Journal of Integrative Agriculture 2017, 16(11): 2597–2611

RESEARCH ARTICLE

Responses of N_2O reductase gene (*nosZ*)-denitrifier communities to long-term fertilization follow a depth pattern in calcareous purplish paddy soil

WANG Ying-yan[1], LU Sheng-e[1], XIANG Quan-ju[1], YU Xiu-mei[1], ZHAO Ke[1], ZHANG Xiao-ping[1], TU Shi-hua[2], GU Yun-fu[1]

[1] *Department of Microbiology, College of Resource, Sichuan Agricultural University, Chengdu 611130, P.R.China*
[2] *Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, P.R.China*

Abstract

The effect of long-term fertilization on soil denitrifying communities was analysed by measuring the abundance and diversity of the nitrous oxide (N_2O) reductase gene, *nosZ*. Soil samples were collected from plots of a long-term fertilization experiment established in 1982 in Suining City, China. The fertilizer treatments were no fertilizer (CK), three chemical fertilizer (CF) treatments (N, NP, NPK), manure (M) alone, and manure with chemical fertilizers (NM, NPM, NPKM). The abundance and diversity of the denitrifying bacteria were assessed by real-time quantitative PCR, terminal restriction fragment length polymorphism (T-RFLP), and cloning and sequencing of *nosZ* genes. The diversity and abundance of *nosZ*-denitrifiers was higher in soil amended with manure and chemical fertilizers (CFM) than in soil amended with CF alone, and the highest in topsoil (0–20 cm). The *nosZ*-denitrifier community composition was more complex in CFM soil than in CF soil. Specific species were detected only in the CFM soil. The abundance of *nosZ*-denitrifier in the NPKM treatment was approximately two times higher than that in the CK, N, and NPK treatments. Most of the cloned *nosZ* sequences were closely related to *nosZ* sequences from Bradyrhizobiaceae and Rhodospirillaceae in Alphaproteobacteria. Of the measured abiotic factors, soil organic matter correlated significantly with the abundance ($P<0.01$); available phosphorus correlated significantly with the topsoil community composition ($P<0.01$), whereas soil organic matter correlated significantly with the subsoil (20–90 cm) community composition ($P<0.01$). This study demonstrated that long-term CFM fertilization affected both the abundance and composition of the *nosZ*-denitrifier community.

Keywords: fertilization, community, abundance, denitrifiers, soil depth

1. Introduction

Denitrification contributes significantly to nitrous oxide (N_2O) emissions, which is involved in the destruction of the stratospheric ozone layer and global warming (Ravishankara *et al.* 2009). Research suggests that, due to denitrification, as little as 2 kg N ha^{-1} yr^{-1} or more than 200 kg N ha^{-1} yr^{-1} could be lost from crop lands (Ryden and Lund 1980; Aulakh *et al.* 1983). The capacity for denitrification has

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Received 18 January, 2017 Accepted 22 June, 2017

WANG Ying-yan, Mobie: +86-13550865352, E-mail: 398766403@qq.com; Correspondence GU Yun-fu, Tel: +86-28-86920982, E-mail: gungyf@163.com

doi: 10.1016/S2095-3119(17)61707-6

been found among many taxonomic groups within bacteria, archaea, and fungi, with more than 50 bacterial genera dominating global denitrification (Zumft 1997). Among them, the phylum Proteobacteria comprises the most dominant denitrifier that found in paddy soil, particularly in the genera *Bradyrhizobium*, *Pseudomonas*, *Azospirillum*, and *Ralstonia* (Dambreville *et al.* 2006a; Chen *et al.* 2012). Denitrifying bacteria are distributed across various kinds of ecological systems, such as sediment (Wang *et al.* 2012, 2014), agricultural soil (Dambreville *et al.* 2006a, b; Horn *et al.* 2006; Chen *et al.* 2012), forest soil (Rich *et al.* 2003; Liu *et al.* 2015), and meadow soil (Rich *et al.* 2003). Owing to the high phylogenetic diversity of denitrifiers, 16S rRNA gene-based approaches are not suitable to explore denitrifying communities (Zumft 1997). Instead, the analysis of functional genes involved in denitrification is a more direct method of detecting or identifying denitrifying bacteria (Wolsing and Prieme 2004). The whole denitrification pathway requires the sequential action of four enzymes. The *nosZ* gene that encodes the N_2O reductase which is needed in the final step of the denitrification process has proven to be a useful genetic marker for studying denitrifying communities (Chen *et al.* 2012; Sanford *et al.* 2012; Orellana *et al.* 2014; Shiina *et al.* 2014).

