

Influence of Long-term Fertilization on AM Fungi Community Structures in a Brown Soil

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Abstract. This study aims to explore changes of community structures of AM (*Arbuscular mycorrhizal*) fungi after a long-term fertilization for 38 years in a brown soil. Soil samples (0-20 cm) were taken from six treatments in May 2016: (1) no fertilization (CK); (2) chemical N input (N); (3) chemical N and P input (NP); (4) chemical N, P and K input (NPK); (5) pig manure input (M); (6) pig manure, chemical N and P input (MNP). Community structures of AM (*Arbuscular mycorrhizal*) fungi were analyzed using PCR-DGGE, cloning and sequencing techniques. In seedling stage, the diversity of AM Fungi was the highest in non-fertilization treatment and the community structures of AM fungi was mainly divided into two groups.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil inhabitants belonging to the phylum Glomeromycota which establish mutualistic symbiotic (mycorrhizal) associations with most land plants [1]. The ecological and physiological benefits of the AM fungi for their host plants have been well documented. Basically, AM fungi enhance plant uptake of minerals and water from beyond the rhizosphere, thereby ameliorating environmental stresses on the plant [2]. A diverse community of AM fungi is important for the development and maintenance of plant diversity of terrestrial ecosystems contributing to plant community productivity [3]. *Arbuscular mycorrhizal* fungi play an important role in the maintenance of agroecosystem stability and sustainable agricultural development [4]. High diversity of AM fungi may favor higher agricultural productivity since different species of AM fungi can adapt to various stress conditions [3] and they exhibit functional diversity [5]. Fertilization is an important abiotic factor influencing growth, colonization, sporulation, community structure, and distribution of AM fungi [6, 7]. Chemical fertilization, especially of phosphorus, depressed both colonization of roots and sporulation by the majority of AM fungi [8]. However, in most of these experiments, treatments were only maintained for a short time. It is more interesting and valuable to investigate effects of long-term fertilization treatments on the diversity of AMF. Long-term fertilization, especially amendments with manure and straw, has beneficial effects on accumulation of soil organic carbon, spore density, GRSP content, and AMF diversity [9]. The influence of contrasting forms of P fertilizers was not significant over 3 years of crop rotation [10]. Inputting of organic matter source can change positively the AMF community composition, and these



results highlight the importance of considering the long-term effect of mineral and organic fertilizers on the AMF community diversity [11]. Joner's study showed that the application of organic fertilizer could have a negative effect on AM fungi [12].

Long-term fertilization test artificially created different soil fertility conditions, in this experiment, the community structures of AM fungi was investigated to explore the effects of long-term fertilization on it.

2. Materials and methods

2.1. Sampling

The study was conducted in Shenyang agricultural university in the semi-humid region of Shenhe district, Shenyang (40°48'N and 123°33'E) of Liaoning Province, China. All fertilizers were applied as base fertilizer before seeding. In this study, a randomized block design was adopted in the experimental field and six treatments with four replicates each were chosen as follows: no fertilizer (CK), N1 (mineral nitrogen fertilizer), N1P (mineral nitrogen and phosphate fertilizer), N1PK (mineral nitrogen, phosphate and potassic fertilizer), pig manure (M), MNP (pig manure, mineral nitrogen and phosphate fertilizer). The mineral fertilizers were applied in the form of urea, calcium superphosphate and potassium sulphate. Basic chemical properties of experimental soil in 1979 and the application rates of fertilizer are described by Luo et al [13].

Soil samples were collected at the maize seeding stage in 2016 by 4 points for each treatment with S-type. The sampling depth is 0-20cm, fully mixed, removed the gravel, plant roots and other sundries.

2.2. DNA extraction and PCR amplification

Total genomic DNA was extracted from soil using Powersoil® DNA isolation kit(MO BIO, Laboratories Inc., German) following manufacturer's instructions, then deposited it in a refrigerator at -20°C. A nested PCR approach was used to amplify the AM fungal 18S rRNA gene. Amplification reactions and conditions of PCR were described in Table 1. The second round of amplification was performed with two different primer sets, NS8-ARCH1311 was for *Paraglomeraceae* and *Archaeosporaceae* families and NS31-AM1 was for the rest of AM fungal families. The concentration of polyacrylamide gel in this experiment was 6%. The range of transsexual agents from 30% to 50%. Electrophoretic buffer solution: 1×TAE, electrophoresis time: 60V, 16h.

Table 1. PCR conditions of AM fungi.

