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Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China

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ABSTRACT

Although diazotrophs are important in the nitrogen (N)-cycle and contribute to the pool of plant available N, the population response to long-term inorganic fertilization is largely unknown. Here, we investigated the diazotrophic populations in both the bulk and rhizosphere soils of maize grown in an acidic farmland soil that experienced 25 years of inorganic fertilization. The fertilization regimes included unfertilized control, N fertilizer alone, N fertilizer with quicklime, phosphorus (P) and potassium (K) fertilizers, N + P + K fertilizers, and N + P + K fertilizers with quicklime. Quantitative PCR and high-throughput pyrosequencing of the *nifH* gene were used to analyze diazotrophic abundance and community composition. All of the fertilizer treatments improved soil nutrient availability, but those without quicklime caused soil acidification. Maize biomasses and *nifH* copy numbers were significantly lower under N and N + P + K treatments but increased under P + K fertilization. Quicklime applications effectively alleviated the inhibitory effect of N input. Fertilization led to decreases in operational taxonomic unit richness and shifts in diazotrophic community composition. Soil pH and nutrient availability had a cooperative effect on diazotrophic abundance, while soil nutrient availability appeared to be the main factor shaping diazotrophic community structure. Rhizosphere effects increased the *nifH* gene copy number but did not obviously change the diazotrophic community composition on the current research scale. Overall, the long-term inorganic fertilization affected both diazotrophic abundance and community composition, and the fertilizer treatment had a greater influence than quicklime remediation or crop cultivation on community composition.

1. Introduction

To meet the food demand of an ever-increasing world population, the resulting intensive agriculture practices have included applying an overload of inorganic fertilizers (Liu et al., 2015; Zeng et al., 2016). In China, nitrogen (N), phosphorus (P), and potassium (K)-based fertilizer consumption increased to 38.94 million tonnes in 2013 from 0.73 million tonnes in 1961 (http://faostat3.fao.org/home) and is expected to continue to increase over the next few decades (Liu et al., 2015). The increased long-term input of inorganic fertilizers, especially N-based fertilizers, in agricultural ecosystem improved soil fertility and crop yields over the past decades (Liu et al., 2015; Zeng et al., 2016) but also had various negative effects such as soil acidification, metal toxicity, lower nutrient use efficiencies, increased greenhouse gas emissions, and groundwater contamination that threaten soil quality, crop growth, biodiversity, and environmental health (Guo et al., 2010; Zhong et al., 2015) and changed the biogeochemical cycles of soil nutrient elements (i.e., carbon (C), N, and P) (Geisseler and Scow, 2014). Thus, there is an increasing concern on how to safely enhance agricultural sustainability.

The N cycle is an important nutrient cycle that influences the productivity and sustainability of the terrestrial ecosystem

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(Geisseler and Scow, 2014; Levy-Booth et al., 2014). Microbes are key players in the soil N cycle, play an important role in regulating soil N availability and transformation, and are closely related to plant productivity. The intensive fertilization has resulted in an unprecedented disturbance in the soil N cycle (Geisseler and Scow, 2014), which involved shifts in a diverse range of the microbial communities and functions (Sun et al., 2015; Zhou et al., 2015). To date, most of the studies have focused on the influences of long-term fertilization on the ammonia oxidizers (He et al., 2007; Wang et al., 2015; Zhong et al., 2015) and denitrifiers (Yin et al., 2015) because they are directly associated with soil acidification, N availability to plants, and N loss (Levy-Booth et al., 2014). The N-fixing bacteria (diazotrophs) are responsible for biological N fixation, another functionally important biogeochemical N cycle (Levy-Booth et al., 2014). Because this bioprocess can supply additional N sources to the ecosystem to improve soil fertility, which is beneficial to plant productivity, it is a potentially sustainable alternative to inorganic N fertilizer use in agricultural systems (Gupta et al., 2006). However, the response patterns of diazotrophic bacteria to long-term fertilization in agricultural ecosystems are not well understood, although some short-term micro-zone experiments (within one complete growing season) have been carried out (Rodríguez-Blanco et al., 2015; Simonsen et al., 2015). However, short-term experiments cannot supply some valuable information that is detected only under long-term fertilization conditions, because changes in soil quality are slow and microbial community structures need time to stabilize (Chakraborty et al., 2011; Reardon et al., 2014). Detailed information on diazotrophic community composition and abundance, as well as their relationships to the soil environment, would improve our understanding of the long-term ecological effects of fertilization and of the biological functions of diazotrophic bacteria in the agroecosystem, as well as reduce inorganic N fertilizer use.

