Effects of Long-Term Fertilizer Practices on Rhizosphere Soil Autotrophic CO₂-Fixing Bacteria under Double Rice Ecosystem in Southern China

Haiming Tang*, Li Wen, Lihong Shi*, Chao Li, Kaikai Cheng, Weiyan Li, and Xiaoping Xiao

Hunan Soil and Fertilizer Institute, Changsha 410125, P. R. China

Introduction

It is generally believed that soil autotrophic bacteria typically exist in agricultural soils [1-3], and play a vital role in helping to regulate the carbon cycle while promoting net uptake of atmospheric carbon dioxide (CO₂) [4, 5]. In the previous studies, results have demonstrated the incorporation of CO₂ into soil microbial biomass at rate of 0.01–0.10 g C/m²/day with soil autotrophic bacteria [4, 6]. Soil autotrophic bacterial composition and diversity were obviously influenced by applying different field practices, such as cropping, fertilization management, crop straw, tillage, etc. [1, 7]. For these reasons, there is a need to explore the impact of different fertilization practices on rhizosphere soil autotrophic bacteria composition and diversity in paddy fields.

Previous results have shown that soil autotrophic microbes were significantly influenced by different fertilization management practices, such as changing soil physical and biogeochemistry characteristics, and soil autotrophic bacterial community and diversity were obviously influenced by applying different field practices, such as cropping, fertilization management, crop straw, tillage, etc. [1, 7]. For these reasons, there is a need to explore the impact of different fertilization practices on rhizosphere soil autotrophic bacteria composition and diversity in paddy fields.

Keywords: Rice, fertilizer treatment, crop residue, soil autotrophic bacteria, paddy field
molecular biology techniques (e.g., RFLP, TRFLP, PLFA and RT–PCR) [7, 12]. Previous studies investigated soil cbbL-carrying bacterial community and diversity by using molecular biology techniques (e.g., RFLP, TRFLP, PLFA and RT–PCR) [6, 7, 14]. According to these approaches, it has been reported that abundances of 16S rRNA and cbbL genes were significantly enhanced by applying straw or biochar treatments, compared to without straw or biochar input treatments [15]. However, rhizosphere soil cbbL-carrying bacterial community and diversity in paddy field in response to different fertilization practices still need further investigation.

Rice is the major grain crop in Asia [16], and double rice cropping (early and late rice planted within a single year) is a common planting system in southern China. The application of organic and inorganic fertilizers is seen as a beneficial practice for enhancing soil physical and chemical properties in paddy fields. Previous studies reported that soil bulk density, soil pH, and soil organic carbon (SOC) content were obviously altered by different fertilization practices [17, 18], which affect soil C sequestration and microbial properties in paddy fields. However, there is also a need to investigate the response of soil C sequestration microbial properties according to different fertilization practices under double rice ecosystem in southern China. Therefore, we set up different fertilizer regimes in paddy fields under double rice ecosystem south China. Our objective in this experiment was as follows: (1) to calculate changes of rhizosphere soil autotrophic bacterial composition and activity by different fertilization practices; (2) to analyze the relationship between soil physiochemical characteristics and soil cbbL-carrying bacterial community, as well as RubisCO activity under double rice ecosystem.

Materials and Methods

Field Experiment Site
The fertilizer experiment was located under a double-cropped rice field near Ningshiang (28°07′ N, 112°18′ E), Hunan Province, in southern China. The related information about climatic characteristics during this field experiment, cropping system, soil chemical properties at plough layer in paddy field at the beginning of fertilizer experiment (1986) was as described by Tang et al. (2018) [18].

Experiment Design
This experiment applied the following fertilizer regime: without any fertilizer input as a control (CK), inorganic fertilizer (MF), straw returning (RF), and organic and inorganic fertilizer (OM). Also utilized was a randomized block design for each fertilizer treatment in paddy field with three replications, and the area of each treatment was 66.7 m² (10.0 × 6.67 m²). We kept the same levels of nitrogen (N), phosphorus pentoxide (P₂O₅) and potassium oxide (K₂O) with OM, RF and MF treatments during the whole growth stage of early rice and late rice, respectively. Other related and more detailed information about the fertilization practices (applied with the kinds and date of fertilizer, total amount of fertilizer) and other field management methods (rice varieties, transplanting density, irrigation pattern) were as described by Tang et al. (2018) [18].