Rice is an important food worldwide; it is grown on approximately 155 million ha for the consumption of more than 50% of the human population (Liesack *et al.* 2000). Paddy fields are a special environment for the transport and transformations of N species because of their semi-artificial, alternating dry and wet conditions (Lüdemann *et al.* 2000; Ishii *et al.* 2011; Li *et al.* 2015). Purplish soils are widely distributed in the hills along the Yangtze River, especially in the Sichuan Basin, China (Zhu *et al.* 2008), where they covering an area of 300 000 km^2. Purplish soils have high mineral nutrient fertility owing to their parent rock composition. However, purplish soil formed in Suining City, China, has low nutrient fertility due to rock weathering (Zhu *et al.* 2008). Thus, a reasonable fertilization system is key to nutrient replenishment of calcareous purplish paddy soils.

In the past, fertilization greatly contributed to food production and fertilizer application was still continuing to increase (Roberts 2009). Fertilization practices, including chemical and organic fertilizer application, can stimulate both nitrification and denitrification, resulting in N loss (Wolsing and Prieme 2004; Enwall *et al.* 2005; Dambreville 2006b; Kastl *et al.* 2015). In China, the application of manure to soil is recommended to enrich the organic matter content (Yu *et al.* 2009). Manure can provide easily degraded organic C and N, as well as considerable amounts of other nutrients to the soil (Surekha *et al.* 2003; Tirol-Padre *et al.* 2005). The combined application of manure and chemical fertilizer to the soil is also recommended for maintaining soil health and sustainability (Plaza *et al.* 2004). N, P, and K are the major chemical elements applied in fertilizers. Urea is the main N fertilizer used in rice paddy fields and it accounts for approximately 60% of the total N fertilizer used in China (FAO 2003).

Previous studies have reported the effect of long-term fertilization on microbial biomass, crop yields, and bacterial community structure under crop rotation in calcareous purplish paddy soil (Gu *et al.* 2009). Chen *et al.* (2012) showed the influence of fertilization regimes on a *nosZ*-containing denitrifying community in rice paddy soil; however, this study did not examine the distribution of *nosZ*-denitrifiers at different soil depths. Given that denitrifiers are facultative anaerobes, it is important to understand the distribution of denitrifiers at different soil depths. Liu *et al.* (2015) reported that denitrifiers which contained *nosZ* were gradually enriched in the deeper soil layers (five soil layers: 0–10, 10–20, 20–40, 40–60, and 60–80 cm) in a wet sclerophyll forest. However, the vertical distribution of the *nosZ* bacterial community responsible for denitrification in calcareous purple paddy soil amended with long-term fertilization is not well known. In this study, the objectives were to (1) investigate the influence of different long-term fertilization practices on the composition and abundance of the *nosZ*-denitrifier community in calcareous purplish paddy soil at various vertical soil depths, and (2) analyse the relationship between the soil physicochemical parameters and *nosZ*-denitrifier community composition and abundance.

2. Materials and methods

2.1. Field site and soil sampling

The experimental site was a N, P and K long-term fertilization field experiment established in 1982 in Chuanshan (30°10´50´´N, 105°03´26´´E), Suining City, Sichuan Province, China. Chuanshan has an annual average temperature of 17.4°C and mean annual precipitation of 930 mm. The calcareous soil was developed from purplish shale. The cropping system was a winter wheat (*Triticum aestivum* L.) and summer rice (*Oryza sativa* L.) rotation. The soil physicochemical parameters were measured prior to the experiment (Appendix A).

Eight fertilizer treatments with three randomly distributed replications were sampled. The treatments were as follows: three chemical fertilizer (CF) treatments (N, NP, NPK), three CFM treatments with manure and CF (NM, NPM, NPKM), manure alone (M), and no fertilizer (CK). Each treatment was established with three replicates and repeated every year. In all M treatments, basal fertilizers contained P as superphosphate and K as K_2SO_4, whereas 70% of N as