Applied Soil Ecology 116 (2017) 23–29

Contents lists available at ScienceDirect

Applied Soil Ecology

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

N_2O emissions dominated by fungi in an intensively managed vegetable field converted from wheat–rice rotation

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ARTICLE INFO

Keywords:
Fungi
Bacteria
Denitrification genes
Vegetable field
N_2O emissions

ABSTRACT

N_2O emissions through fungal pathways have received wide attention and play an important role across various ecosystems. Previous studies have shown that fungi may be more adaptable than bacteria to changeable and extreme conditions. To help identify the fungal roles in N_2O emissions from vegetable fields under intensive management, we investigated the fungal contributions in a degraded vegetable field in China, using the substrate-induced respiration–inhibition method and qPCR. The fungal-to-bacterial biomass ratios were 2.84 and 0.48 in the vegetable field and wheat–rice rotation field soils, respectively; this was in accordance with the abundance ratios of the ribosomal genes. As expected, fungi played a dominant role in N_2O emissions from the vegetable field soils, compared with those from the wheat–rice rotation field soils, regardless of whether or not they were amended with glucose. When glucose was added with a fungicide treatment, almost all of the N_2O emissions were stopped (99.09%). However, accompanying the decline of bacterial abundances and total carbon content in the vegetable field soils, the total N_2O emissions from the vegetable field soils were only 20.6% of those from the wheat–rice rotation field soils, when glucose was not applied. In addition, although the copy numbers of the nitrous oxide reductase gene (*nosZ*) were lower in the vegetable field soils, as expected, the relative abundances (based on the copy number ratios, *e.g.*, *nosZ*/16S) of nitrifiers in the bacterial community increased; this implied that the fungi also indirectly affected the nitrogen cycle. For a more comprehensive understanding of fungal roles in N_2O emissions, further studies are needed that include N_2 determination.

1. Introduction

It is widely accepted that fungi play an important role in N_2O emissions from various ecosystems, such as grasslands, forests, croplands and wetlands (Chen et al., 2014; Crenshaw et al., 2008; Rütting et al., 2013; Seo and DeLaune, 2010). Due to their traits, the potential roles of fungi in N_2O production have received wide attention. Compared with bacteria, fungi exhibit wider pH ranges for optimal growth and utilize more complicated organic substances (Rousk et al., 2010; van der Wal et al., 2006). In addition, fungal denitrification has a higher threshold oxygen demand (Zhou et al., 2001 Zumft, 1997). Such denitrification could be of great importance under aerobic or moderately reducing conditions in sediments (Seo and DeLaune, 2010). More notably, fungi lack nitrous oxide reductase (Shoun et al., 1992), which is a key reductase that regulates the conversion of N_2O to N_2.

Generally, fungi appear to be more adaptable to adverse environmental conditions than bacteria. This may be because the strong cell walls of fungi could help to tolerate drastic changes in soil water potential and reductions in water content, while their mycelial networks can transport nutrients over larger distances (Guhr et al., 2015). These special structures enhance the competitiveness of fungi in extreme situations, such as arid and oligotrophic environments.

Due to high yield requirements, vegetable fields in China are often characterized by intensive management, including high nitrogen fertilizer inputs and multiple cropping. For example, average N fertilization rates (300–700 kg N ha^{-1}) for each vegetable growing season are twice those for non-vegetable crops (150–300 kg N ha^{-1}) (Zheng et al., 2004); sometimes the total rates can reach 1400–1636 kg N ha^{-1} yr^{-1} (Xiong et al., 2006; Zhu et al., 2011). In addition to the heavy nitrogen loads and high cropping intensity, frequent irrigation is common in vegetable fields (Min et al., 2012); this leads to continuous drying–wetting cycles and could affect both organic mineralization and the production of associated biogenic gases (CO_2 and N_2O) (Harrison-Kirk et al., 2013). Furthermore, these intensive management practices not only lead to the acidification and salinization of soils (Zhu et al., 2011), but may also alter the diversity and abundance of the microbial community. In fact, soil enzyme activities, microbial diversity and biomass can significantly decline under such severe conditions (Lei

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http://dx.doi.org/10.1016/j.apsoil.2017.03.021
Received 26 August 2016; Received in revised form 27 March 2017; Accepted 28 March 2017
Available online 11 April 2017
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et al., 2015; Shen et al., 2010). Continuous cropping could also promote the growth of pathogenic fungi (Bai et al., 2015; Lebreton et al., 2004), which may give rise to a microbial community dominated by fungi (Gil et al., 2011).