Fertilization practices strongly influence soil microbial community structure and abundance either directly by altering soil characteristics or indirectly by changing the plant feedback (Geisseler and Scow, 2014; Zeng et al., 2016). In the long-term fertilization regimes, changes in soil physicochemical properties, such as pH, nutrient availability, and carbon quantity and quality, have had positive or negative influences on soil microbial populations (Geisseler and Scow, 2014; Levy-Booth et al., 2014; Li et al., 2014). Likewise, diazotrophic bacteria are very sensitive to soil properties (Pereira e Silva et al., 2013; Rodríguez-Blanco et al., 2015). Additionally, plants, as another important driving factor, have a close relationship to the functions and activities of the rhizospheric microbiome (Berendsen et al., 2012). The growth of crops, such as maize, rice, soybean, and sorghum, leads to a unique diazotrophic communities in the rhizosphere, which are different from the communities in the bulk soil (Coelho et al., 2008; Li et al., 2012; Rodríguez-Blanco et al., 2015; Wang et al., 2012). Conversely, rhizosphere microbes directly impact plant growth through positive or negative traits (Berendsen et al., 2012). Thus, variations in plant—microbe interactions in the rhizosphere are of great significance in agronomy. Previous studies on the impacts of the long-term fertilization practices on the soil microbial community were confined to the bulk soil scale (Geisseler and Scow, 2014; Levy-Booth et al., 2014; Li et al., 2014), which resulted in a lack of a deep understanding of how the plant-associated rhizosphere's microbiome responds to fertilization (Ai et al., 2015).

Soil acidification resulting from inorganic N input has become a major limiting factor for crop yields in the red soil region of southern China (Guo et al., 2010). In agricultural management, lime is applied as an effective way to remediate the acidic soils, maintain crop productivity and alter the microbial population (Xun et al., 2016a, 2016b). Xun et al. (2016b) reported that the addition of lime can quickly increase the soil pH and bacterial diversity in acidic farmland soil. Likewise, our previous studies found that a lime application to an acidic soil significantly improved the abundances of overall bacteria (Wang et al., 2013) and ammonia oxidizers (Che et al., 2015). However, such information is not available for diazotrophic bacteria.

For this study, samples were collected from a long-term fertilized area in southern China that was established in 1990. Some severe acidification regimes were employed in 2010 and part of the area was remediated by adding quicklime (Xun et al., 2016a, 2016b). Previous studies at this site have focused on the influences of long-term fertilization on crop production (Zhang et al., 2009), soil acidification (Cai et al., 2015), remediation approaches to soil acidification (Xun et al., 2016b), soil enzyme activities (Ahamadou et al., 2009), overall bacterial abundance (Ahamadou et al., 2009; Xun et al., 2016a, 2016b), and ammonia oxidizer abundance (He et al., 2007). However, the effects on diazotrophic bacteria are still unknown. Thus, we chose this experimental site, which has undergone 25 years of fertilization, to investigate the shift of soil diazotrophic populations under the different fertilization treatments. The objectives were to: (1) identify the responses of the diazotrophic abundance and community composition to long-term inorganic fertilization, as well as their relationships with soil properties; (2) assess the impact of long-term inorganic fertilization on the rhizosphere effect; and (3) test whether lime remediation can also improve soil diazotrophic populations in the acidic soil. To do so, we chose the dinitrogenase reductase subunit gene *nifH* because it is the most used marker gene for analyzing the abundance and diversity of diazotrophic bacteria (Levy-Booth et al., 2014). Both quantitative-PCR and high-throughput sequencing were applied to determine the abundance and community structure of the *nifH* gene.

2. Materials and methods

2.1. Experimental site and sampling

The fertilization experiment started in 1990 at a site located in the Red Soil Experimental Station at Qiyang, Hunan Province, China (26°45′ N, 111°53′ E). The soil on site originated from the Quaternary red clay soil and is classified as Ferralic Cambisol. The size of each fertilizer treatment was 20 m × 10 m and designed with two replicate plots (Cai et al., 2015; Xun et al., 2016a,b). The cropping system was an annual rotation of summer maize (*Zea mays* L. Yedan 13) and winter wheat (*Triticum aestivum* L. Xiangmai 4). The tillage operation was conventional tillage. The inorganic fertilizers were applied as urea (300 kg N ha^{-1} $year^{-1}$), superphosphate (53 kg P ha^{-1} $year^{-1}$) and potassium chloride (100 kg K ha^{-1} $year^{-1}$). The original soil properties, fertilization methods, natural environment, and field management have been described previously (Cai et al., 2015). Before sowing, fertilizers were applied by banding at a depth of 10 cm. For annual input, 30% of fertilizers were applied for wheat and 70% for maize. As the plots receiving N and N + P + K (NPK) fertilizers showed severe soil acidification, these two plots were each divided into two parts in 2010. One part maintained the same fertilization as before, while the other also received 2550 kg ha^{-1} of quicklime based on the same fertilization protocol, followed by the addition of 1500 kg ha^{-1} of quicklime in 2014. In this trial, six different fertilizer treatments were chosen: unfertilized control (CK), inorganic N fertilizer alone (N), inorganic N fertilizer plus quicklime (NCa), inorganic P + K fertilizers (PK), inorganic NPK fertilizers, and inorganic NPK fertilizers plus quicklime (NPKCa).

Soil samples were collected on June 12, 2015 (at the maize flowering stage). Each fertilizer treatment was established