PCR			
Primer	Primer sequence(5'-3')	Reaction system	Conditions
NS1	GTAGTCATATGCTTGCTC	10×PCR Buffer 5μL, dNTP 4μL (2.5mmol/L), MgCl ₂ 3μL(2.5mmol/L), NS1(10mmol/L)1.5μL,	An initial denaturation of 94°C for 3min; 94°C 1min, 50°C 1min, 72°C 1min, 30cycles; 72°C 7min(The first round)
NS41	CCCGTGTGAGTCAAATTA	NS41(10mmol/L)1.5μL, Taq(2U/μL)1μL, DNA template 1μL, adding ddH ₂ O to 50μL	
GC-NS31 AM1	TTGGAGGGCAAGTCTGGTGCC GTTTCCCCTAAGGCGCCGAA	10×PCR Buffer 5μL, dNTP 4μL(2.5mmol/L), MgCl ₂ 3μL(2.5mmol/L), NS1(10mmol/L)1.5μL,	
GC-ARCH1311	TGCTAAATAGCCAGGCTGY	NS41(10mmol/L)1.5μL, Taq(2 U/μL)1μL, DNA template 1μL, adding ddH ₂ O to 50μL	An initial denaturation of 94°C for 3min; 94°C 30s, 58°C 45s, 72°C 45s, 28cycles; 72°C 7min(The second round)
NS8	TCCGCAGGTTACCTACGGA		

2.3. Recovery of DNA from DGGE gel

DNA was recovered from DGGE gel using the method of Sheng et al. [14]. The sequencing reactions were performed with a DNA sequencing kit, BigDye™ Terminator v3.1

(Applied Biosystems, Foster City, CA, USA), and the reaction products were analyzed with an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Partial 16S rRNA and 18S rRNA sequences were identified based on similarity using the National Centre for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (<http://www.ncbi.nlm.nih.gov/>).

2.4. Statistical analysis

The Shannon-Weaver diversity index (Shannon 1948) was calculated from the number of bacterial and fungal bands detected on DGGE to compare the ribotype diversity between different fertilization treatments using Quantity One. Cluster analysis was performed using Quantity One by UPGMA. The data were subjected to analysis of variance, using IBM SPSS Statistics 19.0 for Windows (IBM, Inc., Armonk, NY, USA). A probability level of 5 % was adopted for accepting or rejecting null hypotheses. Tukey's honestly significant difference test for all-pairwise comparisons was calculated after ANOVA to compare treatment means.

3. Results and discussion

DGGE profiles of AM fungal communities in different fertilization treatments were shown in Figure 1. The quantity, brightness and position of different fertilizer treatments are different. It was shown that the community structures of AM fungi in brown soil are affected by the type of fertilizer applied. Eighteen bands were isolated from the samples, after sequencing, band16 was not belong to AM fungi. The number of bands in the CK treatment and the M treatment were the most, and band7 appeared in all treatments that indicated fertilization do not affect this AM fungi. Band12 only appeared in the CK treatment, fertilization obviously exerted an influence on this type of AM fungi. Band13 and band14 diminutively appeared in organic fertilizer treatment, while the treatments of organic fertilizer with chemical fertilizer did not include it. These kinds of AM fungi may have been brought into the soil by organic fertilizers, but the use of chemical fertilizers inhibited them.

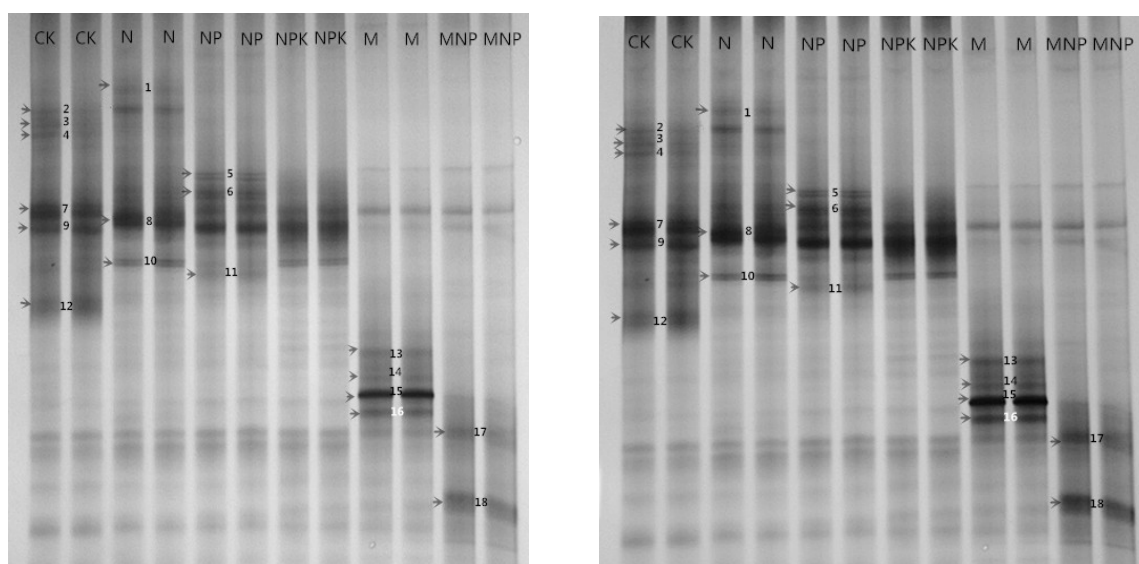


Figure 1. DGGE banding patterns of AM fungi in different treatments.

