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Long-term application of lime or pig manure rather than plant residues suppressed diazotroph abundance and diversity and altered community structure in an acidic Ultisol

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ABSTRACT

Biological fixation of atmospheric dinitrogen (N_2) is an important process that replenishes biologically available nitrogen (N) in soil and helps minimize the use of inorganic N fertilizer in agricultural ecosystems. Diazotrophs are key fixers of atmospheric N_2 in a range of soil types, however, there is uncertainty about how they respond to long-term fertilization. Here, using the *nifH* gene as a molecular marker, we investigated the long-term effects of inorganic and organic fertilization on diazotroph abundance and community structure in an acidic Ultisol. The field experiment ran for 27 years and comprised seven treatments: no fertilization (control); inorganic NPK fertilizer (N); inorganic NPK fertilizer + lime ($CaCO_3$) (NL); inorganic NPK fertilizer + peanut straw (NPS); inorganic NPK fertilizer + rice straw (NRS); inorganic NPK fertilizer + radish (NR); and inorganic NPK fertilizer + pig manure (NPM). Long-term application of fertilizer reduced the abundance and diversity of *nifH* gene compared with the control ($P < 0.05$), while lime and pig manure increased the inhibitory effects ($P < 0.05$). The abundance and diversity of *nifH* genes were negatively correlated with soil pH, indicating that increasing soil pH potentially affect N fixation ability in acidic Ultisols. Community structure of diazotrophs in the NPS, NRS, and NR treatments were similar and shared most operational taxonomic units (OTUs) with the N treatment, with only a minor difference to that of the control. Thus, there was no effect of plant residue types on diazotroph community structure. In contrast, the application of inorganic fertilizer + liming or pig manure altered the diazotroph community structure with shifts in the dominant genus, from *Bradyrhizobium* in the control to *Azohydromonas* in NL and *Azospirillum* in NPM. Soil pH was the key factor correlated with change in diazotroph community structure. Overall, our results suggested that regular use of lime or pig manure rather than different types of plant residue reduced the abundance and diversity and altered community structure of diazotrophs, that may potentially affect N fixation ability in acidic Ultisols.

1. Introduction

Ultisols are widespread in tropical and subtropical regions (Alvear et al., 2005; Chandran et al., 2005; Hauser et al., 2006) and represent 8.4% of global soil types (Lal, 2004). These soils are generally characterized by low cation exchange capacity (CEC) and pH, high nutrient leaching and Al and Mn toxicities, and, low structural stability with little or no mineral reserves (Clair and Lynch, 2010; Uwah and Iwo, 2011). In order to improve crop production (Huang et al., 2010; Onunka et al., 2012), large amounts of fertilizer, particularly inorganic N fertilizer, have been used to amend Ultisols, which in turn has exacerbated soil acidification (Cai et al., 2015), induced metal toxicity and threatened environmental sustainability. The enhancement of biological N fixation is an alternative approach towards meeting the nutritional requirements of plants under low N fertilizer conditions (Cleveland et al., 1999).

Diazotrophs are agents of biological N fixation, that convert atmospheric N_2 into plant available N via the nitrogenase enzyme and contribute 100–290 Tg N yr^{-1} to the biosphere (Cleveland et al., 1999). The highly diverse diazotrophs include members of the Proteobacteria,

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Firmicutes, Cyanobacteria and Archaea, however, most of these groups are recalcitrant to laboratory cultivation techniques (Rösch et al., 2002). The *nifH* gene, which encodes a subunit of the nitrogenase enzyme and is highly conserved across the bacterial and archaeal domains, provides a useful marker for studying the diazotroph communities without the need for cultivation (Collavino et al., 2014). Consequently, the characterization of diazotroph communities by *nifH* genes could be a potential indirect approach to the assessment of levels of biological N fixation in soils (Reardon et al., 2014).

Although studies of diazotroph communities in Ultisols are rare, their activity, abundance and community structure in other soil types have been shown to be influenced by various physical and chemical properties, including soil pH (Nelson and Mele, 2006), oxygen partial pressure (Limmer and Drake, 1996), carbon quantity (Collavino et al., 2014), N availability (Pereira e Silva et al., 2013), soil texture (Pereira e Silva et al., 2011), and soil aggregate size (Poly et al., 2001), but there have been inconsistencies among findings. For example, soil pH was positively correlated with *nifH* gene abundance at all sampling times in the Netherlands (Pereira e Silva et al., 2011), but a negative association was reported in subtropical regions of Australia (Bai et al., 2015). Likewise, N availability has been found to have contrasting effects on *nifH* gene abundance, where stimulatory (Perez et al., 2014; Reardon et al., 2014) and inhibitory effects (Wang et al., 2017a; Zhalnina et al., 2015) have been reported. These inconsistent results may derive from specific diazotroph populations that respond variously to environmental factors in different ecosystems. For example, the relative abundance of *Mesorhizobium* was positively correlated with soil pH in alpine meadows with pH range from 5 to 8 (Wang et al., 2017b) while Brígido et al. (2007) found that some isolates of *Mesorhizobium* showed maximum growth at pH 5 from root nodules of chickpea.

Effects of soil fertilization on diazotroph communities is of growing concern (Meng et al., 2012; Nelson and Mele, 2006; Wang et al., 2017a), due to associated changes in soil physiochemical properties (Berthrong et al., 2014). Long-term experiments are suggested to be particularly useful since the succession and stabilization of microbial community structures requires a long period (Reardon et al., 2014). Long-term experiments have shown that diazotroph communities are affected by established inorganic N and phosphorus (P) fertilization in mangrove sediments (Romero et al., 2012) and by long-term inorganic fertilization in an acidic soil (Wang et al., 2017a). The effect of organic fertilization on diazotroph communities, however, remains unclear, as do the effects of combined applications of inorganic and organic fertilizer that are suggested as the best fertilization practice for enhancing crop yields and improving the quality of Ultisols (Huang et al., 2010).