Soil Sample Collection
Rhizosphere soil samples were collected by randomly taking 20 rice plants from each fertilizer treatment, at maturity stage of late rice, in October 2020. Therefore, three composite soil samples with each fertilizer treatment were collected at sampling time, and these soil samples were divided into two parts. One part of the soil sample was stored at 4°C for investigation of soil chemical characteristics; the other part of soil sample was kept at −20°C for molecular biological analysis.

Soil Physiochemical Characteristics Analysis
Soil bulk density at plough layer in paddy field was measured according to the method as introduced by Blake and Hartge (1986) [19]. Soil pH, soil organic carbon, total nitrogen, available phosphorus and available potassium contents were measured based on the method of Kjeldahl (1996) [20]. Soil dissolved organic carbon content was analyzed based on the method as described by Jones and Willett (2006) [21]. Soil microbial biomass carbon content was measured by using the fumigation–extraction method introduced by Wu et al. (1990) [22]. Meanwhile, soil RubisCO activity was measured based on the method of Ezaki et al. (1999) [23].

Soil DNA Extraction and Illumina High-Throughput Sequencing
Soil microbial DNA was collected from soil sample (0.4 g) by using the Quick Soil Isolation Kit (HuaYueYang Biotechnology Co., Ltd., China). Soil cbbL gene was amplified with primers V2r (5′–barcode–GCCCTTC[G/C][A/GCTT][G/G][G/G][A/G][C/G]) and K2f (5′–barcode–ACCA[G/T][CAAGCC][G/C][AAGCT[T/C][G/G][G/G]GG–3′) and K2f (5′–barcode–ACCA[G/T][CAAGCC][G/C][AAGCT[T/C][G/G][G/G]GG–3′) and K2f (5′–barcode–ACCA[G/T][CAAGCC][G/C][AAGCT[T/C][G/G][G/G]GG–3′) [7] by using a thermocycler (ABI Gene Amp 9700. Oxygen Biosciences, USA). Related and more detailed conditions on the polymerase chain reaction (PCR) were as described by Yuan et al. (2013) [7]. Finally, soil PCR products were sent to OE-Biotech Company (China) for Illumina high-throughput sequencing.

Soil Bacterial cbbL and 16S rRNA Genes
Soil bacterial cbbL gene quantification was done by using real-time quantitative PCR with the same primers as introduced above for cbbL, and soil 16S rRNA gene was amplified with primers Eub518 (5′–ATTACCGCGGCTGCTGG–3′) and Eub338 (5′–ACTCTTACGGGAGGCA GCAG–3′) [24]. Related, more detailed conditions about the
PCR for soil cbbL and 16S rRNA genes abundances were as described by Lu et al. (2019) [24]. Soil cbbL gene abundance (copies/g soil) was analyzed according to soil DNA template (5 ng/μl) to each gram of soil (ng/g soil).

High-Throughput Sequencing Data Analysis
Raw fastq files were quality checked by using Trimmomatic (Version 3.29) and merged by using FLASH (v1.2.7) software, respectively, according to the following standards: (i) These reads were interrupted an average quality score < 20 over 50 bp sliding window. (ii) Sequences were merged based on their overlap (> 10 bp), mismatching below 2 bp was allowed during this step. (iii) Sequences in all soil samples were segregated based on the primers and barcodes, and reads containing ambiguous bases were deleted. The peak areas of terminal restriction fragments with difference of ±1 bp were added and regarded as fragments of the cbbL gene operational taxonomic units (OTUs) in the sample. Soil alpha diversity was analyzed by using Chao1, and diversity was analyzed by using Shannon index. OTUs were clustered with 97% sequence identity by using UPARSE software (Version 7.1), and chimeric filtering was conducted at the same time. The classification of each cbbL sequence was annotated by the Nucleotide database in the National Center for Biotechnology Information (NCBI). Family and genus were designated according to amino acid sequence similarity of 90% and 95%, respectively. All high-throughput sequencing data with soil sample were submitted to NCBI Sequence Read Archive (SRA) under SRA accession number SRP142452.

