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Long-term effects of imbalanced fertilization on the composition and diversity of soil bacterial community

Jinu Eo[a,*], Kee-Choon Park[b]

[a] Climate change and Agro-ecology Division, National Institute of Agricultural Sciences, RDA, Wanju, 55365, Republic of Korea
[b] National Institute of Horticultural & Herbal Science, RDA, Republic of Korea

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ABSTRACT

Repeated fertilization in a monoculture system causes a nutrient imbalance, disturbing the soil bacterial community. To investigate the long-term effects of imbalanced fertilization, we analysed soils under pepper (*Capsicum annum* L.) cultivation for 18 years. The soil was treated with one of five regimens: untreated control, NPK, PK, NP, and NK. Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, and Firmicutes were the dominant phyla. Pyrosequencing revealed that diversity indices were not significantly influenced by different fertilization treatments. We found that the effect of fertilization varied at the genus level even within the same phylum, demonstrating divergent ecological responses of bacterial groups to disturbance at low taxonomic levels. The percentage abundances of Acidobacteria and Nitrospira were decreased in all fertilized plots. The percentages of genus *Sphingomonas* and JF421159 were increased only with NPK treatment, and our results suggested that bacterial community composition is altered by fertilization lacking one of three components. The percentages of Chloroflexi and Planctomycetes were decreased in the plots receiving N, while the percentage of candidate division TM7 showed an increase with N. The percentages of these genera were correlated with soil chemical parameters such as nitrate content and pH. Our study suggests that N promotes some bacterial groups, which are involved in the degradation of materials; however, it has an overall negative impact on the percentages of some other groups due to changes in the soil chemical properties. The percentages of *Koribacter* and *Pseudolabrys* were increased with NK treatment, likely due to a lack of P. Our results implied that N and P exert substantial effect on specific bacterial groups; in contrast, K has minimal effect. We suggested that an imbalanced N-P-K ratio caused by repeated fertilization could be a driving force altering the bacterial community composition not its diversity.

1. Introduction

Soil bacteria are fundamental to the productivity of soil. They play pivotal roles in soil chemical and physical properties, including nutrient cycling, decomposition of organic matter, and formation of soil aggregates (Six et al., 2004). Some bacteria are responsible for supplying nutrients and plant growth-promoting materials (Franche et al., 2009; Esitken et al., 2010). The addition of inorganic materials and subsequent changes in bacterial community composition alter these processes (Brant et al., 2006; Ramirez et al., 2012).

The nutrient budget in soil is determined by the input and output of materials, and nutrient imbalance is a worldwide phenomenon (Vitousek et al., 2009). The added nutrients are mainly composed of nitrogen (N), phosphorus (P), and potassium (K), and are largely dependent on inorganic fertilizers (Stewart et al., 2005). The application ratio of three components in fertilizer differs according to crop and soil type. Imbalance in N-P-K the ratio in soil can be exacerbated by repeated fertilization in monoculture systems, and it intensifies disturbances to the soil ecosystem. Soils under repeated inorganic fertilization experience changes in abundance, community structure, and function of soil bacteria (Ramirez et al., 2010a; Kamaa et al., 2011).

Inorganic fertilization disturbs soil ecosystems through nutrient addition and changes in soil properties, both of which affect plant growth. Some bacteria are involved in the use and degradation of added fertilizers (Tian et al., 2004). Changes in chemical properties that accompany fertilization can influence the diversity and composition of bacterial communities (Lauber et al., 2008; Zhang et al., 2013). Increased plant growth accelerates the

* Corresponding author.
E-mail address: eojiny@korea.kr (J. Eo).

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accumulation of organic matter via the input of rhizodeposits and plant litter. Hence, there is a need to investigate these factors together to determine the impact of imbalanced fertilization.

Previous studies have emphasized the importance of long-term field experiments (Marschner et al., 2003; Chakraborty et al., 2011; Pan et al., 2014). The effects of inorganic fertilizers can differ according to its application period, and it may take a long time for a soil ecosystem to reach an equilibrium state (Fauci and Dick 1994; Moscatelli et al., 2008). To investigate the long-term effects of inorganic fertilization, we performed this study using an 18-year field experiment. Many studies have addressed the individual effects of N, P, and K on microbial communities (Bates et al., 2010; Joa et al., 2014; Ramirez et al., 2012); however, little information is available on the effect of the ratio of these three components. We hypothesized that imbalanced fertilization would have negative effects on soil bacterial diversity with changes in their community composition by disturbing the soil nutrient balance. To test this hypothesis, we manipulated five treatments (NPK, PK, NP, NK and untreated soil) by creating imbalanced N:P:K ratio conditions in the soil. Changes in the composition and diversity of bacterial communities were compared using pyrosequencing analysis.

