Science of the Total Environment 649 (2019) 686–694

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Impacts of Mo application on biological nitrogen fixation and diazotrophic communities in a flooded rice-soil system

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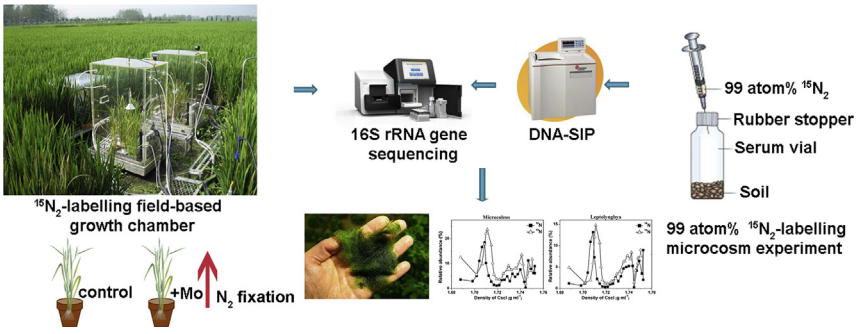
HIGHLIGHTS

- Mo application enhanced N_2 fixation in a rice-soil system under no N fertilization.
- Mo application increased the number of *nifH* gene copies in paddy soil.
- Mo application stimulated the growth of cyanobacteria in paddy soil.
- Non-heterocystous cyanobacteria *Leptolyngbya* and *Microcoleus* were sensitive to Mo.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 June 2018
Received in revised form 23 August 2018
Accepted 23 August 2018
Available online 24 August 2018

Editor: Charlotte Poschenrieder

Keywords:
Biological nitrogen fixation
Molybdenum
Rice
16S rRNA gene
DNA-SIP
Cyanobacteria

ABSTRACT

Molybdenum (Mo) deficiency in the farmland of China may limit biological nitrogen fixation (BNF), however, the impact of Mo application on BNF capacities and diazotrophic communities in rice-soil systems is unclear. In this experiment, treatments in a 6.7 atom% $^{15}N_2$-labelling field-based growth chamber for 74 days and treatments in a 99 atom% $^{15}N_2$-labelling microcosm experiment for 40 days combined with 16S rRNA gene sequencing and DNA-stable isotope probing (SIP) were used to investigate the impacts of Mo application on BNF and diazotrophic communities. Our results showed that under the condition that no nitrogen (N) fertilizer was applied, Mo application (500 g sodium molybdate ha^{-1}) significantly increased N_2 fixation in a rice-Inceptisol system, from 22.3 to 53.1 kg N ha^{-1}. Mo application significantly increased the number of *nifH* gene copies and the relative abundance of cyanobacteria in both growth chamber and microcosm experiments. Among cyanobacteria, the relative abundances of the most abundant genera *Leptolyngbya* and *Microcoleus* were significantly increased by Mo application. $^{15}N_2$-DNA-SIP further demonstrated that *Leptolyngbya* and *Microcoleus* incorporated $^{15}N_2$. Mo application greatly increased BNF in Mo-deficient paddy field (≤0.068 mg kg^{-1}) and stimulated the growth of cyanobacteria. These results indicated that Mo application in Mo-deficient paddy field could be a useful measure to increase soil N input under no N fertilization.

1. Introduction

Rice fields have long been known to fix dinitrogen (N_2). Ladha et al. (2016) suggested that non-symbiotic N_2 fixation could contribute up to 22 kg Nitrogen (N) ha^{-1} $year^{-1}$ for rice by using the difference between

Abbreviations: BNF, biological nitrogen fixation; OTU, operational taxonomic unit; SIP, stable isotope probing; NGS, next-generation sequencing; NMD, non-metric multidimensional scaling; HSD, honestly significant difference; d.w.s, dry weight of soil.

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https://doi.org/10.1016/j.scitotenv.2018.08.318
0048-9697/

the total harvested N in cereals and fertilizer N sources during a 50-year period (1961 to 2010). By direct $^{15}N_2$-labelling, Bei et al. (2013) assessed a biological nitrogen fixation (BNF) of 45 kg N ha^{-1} over 70 days under no N fertilizer application and found that 49% of the fixed N_2 was allocated in rice and 51% in soil. China owns 20% of the world's rice planting area and consumes nearly 40% of the total N fertilizer (Yan et al., 2006). To reduce the environmental problems caused by excessive application of N fertilizer, the improvement of BNF in paddy soils is of great importance and necessity.