The Shannon-Wiener index (H), also known as the diversity index, is used to predict species diversity. The diversity index of AM fungi in different fertilization treatments were shown in Figure 2. The H index of non-fertilizer treatment was significantly higher than that of any other fertilizer

treatments, indicated that fertilization can inhibit the growth of AM fungi in the brown soil. In the seedling stage, NPK and MNP had the lowest index, and possibly application of potash fertilizer inhibits AM fungal growth and reduces the number of AM fungi.

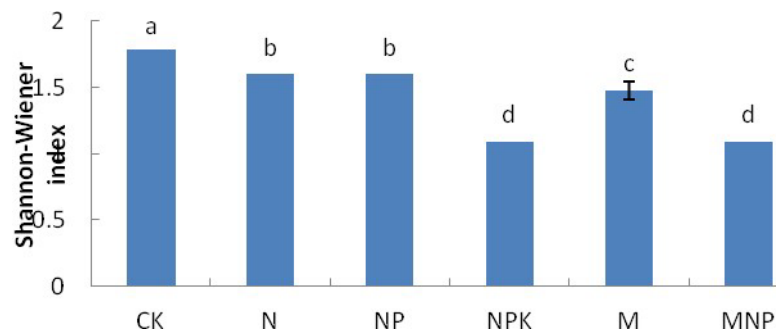


Figure 2. The diversity index of AM fungi in different fertilization treatments.

UPGMA dendrogram of AM fungi in different fertilization treatments was shown in Figure 3. In the seedling stage, Cluster analysis is showed that community structures of AM fungi were divided into two groups, namely, organic fertilizer group and inorganic fertilizer group, and their similarity was only 13% under a long-term fertilization in a brown soil. Inorganic fertilizer group also was divided into two groups, chemical fertilizer group and non-fertilizer group, and the similarity of them was 38%, indicating the different fertilization treatments have significantly affected the community structures of the AM fungi. The highest similarity between NPK and NP had reached 71%, indicating that potash fertilizer has no significant influence on the community structures of AM fungi in brown soil.

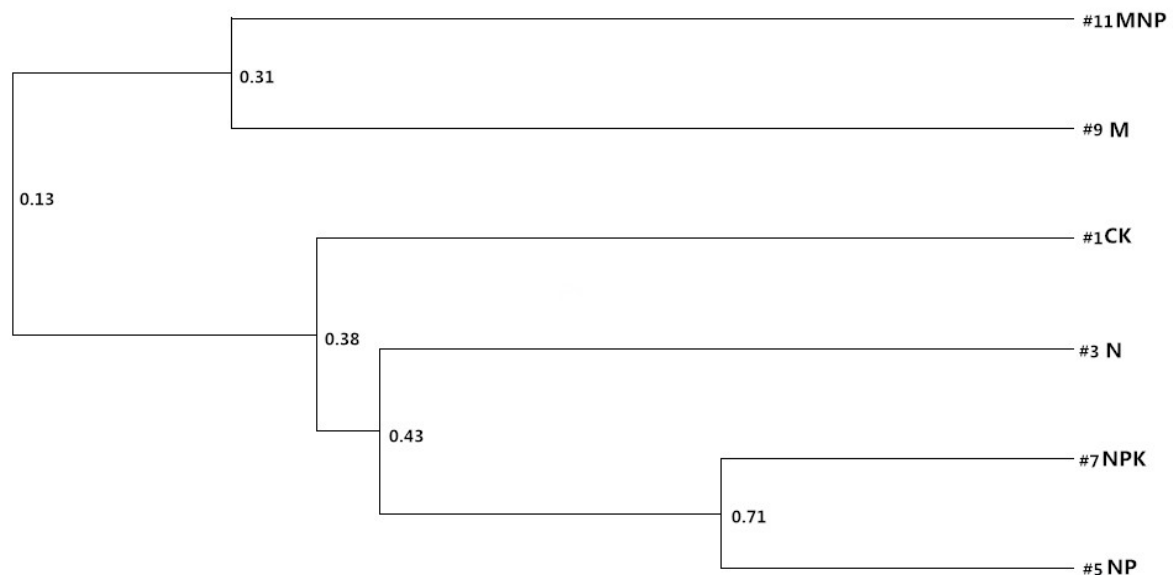


Figure 3. UPGMA dendrogram of AM fungi in different fertilization treatments.

4. Conclusion

This study demonstrated that community structures of AM fungi were significantly changed by long-term fertilization in a brown soil. In the future study, our researches will emphasis on the factors which change community structures of AM fungi. In seedling stage, the diversity of AM Fungi was the highest in non-fertilization treatment and the community structures of AM fungi was mainly divided into two groups.

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