2. Materials and methods

2.1. Experimental site

The study was performed at an experimental field located in Suwon, Republic of Korea (37°15′51″N, 126°58′25″E). The experimental site was in a typical temperate zone, with an annual mean temperature of 12.0 °C and an annual precipitation of 1312.3 mm. The treatments were initiated in 1994 to investigate the long-term effects of inorganic fertilization. Concrete blocks (W 3.0 m × L 3.6 m × D 1.0 m) were constructed underground, and silt loam soil (sand 68.0%, silt 26.0%, and clay 6.0%) was placed in them. A randomized block design with three replicates was set up with the same 5 treatments, namely, NPK, PK, NK, NP, and an untreated control. Fertilization was conducted according to Lee and Choi (1977). N was applied as urea at 190 kg ha^{-1}, P was applied as fused superphosphate at 101.6 kg ha^{-1}, and K was applied as potassium sulphate at 166.7 kg ha^{-1}. For N and K, 55% was applied as basal fertilization and 45% was applied as additional fertilization. P was all applied as basal fertilization. Compost was applied at 20 Mg ha^{-1} to all plots; the chemical composition of the compost was as follows: N (0.95%), P_2O_5 (0.55%), K_2O (1.17%), CaO (0.58%), and MgO (0.45%). The basal chemical fertilizer and compost were broadcast on the surface and tilled into the soil before planting. Additional fertilization was performed by applying 15% of N and K fertilizer, three times (total of 45%) to spaces between plants, near the end of May to late in June. To prepare for cultivation, furrows were made at 75-cm intervals, and the soil surface of the furrow was mulched with a black plastic film. Pepper (*Capsicum annuum* cv. Gumtop) seedlings grown in commercial soil were transplanted at a distance of 75 cm × 45 cm in early May.

2.2. Soil sampling and chemical analysis

We collected 4–5 soil samples at a depth of 0–10 cm from each plot by using a soil sampler and homogenized them on September 7 in 2011. For pyrosequencing analysis, the soil samples were freeze-dried and screened through a 2-mm mesh sieve. Soil pH in the saturation extract (1:5 w/v) was measured using a pH meter. The C/N ratio was determined in a C/N analyser (Vario Max CN; Elementar, Germany). The analysis of nitrate content was performed using an autoanalyser (Auto-Analyser 3; Bran Luebbe, Germany). The exchangeable cations in the soil were analysed using inductively coupled plasma (ICP) analysis (Integra XMP; GBC, Australia).

2.3. DNA extraction and PCR

Genomic DNA was extracted from 0.5 g of freeze-dried soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, USA). DNA was amplified using PCR (PTC-200, Peltier Thermal Cyclerm, PharmaTech & GeneAmp PCR System 9700, Applied Biosystems). The V1-3 region of the bacterial 16 S rRNA gene was amplified. The forward primer was V1–9 F (5′-CCTATCCCCTGTGTGCCTTGG-CAGTC-TCAG-AC-AGTTTGATCMTGGCT CAG-3′) and the reverse primer was V3–541 R (5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGAGCTG-AC-WTTACCGCGGCTGCTGG-3′). Each 50 μL PCR mixture contained 1 μL (1 : 10 dilution) community DNA, 5 μL PCR buffer (1X), 1 μL of each deoxyribonucleoside triphosphate (dNTP) (100 mM), 2 μL of forward and reverse primers (20 pM), and 0.25 μL Taq polymerase (5 U/μL). The amplification cycle consisted of an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, and 90 s at 72 °C. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany).

2.4. Pyrosequencing

Pyrosequencing was performed according to Chun et al. (2010). An aliquot (0.5 μg) of DNA obtained after PCR was used for pyrosequencing. The sequences were determined using a 454 GS FLX Titanium Sequencing system (Roche, Branford, CT, USA). Sequencing reads shorter than 300 bp in length or containing two or more unresolved nucleotides were removed. AmpliconNoise pipeline was used to correct pyrosequencing errors. OTU clustering was based on CD-HIT's algorithm with a 97% cut-off, and classification was conducted using Ez Taxon database (www.ezbiocloud.net). Taxonomic classification was determined using a criterion of ≥94% identity for genus and ≥75% identity for phylum.

2.5. Data analysis

The differences among the treatments were compared by means of a least significant difference (LSD) test when significant differences existed in the ANOVA. Spearman's rank correlation coefficient was used to assess statistical dependence among soil parameters and OTUs assigned to a phylum or genus. These tests were performed using SAS v9.1 (SAS Institute Inc., Cary, NC, USA). To estimate alpha diversity, Shannon and Simpson indices were calculated using the algorithm of Mothur package (www.mothur.org). Similarity between bacterial communities was determined using the Fast Unifrac online tool for principal coordinate analysis (PCoA). All analyses of pyrosequencing results were conducted using CLcommunity ver. 3.31 (ChunLab Inc., Korea).

3. Results

3.1. Soil properties and pepper growth

The analysis of chemical properties of the soil showed apparent effects of fertilization according to the combination of inorganic fertilizers (Table 1). The concentration of nitrate and available phosphate increased with the use of fertilizers containing N and P, respectively. In particular, pH was lowered and EC increased in the plots treated with N. The fertilization treatment containing N increased the shoot length of peppers (Fig. 1). In the analysis of serial correlation among parameters, several correlationships were found. Organic matter content, nitrate concentration, and shoot length were all correlated with each other ($P < 0.05$). However,