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Pyrosequencing analysis of bacterial community diversity in long-term fertilized paddy field soil

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ABSTRACT

Analysis of soil bacterial community and its diversity in conditions of intensive fertilization using high-throughput sequencing in paddy field soil has to date, not been extensive. Using 454 pyrosequencing of 16S rRNA genes, the current study investigated how bacterial succession changed under seven different fertilizer regimes (NP, NK, PK, NPK, compost, NPK + compost and unfertilized) in a 45-year old paddy field trial. Of the selected treatment variables the application of compost best enhanced soil fertility. Unexpectedly, long-term fertilization had no significant effects on soil microbial structure in paddy soils. The bacterial communities were dominated by *Proteobacteria* and *Chloroflexi*. *Actinobacteria* and *Firmicutes* were substantially abundant in the compost and NPK + compost treatments. Our findings highlight the fact that organic fertilizer amendment activates diverse groups of Gram-positive microorganisms when compared to conventionally used chemical fertilizers. Abundance of *Rhizobiales* that directly influences rice growth through symbiosis or indirectly through nutrient cycling, and *Methylococcales* that combat greenhouse gas (methane) emissions, were high in treatments that received compost, in comparison to inorganic fertilizer amended and unfertilized treatments. Consequently, the application of long-term organic fertilizer has a reasonable and beneficial impact on the bacterial community inhabiting the soil, and can lead to a good crop yield.

1. Introduction

Understanding soil microbial ecology is very important for sustainable agriculture. Maintenance of microbial diversity and composition directly influences the soil ecosystem, nutrient cycling and crop production. Anthropogenic intervention for the management and treatment of agricultural soils, notably fertilizer applications provides excess nutrients and organic matter to soil microorganisms, affecting soil microbial abundance, activity, community structure and metabolism, and thus short-term biogeochemical processes (Zhong et al., 2010). According to several field reports (Ros et al., 2006; Zhong et al., 2010; Sun et al., 2014) both inorganic and organic fertilizer applications increase microbial biomass and diversity. Ros et al. (2006) in their study indicated that employing composts combined with inorganic fertilizers (NPK) brings benefits in terms of better agricultural yield, soil protection, biodiversity (bacteria) and C sequestration. Zhong et al. (2010) found that bacterial and fungal community structure and diversity were significantly influenced by long-term fertilization regimes. In particular, Gram-positive bacteria were sensitive to organic manure, whilst soil fungi were sensitive to inorganic fertilizers. Sun et al.'s (2014) experimental results suggested that 10% compost treatment resulted in significantly higher soil enzyme activity and a more diverse bacterial community composition. However, Sarathchandra et al. (2001) did not witness any change in the biological properties in pasture soil following the application of inorganic fertilizer. In the long-term these changes are believed to reverse due to the effects of time (both either increase or decrease in microbes might occur). Hence many studies (Ahn et al., 2012; Chaudhry et al., 2012; Cui et al., 2012) have been conducted to understand the long-term effects of fertilizer applications on the soil bacterial communities located in field soils.

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Rice is one of the world's most important food crops. In 2015, global rice production accounted for around 743 million tons and 95% of which were harvested in Asia (FAO, 2015). This year South Korea has produced a total of 4 million tons (Statistics Korea, 2015) and it has long been the staple food for the nation's population. Continuous cropping over the centuries, however, has resulted in nutrient deprivation, and subsequently much effort has been made since the 1960s to replenish the soil nutrients through long-term fertilization. In 2012, Cui and co-workers showed that a long-term supply of fertilizers (inorganic) alters the bacterial community's structure and functions in rice fields. Using terminal restriction fragment length polymorphisms and 454 pyrosequencing of 16S rRNA genes, Cui et al. (2012) confirmed that soil development and bacterial succession occurred, which in turn was associated with soil's physico-chemical changes over time. Ahn et al. (2012) confirmed the same trend, stating that the abundance and diversity of microbes in rice fields that influences both crop growth and greenhouse gas emissions are not well documented.

Other studies (Watanabe et al., 2010; Wu et al., 2011) have attempted to understand the long-term effects of inorganic and organic fertilizers on the soil bacterial communities in rice fields. Although these analyses have produced useful information on the overall change in soil physico-chemical and biological properties via long-term fertilizer application, the techniques employed for bacterial community analyses (PCR amplification and subsequent sequence analysis of clone libraries, DGGE and terminal restriction fragment length polymorphism/T-RFLP) were insufficient for surveying the full extent of microbial richness and diversity. For instance, sequence analysis of clone libraries provides a snapshot of only the predominant bacterial communities, but not the phylogenetic groups which are not very abundant, so that some of the bacteria with important ecosystem functions have been missed. This has led to invalid assumptions. On the other hand, genetic profiling techniques such as T-RFLP and DGGE have a throughput capability and make it possible to unravel differences in community structure. However, they are limited only to assessing the level of microbial diversity (Kuppusamy et al., 2016). To more deeply investigate the composition and diversity of bacterial communities in soils, pyrosequencing analysis of 16S rRNA genes has been recently employed (Yergeau et al., 2012). However, only limited pyrosequencing studies have been conducted on long-term fertilized paddy soils. Hence, the present study undertook 454 pyrosequencing analysis in a long-term fertilized paddy field soil, the objective being to: firstly, examine the composition and diversity of bacterial communities; and secondly, identify the differential effects of organic and inorganic fertilizer application.

