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Temporal variation of diazotrophic community abundance and structure in surface and subsoil under four fertilization regimes during a wheat growing season

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ABSTRACT

Biological nitrogen fixation (BNF) is an initial process of the nitrogen cycle (Cleveland et al., 1999) by the specialized microorganisms known as diazotroph. However, little information is available concerning the dynamic changes of diazotrophic communities in surface and subsoil layers. In this study, five representative season soil samples were collected from surface (0–20 cm) and subsoil (20–40 cm) in the long term experimental field plots that received no nitrogen fertilizer (CK), chemical fertilizers (CF), organic-inorganic mixed fertilizer (OIMF) and organic fertilizer (OF) since 2005. Real-time PCR was used to determine the *nifH* and total bacterial 16S rRNA gene abundance. Terminal restriction fragment length polymorphism (T-RFLP) of *nifH* gene was used to analyze the changes of diazotrophic communities. Results revealed that the abundance of *nifH* and 16S rRNA genes declined with increased soil depth regardless of fertilization regimes, although considerably high abundance was also observed in subsoils. The abundance of *nifH* genes was significantly positively correlated with the total bacterial abundance in oligotrophic subsoil layers, but not in the topsoil. Ammonium contents showed significant correlation to the *nifH* gene abundance in both surface and subsoil. Clustering analysis of the T-RFLP profiles showed that diazotrophic structures were clearly separated by surface and subsoil habitats. Permutational multivariate analyses demonstrated that soil depth, rather than the sampling time or fertilization regime, was the important factor that influence the diazotrophic structures. In the surface soil, soil organic carbon (SOC) and ammonium contents were significantly positively correlated to the diazotrophic community structures. These results suggest soil physiochemical properties selected for distinct diazotrophic communities inhabit in the topsoil and subsoil.

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1. Introduction

Wheat is the third most-produced cereal in the world, and China is the world's largest producer of wheat (Tao and Zhang, 2013). Over the past several decades, the application of synthetic N fertilizer has significantly increased to meet the demand for wheat production in China. However, excessive N use has caused the environmental problems of eutrophication, nitrate leaching and groundwater contamination (Vitousek et al., 1997). Alternatively, exploiting biological nitrogen fixation (BNF) can also obtain the desired plant outcomes, as reviewed by Hayat et al. (2010). BNF is an initial process of the nitrogen cycle and an important N source for the ecosystem (Cleveland et al., 1999), which transforms the dinitrogen to ammonium and is performed by the specialized microorganisms known as diazotroph (Martinez-Romero, 2006). Every year, N entry from BNF is the second largest factor after mineral fertilizer and contributes up to 16% of the total global N input (Ollivier et al., 2011). Understanding and utilizing the ecological significance of the diazotroph community are useful for the development of sustainable agriculture. However, for the reason that many diazotroph are not successfully cultured, the laboratory studies on these unculturable organisms are limited. Recently, molecular methods analyzing the *nifH* gene, which encodes a subunit of nitrogenase, have been widely used to investigate the diversity and composition of diazotrophic organisms in several soil systems (Rösch et al., 2002; Hsu and Buckley, 2008; Wartiainen et al., 2008).

N fixers are sensitive to agricultural practices such as fertilization, crop rotation and field tillage. Different fertilization practices showed significant influence on the diazotrophic community size, diversity and structure, but many results are

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equivocal. For example, Berthrong et al. (2014) reported that the addition of N fertilizer significantly changed the structure of the diazotrophic community in forest soils. Tan et al. (2003) found that the diazotrophic community structure rapidly changed after N fertilizers were added to paddy soils. However, Wakelin et al. (2007) revealed that the application of urea did not necessarily affect the diazotrophic communities. These inconsistent results might be due to the multiple secondary effects of fertilization regimes on the soil properties, such as soil pH (Limmer and Drake, 1996), soil carbon (Keeling et al., 1998; Wakelin et al., 2010), N availability (Poly et al., 2001) etc. Additional factors, such as different crops (Tan et al., 2003), soil moisture (Nelson and Mele, 2006), the land use and the soil types (Hayden et al., 2010) may also cause the inconsistent results when evaluating the effects of fertilizers on diazotrophic communities. Furthermore, the majority of these investigations only focused on one site or one time, and recent studies have revealed that diazotrophic communities changed with different sampling months (Pereira e et al., 2011, 2013). It is not surprising that the results obtained in different sites and different sampling time are inconsistent.