Statistical Analysis
Data for each investigated item in all fertilizer treatments were analyzed by using one-way analysis of variance (ANOVA) (p-value < 0.05). The results for each item were shown as mean ± standard error. Statistical analysis was done with SAS software (Version 9.3) [25]. The relationship between soil physiochemical characteristics and soil microbial composition was analyzed with canonical correspondence analysis (CCA). Meanwhile, soil microbial community change at OTU level was evaluated with principal component analyses (PCA). The correlation test, CCA and PCA analyses were conducted with ‘vegan’ package (Version 3.20).

Results
Abundance of Rhizosphere Soil Bacterial cbbL and 16S rRNA Genes
The results indicated that abundance of rhizosphere soil cbbL gene with all fertilizer treatments (MF, RF, OM and CK) ranged from 0.54 to 2.95 × 10^8 copies/g. Therefore, abundance of rhizosphere soil cbbL gene with MF, RF and OM treatments increased by 2.72, 3.44, and 5.46 times higher than that of CK treatment, respectively. The results also showed that abundance of 16S rRNA gene with MF, RF, OM and CK treatments ranged from 6.69 to 24.38 × 10^9 copies/g. Therefore, abundance of 16S rRNA gene with MF, RF and OM treatments increased by 2.06, 2.93, and 3.64 times higher than that of CK treatment, respectively (Table 1).

| Genes                  | MF       | RF       | OM       | CK       |
|------------------------|----------|----------|----------|----------|
| cbbL abundance (×10^8 copies/g) | 1.47 ± 0.06c | 1.86 ± 0.07b | 2.95 ± 0.10a | 0.54 ± 0.03d |
| Bacterial abundance (×10^9 copies/g) | 13.72 ± 0.68c | 19.63 ± 0.97b | 24.38 ± 1.05a | 6.69 ± 0.33d |
| RubisCO activity (nmol CO₂/g/min) | 4.27 ± 0.16b | 5.17 ± 0.21a | 5.36 ± 0.22a | 3.56 ± 0.16c |

MF: inorganic fertilizer; RF: straw returning; OM: organic and inorganic fertilizer; CK: without any fertilizer input as a control. Values expressed as mean ± standard error. Different lower case letters indicate significant difference among fertilizer treatments at p < 0.05.

There were positive correlations (p < 0.05) between abundance of rhizosphere soil cbbL gene and dissolved organic carbon content, but there were negative correlations (p < 0.05) between abundance of rhizosphere soil cbbL gene and soil bulk density (Table 2). Meanwhile, there were positive correlations between abundance of rhizosphere soil 16S rRNA gene and soil dissolved organic carbon content, and abundance of rhizosphere soil cbbL gene, but the correlations were not significant (p > 0.05).

| Genes                  | MF       | RF       | OM       | CK       |
|------------------------|----------|----------|----------|----------|
| pH                     | 0.172    | -0.365   | -0.306   | -0.385   |
| Available P            | -0.385   | 0.363    | 0.836    | 0.803    |
| Available K            | -0.385   | 0.363    | 0.836    | 0.803    |
| SOC                    | 0.836    | 0.803    | 0.431    |
| DOC                    | 0.363    | 0.803    | 0.431    |
| BD                     | 0.836    | 0.803    | 0.431    |
| MBC                    | 0.431    | 0.431    | 0.431    |
| Abundance of cbbL gene | 0.172    | -0.365   | -0.306   |
| Abundance of 16S rRNA gene | -0.462 | -0.447   | -0.103   |
| RubisCO activity       | -0.116   | -0.407   | 0.539    |
| Abundance of cbbL gene | -0.462   | -0.447   | -0.103   |
| Abundance of 16S rRNA gene | -0.462 | -0.447   | -0.103   |
| RubisCO activity       | -0.116   | -0.407   | 0.539    |