2. Materials and methods

2.1. Experimental site, plots and design

Experiments were conducted at the Department of Functional Cereal Crop Research Farm, Miryang, South Korea (latitude, 36°36′N; longitude 128°45′E; elevation, 12 m). The soil at this site is classified as a fine silty mixed mesic Typic Haplaquepts. The experimental plots (10 m × 10 m each) were arranged in a randomized manner consisting of three replicates. The following seven different fertilization treatments were selected for this study: inorganic N and P fertilizers (NP); inorganic N and K fertilizers (NK); inorganic P and K fertilizers (PK); combination of inorganic fertilizers (NPK); organic fertilizer (compost); combination of both organic and inorganic fertilizers (NPK + compost); and no fertilizer (control). Inorganic fertilizers include urea, super phosphate and potassium chloride as the sources of N, P and K, respectively. Organic fertilizer used was straw compost (431, 19.8, 5.2 and 29.1 g/kg of total C, N, P and K, respectively) mixed with cattle manure and composted outdoors for more than 6 months. The long-term fertilization experiment started in 1967 and rice was cultivated as a single crop. Plots with inorganic fertilizer input were amended with 120, 80 and 80 kg/ha N, P and K, respectively, during 1967–1972. From 1973 onwards, those plots were treated with 150, 100 and 100 kg/ha N, P and K, respectively. In the case of organic fertilizer input, each plot was treated with 10 Mg/ha of compost annually.

2.2. Soil sampling and chemical analysis

Three sub-samples (0–15 cm depth) were collected during April 2012 from each of the three replicates before the application of fertilizer for the next cropping season. Sub-samples were pooled to make composite samples at room temperature, pulverized, sieved through a 2 mm sieve and stored at −80 °C until they were subjected to further molecular analyses. Chemical properties of soil samples were analyzed as follows: pH (1:5 water extraction), organic matter (Tyurin method) (Heczko et al., 2011), total N (Kjeldahl method), available P_2O_5 (Lancaster method) (NIAST, 2000), exchangeable cations – Ca^{2+}, Mg^{2+} and K^+ (1 M NH_4-acetate pH 7.0, ICP-MS, PerkinElmer Optima, US) (RDA, 1988).

2.3. DNA extraction, PCR and pyrosequencing

Genomic DNA was extracted using the FastDNA SPIN kit (MP Biomedicals, South Korea) as per the manufacturer's instructions. The extracted DNA was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, US). Bacterial 16S rRNA genes were amplified using primers V1-9F (5′-X-AC-GAGTTTGATCMTGGCTCAG-3′) and V3-541R (5′-X-AC-WTTACCGCGGCTGCTT-3′), where X denotes a 7–11 nucleotide long barcode followed by a common linker AC (Chun et al., 2010). Each PCR mixture (50 μl) contained 1 x PCR buffer, 0.2 mM of dNTPs, 400 μM of each primer, 1 mg/ml of bovine serum albumin (Sigma-Aldrich, USA), 1.25 U of *taq* polymerase (Roche, Germany) and 1 μl of 1/10 diluted DNA template. The PCR reactions were executed using the following program: initial denaturation of 94 °C for 5 min; 10 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 90 s (the annealing temperature was reduced by 0.5 °C per cycle from the preceding cycle), and 20 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s. The PCR products were purified with QIAquick Gel Extraction kit (QIAGEN, Germany). Pyrosequencing was done with 454 GS FLX Titanium (454 Life Science, South Korea) either at ChunLab, Inc. (Seoul, South Korea) or National Instrumentation Center for Environmental Management (Seoul, South Korea) according to the manufacturer's instructions.

2.4. Pyrosequencing data and statistical analyses

Post-run analysis of the 16S rRNA gene amplicon sequences was performed using Mothur software (Schloss et al., 2009). After the barcodes and primers were trimmed, sequences having unidentified bases (Ns) or being shorter than 200 bp were discarded. The quality-filtered reads were denoised. Sequences were classified using the Silva database (Pruesse et al., 2007) at a bootstrap value of 80%. Mothur was used to conduct refraction analysis, construct the distance matrix and assign sequences to operational taxonomic units (OTUs, 97% similarity). Clustering of sequence data was done using CD-HIT (Fu et al., 2012). Species richness and diversity were estimated by the abundance-based coverage estimator (Ace), Chao 1 estimator (Chao 1), non-parametric Shannon diversity index (Np-Shannon) and Shannon diversity index (Shannon). Cluster analysis was conducted using Bray–Curtis metrics. Statistical analysis was carried out using SAS software version 9.1 (SAS