The soil vertical profile is usually considered an ecological filter for the environmental diversification and microbes, and many surface living microorganisms cannot grow in the deeper soil layers (Eilers et al., 2012). Diazotrophs also decrease with soil depth. Reardon et al. (2014) reported that the abundance of the *nifH* gene was stratified by soil depth, which was less in the 10–20 cm soil layer than in the 0–5 cm and 5–10 cm layers. Not so many studies can be documented on the N_2-fixer in deeper soil layers. Dawson et al. (1989) reported that the N_2-fixing *Frankia* was present on the *Casuarina* and *Allocasuarina*'s seeding nodulation at the 0–80 cm soil depth. Nalin et al. (1997) found the abundance of *Frankia* decreased at a soil depth of 60 cm. However, knowledge about N-fixers in the deeper soil horizons is not fully explored, and little information is available about the temporal variation of the diazotrophic abundance and structure in the subsoil layers, which is important for better understanding the roles of diazotroph in the soil ecosystem.

Long-term field experiments with different fertilization regimes since 2005 were used to characterize the temporal variation of diazotrophs in different soil depths and determine the effects of soil depth, sampling time and fertilization on the diazotroph structures in rice-wheat rotation system in this study. Surface and subsoil sampling were performed during wheat growing season from October 2012 to June 2013. Quantitative PCR was used to determine the abundance of both diazotrophic *nifH* and bacterial 16S rRNA genes. Terminal restriction fragment length polymorphism (T-RFLP) was performed to determine the structure of the diazotrophic communities.

2. Materials and methods

2.1. Site description

The long-term rice-wheat rotation field experimental site is situated in Changshu, Jiangsu province, China (31°18′N, 120°37′E, 6 m above sea level), and was established in 2005. The site has a humid subtropical monsoon climate with an average annual rainfall of ≈1063 mm and annual mean minimum and maximum temperatures of 3.1 °C and 33 °C, respectively (Wang et al., 2012). 150 kg wheat seeds were sown per hectare after the rice were harvested in October every year. Fertilizers were applied as basal fertilizer after both the rice and wheat were harvested. Rice growth seasons were flooded with 5 cm of standing water, while rain was the only water source for wheat growth.

2.2. Experimental design and soil sampling

Four treatments with three replicates were established in a randomized block design: (1) control without nitrogen fertilizer (CK), (2) NPK chemical fertilizer (CF), (3) organic–inorganic mixed fertilizer (OIMF) and (4) organic fertilizer (OF). The randomized block plots were 6 × 7 m in size. The details of experimental design and the nutrient contents of fertilizers are shown in Table 1.

Soil samples of the surface soil (0–20 cm) and subsoil (20–40 cm) were collected on 25th October 2012 (Oct_{12} at wheat seeding), 1st March (Mar_{13} at wheat tillering), 1st April (Apr_{13} at wheat jointing), 1st May (May_{13} at wheat heading) and 4th June 2013 (Jun_{13} at wheat ripening), respectively. Deeper soil layers of 40–60 cm, 60–80 cm and 80–100 cm were also collected on Oct_{12}. For each plot, 4 cores (5 cm in diameter) of surface soil, and 3 cores of subsoil were collected. Then, the samples from different layers and treatments at each sampling time were mixed separately and sieved (2 mm) to remove roots. To minimize changes in soil communities after sampling, soils are immediately processed and DNA extracted.

2.3. DNA extraction

Soil DNA was extracted from 0.25 g fresh soil using the MoBio Powersoil™ DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA, USA) using the bead-based homogenizer protocol according to the manufacturer's instructions. The quantity and quality of DNA extracts were assayed by a Nanodrop ND-2000 UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the DNA was stored at −80 °C before analysis.

Table 1
Experimental design and nutrient contents of fertilizers.

Treatment[a]	Fertilizer[b] (kg ka^{-1}, every wheat and rice season)				
	Urea (46% N)	Superphosphate (12% P_2O_5)	KCl (60% K_2O)	OIMF[c] (11.0% SOC, 12.0% TN, 4.1% P_2O_5, 4.1% K_2O)	OF (26.4% SOC, 2.3% TN, 2.9% P_2O_5, 1.3% K_2O)
CK	0	750	183	0	0
CF	391	750	183	0	0
OIMF	0	28.5	48.5	1500	0
OF	0	0	0	0	4500

[a] CK (control without nitrogen fertilizer), CF (chemical fertilizer), OIMF (organic–inorganic mixed fertilizer) and OF (organic fertilizer).
[b] Fertilizers were made by Tianniang Ltd. of Changshu, China.
[c] Organic fertilizers in both OIMF and OF were made of composted pig manure and rice straw.

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