(*) indicated significant difference at 0.05 level. SOC: soil organic C; DOC: dissolved organic C; MBC: microbial biomass C; BD: soil bulk density.
Diversity of Rhizosphere Soil Bacterial *cbbL* and 16S rRNA Genes

The results indicated that rhizosphere soil Chaoh1 and Shannon indices for *cbbL* libraries with MF, RF and OM treatments were enhanced, compared with CK treatment. Compared with CK treatment, Chaoh1 index with OM and RF treatments was significantly increased \((p < 0.05)\). However, there was no significant \((p > 0.05)\) difference in Chaoh1 index between OM and RF treatments (Fig. 1A). This result showed that Shannon index with MF, RF and OM treatments was significantly \((p < 0.05)\) higher than that of CK treatment. However, there was no significant difference \((p > 0.05)\) in Shannon index between MF, RF and OM treatments (Fig. 1B).

Principal component analysis (PCA) result showed that first principal component analysis (PCA1) of soil *cbbL* gene was explained 67.25%, indicating that difference in fertilizer regime was the most vital factor influencing soil *cbbL*-carrying bacteria community (Fig. 2). Our result showed that second principal component analysis (PCA2) of soil *cbbL* gene was explained 20.06%, indicating that rhizosphere was the second important factor influencing soil *cbbL*-carrying bacteria community (Fig. 2).

In the present study, rhizosphere soil *cbbL*-carrying bacteria with all fertilizer treatments mainly belonged to *Actinobacteria* and *Proteobacteria*. The top 11 abundant species of rhizosphere soil *cbbL* with MF, RF, OM and CK treatments were identified. However, there were the same top five abundant species of rhizosphere soil *cbbL* with all fertilizer treatments, including *Variovorax paradoxus*, uncultured proteobacterium, *Ralstonia pickettii*, *Thermononaspora curvata*, and *Azoarcus* sp.KH33C. This result showed that abundance of *V. paradoxus* with MF, RF and CK treatments was significantly decreased \((p < 0.05)\), compared to OM treatment. Compared to RF, OM and CK treatments, abundance of *Sphingomonas* sp.MM and *T. curvata* with MF treatment was significantly increased \((p < 0.05)\). However, the abundance of *Ralstonia pickettii* with MF, OM and CK treatments was significantly lower \((p < 0.05)\) than that of RF treatment (Fig. 3).

**Rhizosphere Soil RubisCO Enzyme Activity**

Rhizosphere soil RubisCO activity with all fertilizer treatments (MF, RF, OM and CK) ranged from 3.56 to 5.36 nmol CO₂/g/min (Table 1). Rhizosphere soil RubisCO activity with MF and CK treatments was significantly lower \((p < 0.05)\) than that of OM and RF treatments (Table 1). Rhizosphere soil RubisCO activity showed negative correlations \((p < 0.05)\) with soil bulk density and microbial biomass carbon content, but positive correlations \((p < 0.05)\) with soil organic carbon content and soil pH.
L (p < 0.05) with abundance of soil *cbbL* gene (Table 2). These results indicated that RF and OM treatments were beneficial fertilizer practices for enhancing rhizosphere soil CO$_2$ fixation activity under more abundance of *cbbL*-carrying bacteria and smaller soil bulk density conditions.