BNF is the process of reducing N_2 to ammonia via catalysis by nitrogenase. Molybdenum (Mo) is the central element of the Mo-nitrogenase enzyme, which is more efficient than other forms of the nitrogenase in the conversion of N_2 into NH_4^+ at high temperature (30 °C) (Miller and Eady, 1988; Walmsley and Kennedy, 1991). However, Mo is the rarest (i.e., 1.1 ppm) of all biometals in the Earth's crust, soil and plants (Kabata-Pendias, 2010; Wedepohl, 1995). Mo deficiency limits the N-fixation efficiency of symbiotic fixers in natural ecosystems, managed pastures and legume crops (Gupta, 1997; Kaiser et al., 2005) and asymbiotic fixers in highly leached temperate forest soil (Jean et al., 2013; Silvester, 1989). Rousk et al. (2017) found that moss BNF reached 4 times higher levels shortly after Mo addition. Previous work shows that when the available Mo concentration of soil is lower than 0.15 mg kg^{-1}, N_2 fixation is inhibited for legume plants (Zou et al., 2008). According to this standard, approximately 47% of the agricultural area in China is Mo deficient, and it is mainly distributed in the east of China (Zou et al., 2008). However, little work has examined the influences of molybdenum application on BNF capacities in rice-soil systems.

Diazotrophs drive N_2 fixation, and some work has been done on their community composition, BNF activities and influencing factors in rice field. Islam et al. (2012) found that there were 16 known genera of putative diazotrophs in a Korean paddy soil, with the two most populous being *Burkholderia* and *Sphingomonas*. Twenty-one known genera of putative diazotrophs in rhizospheric soil and roots of rice have been found in a Brazilian paddy soil, with the two most abundant being *Burkholderia* and *Enterobacter* (Costa et al., 2013). Tang et al. (2017) found that nitrogen, phosphorus, potassium and straw amendment influenced diazotroph populations and acetylene reduction activity in paddy field. Cyanobacteria are important BNF contributors, contributing 19–28 kg N kg^{-1} $crop^{-1}$ in acidic, saline and neutral soils (Hashem, 2001). Rousk et al. (2017) found that cyanobacteria biomass colonizing moss was increased by Mo application. However, the impacts of Mo application on microbial communities and abundances in paddy field are elusive.

^{15}N-DNA-stable isotope probing (SIP) represents an appealing method for examining N_2-fixing organisms (Buckley et al., 2007a). Only those organisms that are actively engaged in N_2 fixation would become heavily labelled with ^{15}N in response to incubation with $^{15}N_2$ (Chalk et al., 2017). By $^{15}N_2$-DNA-SIP, Buckley et al. (2008) explored linkages between different C sources and N_2 fixation by specific diazotrophic populations in soil. Pepe-Ranney et al. (2016) found that Clostridiaceae and Proteobacteria (such as *Pseudomonas*, *Klebsiella*, *Shigella* and *Ideonella*) were the main N_2-assimilating microorganisms during early crust formation. However, the diazotrophs that respond sensitively to Mo addition and whether they play roles in N_2 fixation in rice field are unknown.

N is the most difficult of the elements to use in SIP because ^{15}N-labelled nucleic acids are only slightly denser than ^{14}N-labelled nucleic acids. Cadisch et al. (2005) revealed that 40 atom% ^{15}N was the lowest amount of ^{15}N-DNA enrichment required for ^{15}N-DNA-SIP experiments. Tag-SIP (combining 16S rRNA gene sequencing and SIP) can easily identify and compare the density shifts of DNA of individual operational taxonomic unit (OTU) (Connelly et al., 2014; Morando and Capone, 2016). Therefore, in this paper, a 6.7 atom% $^{15}N_2$-labelling growth chamber was used to investigate the impacts of Mo addition on BNF capacities and microbial communities, and a 99 atom% $^{15}N_2$-labelling microcosm DNA-SIP experiment was used to study diazotrophs that incorporated $^{15}N_2$. The experimental flowchart was displayed in Fig. 1. The objectives were (i) to assess the effects of Mo application on BNF in rice-soil systems, (ii) to explore the influence of Mo application on microbial communities of paddy soils, and (iii) to find the main diazotrophs that fix N_2 after Mo application.