Vegetable production has gradually become a very important part of agriculture in China, in-line with rapid urbanization. In the 1990s, an average of 6.18% of the cultivated area in China consisted of vegetable fields, which received 17% of the anthropogenic reactive nitrogen applied; the vegetable-cultivated area ratio more than doubled (to 12.7%) by 2013 (National Bureau of Statistics of China, 2014; Zheng et al., 2004). Vegetable cultivation significantly contributes to national N_2O budgets in China; this has received attention, leading to several field assessments (Diao et al., 2013; Min et al., 2012; Xiong et al., 2006; Zhang et al., 2015; Zhu et al., 2011). However, the roles of fungi in the N_2O emissions that vegetable fields produce have not been identified.

In this study, we explored the roles that fungi play in N_2O emissions within a greenhouse vegetable field (VF), which had degraded seriously to the point that plants were not able to grow in it. We hypothesized that there would be large differences in the microbial community and abundance, especially in terms of the ratios between fungi and bacteria, of the VF soils in comparison with the community present in a wheat–rice field (WF). The fungal and bacterial contributions to N_2O emissions were assessed using a fungicide and bactericide. The abundances of related functional genes (*narG*, *nirK*, *nirS*, and *nosZ*, respectively encoding nitrate reductase, cytochrome cd_1 nitrite reductase, copper-containing nitrite reductase and nitrous oxide reductase) were measured. We hypothesized that, compared to the WF: (1) fungi would dominate the N_2O emissions in the soil of the VF, and (2) because fungi lack nitrous oxide reductase, the VF would produce more N_2O.

2. Materials and methods

2.1. Soil sampling and site description

The two study sites, a VF and a WF, were located in Suzhou, Jiangsu province, China (31°31′N, 21°05′E). The VF had an 8-m tall polytunnel greenhouse and was converted from a wheat–rice field 10 yrs ago. The distance between the two fields was about 200 m. The VF had been left fallow for one year (2012–2013), because it was seriously degraded and plants were unable to grow healthily. Before the no-tillage period, green pepper (*Capsicum*) and cucumber (*Cucumis sativus* Linn.) were cultivated several times each year and fertilizer was applied at about 1000 N kg ha^{-1} $year^{-1}$. During cultivation, the water used to irrigate the field was sourced from an underground reservoir.

Soil samples (Hydragric Anthrosols, based on the FAO World Reference Base) were collected (at 0–10 cm depth) randomly from each field in December 2013. The samples were sieved through a 2 mm mesh, and stored at −20 °C for the soil incubation experiment and DNA extraction.

2.2. Optimum inhibitor concentrations for substrate-induced respiration–inhibition

The substrate-induced respiration–inhibition method (SIRIN) was used to distinguish the contributions of bacteria and fungi to the total metabolic activity of the soils, according to Anderson and Domsch (1973). In this method, cycloheximide (a fungicide) and streptomycin (a bactericide) were used to selectively inhibit the fungal and bacterial activity in the soil. The inhibitors could affect non-target microorganisms, so it was necessary to run a pilot study and determine the optimum inhibitor concentrations; we evaluated the effects of five concentrations of streptomycin (1, 4.5, 6, 10, and 15 mg g^{-1} dry soil) and cycloheximide (1, 4.5, 6, 10, and 15 mg g^{-1} dry soil). Subsamples of the soil (20 g dw) were preincubated for 48 h at 25 °C in 250 mL flasks. Then, inhibitors (streptomycin, cycloheximide or both) mixed with talc powder were added to the soils and the flasks were sealed with Parafilm, before incubation at 5 °C for 12 h. After 12 h, the soil water content was adjusted with glucose solution, to achieve 60% water holding capacity and a glucose concentration of 6 mg glucose-C g^{-1} dry soil, which meant any microorganisms present could obtain enough carbon for metabolism (Lin and Brookes, 1999). The flasks were again sealed with Parafilm. CO_2 emissions were sampled at 4 h and 8 h after the addition of glucose; because the CO_2 emissions remained stable 4–8 h after glucose solution addition, they represented the biomass in the soil. Each inhibitor treatment and control treatment (without any inhibitor addition) was performed in triplicate.