**Discussion**

In previous studies, soil bacterial community were positively affected by organic manure and crop straw practices, resulting in increased soil bacterial abundance and activity, compared with inorganic fertilizer management [3, 7, 10, 15]. In this study, our results demonstrated that rhizosphere soil *cbbL*-carrying bacteria abundance and soil carbon dioxide (CO$_2$) fixation activities were promoted with OM and RF treatments. Meanwhile, our own previous study results indicated that rhizosphere soil organic carbon (SOC) and soil
we found that most soil autotrophic CO$_2$-fixing bacteria belonged to carbon substrate, and soil nutrient content [7, 15]. Beneficial to increase soil bacteria activity under smaller soil bulk density condition with organic manure input practices for increasing growth of rhizosphere soil cbbL-carrying bacteria under higher soil DOC content and lower soil bulk density conditions. Meanwhile, this result showed that rhizosphere soil cbbL gene abundance and soil RubisCO activity with OM treatment were more than that of RF and MF treatments, which indicated that soil quality and fertility in paddy field were enhanced with organic manure practice resulted in soil porosity decrease and soil bulk density increase [15]. Meanwhile, soil gas diffusivity was significantly increased with organic manure input practice, which implied more available CO$_2$ for cbbL-carrying bacteria [10]. As a result, it was found beneficial to increase soil bacteria activity under smaller soil bulk density condition with organic manure input practice, including soil autotrophic bacteria, endoglucanases, cellobiohydrolases, $\beta$-glucosidases, and fungal and actinobacterial cellulolytic and nitrogenase activities [27, 28], all of which enhanced soil ecology condition, carbon substrate, and soil nutrient content [7, 15].

Previous studies results showed that soil cbbL-carrying taxa belonged to Alcaligenes utrophus, Ralstoniaeutropha, Thiobacillus denitrificans, Nitrobacter vulgaris and Nitrobiacter winogradskyi [7, 10]. In the present study, we found that most soil autotrophic CO$_2$-fixing bacteria belonged to Proteobacteria, while some belonged to Actinobacteria. Our results also demonstrated that dominant cbbL gene sequences with all fertilizer practices were related to V. paradoxus, uncultured proteobacterium, R. picketti, T. curvata, and Azoroocus sp. KH33C, which were consistent with the previous results [10, 29]. These abundant species were closely attached to those previously found taxa, with the reason mainly attributed to the fact that there are more available nutrients in organic manure and crop straw soils, compared to without any fertilizer input soil [10]. Therefore, the appearance of organic manure and crop straw related to cbbL-carrying bacteria was induced by the presence of these soil nutrient contents. Shannon index for cbbL libraries with OM and RF treatments showed significantly increased soil autotrophic microorganism diversity, which were promoted by the process of soil autotrophic CO$_2$-fixing. At the same time, these results indicated that relative abundance of V. paradoxus with OM and RF treatments was significantly increased, suggesting that V. paradoxus was usually in wide existence among soil mesophilic bacteria and obligate heterotrophic bacteria under organic manure and crop straw conditions. However, the relative abundances of Mesorhizobium australicum, Sphingomonas sp.MM and T. curvata in rhizosphere soil with inorganic fertilizer practice were increased, and these patterns were also reported in the previous study [10].

The present study demonstrated that rhizosphere soil cbbL gene abundance was positively correlated with soil chemical characteristics (soil pH, SOC, DOC, MBC, soil total N, soil available P, soil available K contents), while being negatively correlated with soil bulk density. Some results found that soil pH and other soil physicochemical characteristics play a vital role in limiting or co-limiting soil autotrophic bacteria growth [15]. Our results also suggested that soil pH and soil physicochemical characteristics provide more available nutrient for soil microbes to multiply [6, 30], suggesting that growth of soil autotrophic microbes was significantly associated with those soil physicochemical properties. These results were in accordance with Selesi et al. (2007) [31], who found that copy numbers of cbbL gene were positively correlated with soil DOC content and negatively correlated with soil bulk density. The observed correlations were related to the activities of soil autotrophic CO$_2$-fixing bacteria and cellulolytic microbes being stimulated under soil DOC content conditions [27, 32]. Meanwhile, the CCA result also found that soil bulk density, MBC content, abundance of cbbL, and 16S rRNA genes were vital triggers for changing soil cbbL-carrying bacterial community (Fig. 4), indicating that it was beneficial for promoting soil autotrophic CO$_2$ fixation under a lower soil bulk density environment [33]. Therefore, our results demonstrated that lower soil bulk density, more soil pH, DOC, SOC, and MBC contents were the important factors influencing rhizosphere soil autotrophic microorganism community.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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