2. Materials and methods

2.1. 6.7 atom% $^{15}N_2$-labelling growth chamber

2.1.1. Site description and growth chamber control system

Detailed information of the study site and soil characteristics can be found in Bei et al. (2013) except that the total Mo of soil was 0.6 mg kg^{-1} and the available Mo of soil was 0.068 mg kg^{-1}, which was extracted by oxalic acid-oxalite ammonium and determined by Inductively coupled plasma mass spectrometry (ICP-MS). The design of the airtight growth chamber and control systems generally followed Bei et al. (2013) and was modified with a device that consumed O_2 by burning it to maintain the O_2 concentration at approximately 21%. The O_2 concentration-controlling system contains an oxygen-consuming burning lighter installed into the chamber with butane as fuel and a portable oxygen-measuring instrument (CY-12C, Aipu Equipment Co., Ltd., China). The oxygen content in the growth chamber was monitored once a week. When the oxygen content in the growth chamber was higher than 21%, the lighter was ignited to burn the excessive O_2 in the growth chamber.

2.1.2. Experiment setup

In this experiment, there were two main treatments (enriched $^{15}N_2$ and normal N_2) and two sub-treatments (with Mo addition and without Mo addition). $^{15}N_2$ treatments were realized by the abovementioned airtight growth chamber technique. There were two growth chambers (one for the 6.7 atom% $^{15}N_2$, and one for normal N_2), which were placed side by side with a distance of 0.6 m between them. In each chamber, there were 6 pots (each with a dimension of length × width × height of 12 × 12 × 20 cm), 3 for Mo addition and 3 for no Mo addition. In each pot, 2.6 kg of air-dried soil was packed to 15 cm at a bulk density of 1.2 g cm^{-3}. Before rice planting, pots were flooded with water for 10 days to pick out vascular weeds (but retaining the autotrophic biofilms at the water surface). Before rice seedling transplantation, 166 mg superphosphate pot^{-1} (equivalent to 70 kg P_2O_5 ha^{-1}), which contained about 0.73 μg Mo pot^{-1} (equivalent to 0.5 g Mo ha^{-1}) according to McBride and Spiers (2001) and 160 mg potassium chloride pot^{-1} (equivalent to 70 kg K_2O ha^{-1}) following local application rates were dissolved in water and applied to each pot. Sodium molybdate [0.72 mg pot^{-1}; equivalent to 500 g ha^{-1}, a suitable concentration to support algal growth (Venkataraman, 1972)] was applied to the Mo-application treatment. The applicated dosage of superphosphate, potassium chloride and sodium molybdate in each pot was calculated according to the area of pot ($12 * 12 = 144\ cm^2$). One-month old rice seedlings [*Oryza sativa* L. Wuyunjing 23 (W23)] were obtained from a local farmer's field. Rice seedlings with similar height and thickness were chosen, and one rice seedling was transplanted into each pot after soil on the seedling root had been thoroughly washed away. After one week of rice seedling recovery, pots were placed into the two chambers (each with 6 pots, 3 with Mo addition and 3 without Mo addition). The treatment with Mo was hereafter referred to as W23 + Mo, and the control treatment without Mo was referred to as W23.

One week after the pots were placed into the chambers, 21 l of air in a chamber was replaced by 21 l of $^{15}N_2$ (99 atom% ^{15}N), and the air in the other chamber was retained as normal air. $^{15}N_2$ was produced and purified following the method of Ohyama and Kumazawa (1981). $^{15}N_2$ was generated by adding sodium hypobromite (NaOBr) to ^{15}N-labelled ammonium sulphate $(^{15}NH_4)_2SO_4$ (99.14 ^{15}N atom%, Shanghai Engineering Research Center of Stable Isotopes), and the products were passed through a liquid N_2 cold trap, a $KMnO_4$-KOH solution and a