Liming served as an effective way to ameliorate soil acidity, is also an important agricultural management in Ultisols, and has been shown to increase kernel yield of peanuts (Chang and Sung, 2004; Basu et al., 2008). The impact of liming on peanut nodulation was inconsistent from acidic soils, stimulation (Basu et al., 2008) and suppression (Van Rossum et al., 1994) were both reported. However, much less was known about the impact of liming on diazotroph communities in acidic soils, although some efforts have been made by Wang et al. (2017a) in a maize-wheat rotation system.

We established a long-term field experiment in April 1988 to monitor the influences of inorganic and organic fertilizers, and their combination, on crop yield and physicochemical and diazotroph properties of an Ultisol. Using quantitative PCR and high-throughput sequencing, soil samples were analyzed to understand long-term fertilization effects on diazotrophs in this study. Specifically, the objectives of this study were to: (1) evaluate whether long-term fertilization affected diazotroph abundance, diversity or community structure; and (2) determine the response of dominant diazotroph genera to key influencing factors in an acidic Ultisol.

2. Materials and methods

2.1. Experimental site and soil sampling

The fertilization experiment was established in April 1988 at the Yingtan Red Soil Ecology Experimental Station at the Chinese Academy of Sciences, Jiangxi, China (28°15′20″N, 116°55′30″E) in a region that has a subtropical monsoon climate, with a mean annual temperature of 17.6 °C and a mean annual precipitation of 1795 mm. The soil was derived from quaternary red clay and is classified as a Typic Plinthudult (Ultisols) based on USDA Soil Taxonomy (Soil Survey Staff, 1998) and comprised 41.2% clay, 33.2% silt, and 25.6% sand.

The field experiment was arranged as three replicates of seven treatments in a randomized block design, where plots measured 34.6 m^2 and were separated by a concrete wall embedded 100 cm into soil. The treatments comprised Control: no fertilizers; N: inorganic NPK fertilizer; NL: N + lime ($CaCO_3$); NPS: N + peanut straw; NRS: N + rice straw; NR: N + radish residues; and, NPM: N + pig manure aged for 3 months. The field was cultivated with continuous peanut monocropping in summer and fallow in winter. NPK was applied annually as 120 kg N ha^{-1} as urea, 68.7 kg P_2O_5 ha^{-1} as calcium magnesium phosphate, and 108.4 kg K_2O ha^{-1} as potassium chloride. In the NPS, NRS, NR, and NPM treatments, 30% of the inorganic N fertilizer was replaced by organic N. All treatments received the same rates of total N, P, and K, and calcium magnesium phosphate and potassium chloride were added to the organic fertilizer treatments to achieve the same rate of P or K. For the NL treatment, 1500 kg ha^{-1} $CaCO_3$ was applied annually. For all treatments, fertilizer and organic materials were evenly spread onto the soil surface by hand and immediately tilled into the plowed soil prior to sowing. Each year, peanut (cv Ganhua 5) was sown on 10 April by placing two seeds per hole to give a 20 cm plant-to-plant spacing and 30 cm row-to-row spacing per plot.

Soil samples were collected on 13 December 2014, where ten soil cores (10 cm diameter, 0–20 cm depth) were randomly collected from each plot and combined to form one composite sample per plot. Soil samples were transported from the study site to the laboratory in a constant temperature box containing ice. After visible stones and plant residues were removed using forceps, the soil samples were gently broken apart along natural-break points, passed through an 8-mm sieve and thoroughly mixed before being divided into two parts. One part was analyzed for soil physicochemical properties, while the other part was sieved to < 2 mm, to increase homogeneity, and immediately stored at −80 °C for subsequent DNA extraction.

2.2. Soil physicochemical analysis

Air-dried soils were used to determine soil organic carbon (SOC), total nitrogen (TN), available P (AP), available K (AK), and pH, and fresh soil samples were analyzed for content of soil ammonium (NH_4^+-N), nitrate (NO_3^--N), dissolved total N (DTN), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON). Soil pH was measured using a glass electrode in a 1:5 soil to water ratio. Concentrations of SOC and TN were determined by the wet oxidation redox titration and micro-Kjeldahl methods, respectively (Lu, 2000). DOC was extracted by incubating 10 g of fresh soil (on an oven-dried basis) with 50 ml deionized water for 30 min on an end-over-end shaker at 25 °C. The extracted samples were centrifuged at 10000 rpm for 10 min at 4 °C and resulting supernatants were filtered through a 0.45-μm membrane filter (Whatman, Clifton, NJ, USA) and DOC content was quantified using a Shimadzu C analyzer (TOC Vcph, Shimadzu, Kyoto, Japan) (Liu et al., 2014). We extracted NH_4^+-N, NO_3^--N, and DTN in 2.0 M KCl before measurement in a continuous flow analyzer (San^{++}, Skalar, Holland) and DON was calculated as DTN − NH_4^+-N − NO_3^--N. AK in soil was extracted with 1 M ammonium acetate and analyzed by flame photometry (FP640, INASA, China), while AP was extracted with 0.0125 M H_2SO_4 in 0.05 M HCl and determined using the molybdenum blue