The optimum inhibitor concentrations were calculated based on the inhibitor additivity ratios (IAR), $[(A - B) + (A - C)]/(A - D)]$, where A is the CO_2 released from soil amended with glucose only, B is the CO_2 released from soil amended with glucose and streptomycin; C is the CO_2 released from soil amended with glucose and cycloheximide; and D is the CO_2 released form soil amended with glucose, cycloheximide and streptomycin. The optimum combined concentrations were selected when the ratio approached 1 (Anderson and Domsch, 1973).

2.3. The contribution of fungi and bacteria to N_2O emissions

After selecting the optimum inhibitor concentrations, the relative contributions of the fungi and bacteria to the N_2O emissions were determined. The experiment consisted of four treatments which were the same as the pilot study above, but after the addition of the inhibitors, the soil water content was adjusted with NH_4NO_3 solution (instead of glucose solution) to achieve 60% water holding capacity and a NH_4NO_3-N concentration of 100 μg g^{-1} dry soil. The flasks were again sealed with Parafilm. Each inhibitor treatment and control treatment (without any inhibitor addition) was performed in triplicate. Gases produced were sampled with syringes at 2 h, 26 h, 50 h, 98 h, and 146 h after NH_4NO_3 addition, and were measured within 12 h or stored in vacuum flasks. Before gas sampling, the Parafilm was removed from each flask for 30 min, to balance the gas inside, and then the flasks were sealed for 6 h with rubber stoppers.

We also designed a group of treatments with a carbon source. To avoid excessively rapid growth of microorganisms, we decreased the glucose concentration (compared with that used in the pilot study) to 2 mg g^{-1} dry soil. Because the glucose was consumed quickly, within 24 h, sampling was conducted at 4 h, 8 h, 12 h, and 24 h after glucose/NH_4NO_3 addition. The sealing time (with rubber stoppers before sampling) was 2 h.

2.4. Soil properties and gas analysis

Extractions of fresh soil subsamples were performed with 2 M KCl solution, under a soil/water ratio of 1:5, and the contents of NH_4^+ and NO_3^- were determined with a continuous-flow analyzer (Skalar, Netherlands). Fresh soil subsamples were air-dried and sieved with a 150-μm mesh, before measuring total C and N contents using a Vario MAX CN elemental analyzer (Elementar, Germany). Fresh soil subsamples sieved with a 2-mm mesh were used to determine the soil particle size, using a laser particle analyzer (LS 13 320, USA). The soil pH was measured in 0.01 M $CaCl_2$ solution, at a soil/solution ratio of 1:5, using a pH meter (Sartorius, USA). Major characteristics of the soils are shown in Table 1.

The concentrations of the headspace gases, N_2O and CO_2, were analyzed with an Agilent 7890A gas chromatograph. The column temperature was set at 55 °C and the carrier gas was high-purity N_2, with a flow rate of 25 mL min^{-1}. For N_2O determination, a stainless steel column (2 mm ID, 3 m length) was packed with Porapak Q (80/100 mesh); the electron capture detector was set at 330 °C. To determine CO_2, the flame photometric detector (250 °C) was used, separated by a stainless steel column (2 mm ID, 2 m length) packed with Porapak Q (60/80 mesh).

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