen availability (Galloway 2008). Ammonia oxidation is the rate-limiting step of nitrification and plays a crucial role in soil nitrogen cycle. Therefore, as the main contributor to ammonia oxidation, AOA in soil has been a hotspot in agricultural ecosystem research. The ammonia-oxidizing archaea in soils treated with different biological fertilizers at the soybean seedling stage were analyzed in the present study. The results showed that the content of available nitrogen in soil was the highest under the treatment of nitrogen-fixing bacterium in the pot experiment and field experiment, and this followed by MI and *P. indica*. The application of MI, NF and PI can significantly increase the soy yield and its components. The number of soybean root nodules under NF treatment increased most significantly, followed by the MI treatment and PI treatment. The effects of the different biofertilizer treatments on root nodule weight were similar to the number of nodules. These results revealed that the biofertilizer has important effects on the nitrogen fixation capacity of soils (Chakraborty and Tribedi 2019). These promote the uptake and utilization of nitrogen by plants, and ultimately promote the formation and development of seeds. So, do these biofertilizers have an effect on the ammonia-oxidizing archaea in the soil?

In fact, the community composition of AOA in soil is influenced to different extent by the applied fertilization treatments. For example, the long-term chemical fertilization had significant effects on the community composition of AOA in soil (He *et al.* 2007). However, organic fertilization had little effects on AOA (Wang *et al.* 2014; Tao *et al.* 2017). In this study, the changes in ammonia-oxidizing archaea in soil under different bacterial fertilizer treatments were analyzed using high-throughput sequencing technology. The results revealed that the number of unique OTUs in the MI treatment was the highest among all treatments. The Chao1 and ACE indices focus on the analysis of community richness (Chao 1984; Chao and Yang 1993). Compared to the control, the single-bacteria fertilizer treatments had little effect on the Chao1 and ACE indices, suggesting the stability of the soil community richness.

However, the mixed bacterial fertilizer greatly influenced the Chao1 and ACE indices. The effects of all kinds of treatments on the Shannon index were evaluated as community evenness, and the results were similar to those for the Chao1 and ACE indices. These results suggest that mixed biofertilizer might better improve the community richness and evenness, when compared to single-bacteria fertilizers. These differences from the applied fertilization treatments could be closed relative to the different soil conditions and fertilezer varieties.

Archaea are important components of complicated microbiomes in the holobiont environment. These interplay closely with viruses, microorganisms and holobionts, such as plants and animals (Moissl–Eichinger *et al.* 2018). Therefore, the changes in microbe species in soil must influence archaea, to some extent. These present results revealed the changes in archaea taxa at the genus and species level. Furthermore, *Nitrososphaera* was dominant at the genus-level in the different soil samples, implying the greater functional importance of the *Nitrososphaera* group, when compared to other archaea, in the present study. The heatmap analysis also revealed that different bacterial fertilizer treatments may influence the soil microbe species. These results are consistent with the report of Wu *et al.* (2019). In addition, the correlation network analysis result revealed that *Candidatus Nitrosotalea* and *Nitrosopumilus* were positively correlated. Therefore, on the basis of this study, it is very sinificant to analyze the interaction between the dominant archaea group and three bio-bacterial fetilizers in soil.

Conclusion

Overall, the NF, PI and MI treatments were not only beneficial for increasing the soybean yield, but also the growth and development of soybean root nodules. the MI treatment significantly increased the diversity of AOA in soil. The composition of AOA was only somewhat different at the genus and species level under different biofertizer treatments. In addition, *Nitrososphaera* was dominant at the genus level among the different soil samples. The communities in soil treated with nitrogen-fixing bacterium and the mixture of nitrogen-fixing bacterium (MI) were different from that in sterile water. These results confirmed that different biofertizers could influencd the diversity of AOA. However, the interactive mechanisms between these biofertilizers and the dominant group of ammonia-oxidizing archaea are not elucidated well at present. Therefore, these research on ammonia-oxidizing archaea in soil needs to be further enhanced in the future in order to lay the foundation for the effective utilization of biofertilizers.

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Author Contributions

Yazhen Yang and Jianqiang Zhu conceived and designed the experiments; Mingke Fang and Yazhen Yang performed the experiments; Meiyan Wu and Jun Hou analyzed the data, Yazhen Yang wrote the manuscript. Jianqiang Zhu revised the manuscript. All authors read and approved the final draft.

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