Soil Biology & Biochemistry 69 (2014) 157–167

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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

N_2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer

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ARTICLE INFO

Article history:
Received 22 July 2013
Received in revised form
29 October 2013
Accepted 30 October 2013
Available online 11 November 2013

Keywords:
Surface fertilization
Organic fertilizer
Nitrous oxide
Fungal denitrification
Fungal community
Andisol

ABSTRACT

Agricultural fields are one of the largest anthropogenic sources of atmospheric nitrous oxide (N_2O). Fungi have been suggested to contribute substantially to N_2O emission in terrestrial environments; however, the extent of the fungal contribution and the phylogenetic and physiologic nature of related fungal communities in agricultural fields are largely unknown. This study focused on the large N_2O emission from cropland soil that occurs after granular organic fertilizers were applied to the surface. The granular organic fertilizers applied were evidently covered by fungal mycelia. An experiment using a soil microcosm was established to imitate the field observations. N_2O emissions following surface organic fertilization were suppressed by 84 and 20% after the addition of cycloheximide (a fungal inhibitor) and streptomycin (a bacterial inhibitor), respectively, suggesting that fungi provide the main contribution to the observed N_2O emission. The population density and community composition of fungi in the surface-fertilized and non-fertilized soils in the field were determined using colony counting, denaturing gradient gel electrophoresis and subsequent phylogenetic analyses. Thirty-four fungal strains were isolated from the soils, and their N_2O producing activities were analyzed. Fungal population density in the surface-fertilized soil (2.6×10^6 CFU/g) was much higher than that in the non-fertilized soil (1.0×10^5 CFU/g). In addition, the fungal community compositions of the soils differed. *Actinomucor elegans*, *Bionectria ochroleuca*, *Fusarium avenaceum*, *Fusarium equiseti*, *Fusarium oxysporum*, *Fusarium solani* and *Nectria* sp. dominated the surface-fertilized soil, and their activity in producing N_2O was confirmed. These results suggested that N_2O emission after the surface application of granular organic fertilizers in the cropland field mainly resulted from fungal denitrification.

1. Introduction

Agricultural fields are one of the largest anthropogenic sources of atmospheric nitrous oxide (N_2O) which contributes to approximately 6–11% of global total N_2O emissions (IPCC, 2007; Davidson, 2009). N_2O generates a 298-fold stronger effect on global warming than carbon dioxide (CO_2) and is a potent greenhouse trace gas (IPCC, 2007); N_2O is also involved in stratospheric ozone depletion (Ravishankara et al., 2009). N_2O emissions are greatly stimulated after nitrogen fertilization because N input enhances the microbial N_2O-producing activities in soils (Sánchez-Martín et al., 2008). Organic fertilizers are now widely employed instead of chemical fertilizers for the development of sustainable agriculture and the integration of crop nutrition (Inubushi et al., 2000). In particular, the granular organic fertilizers are commonly used due to their ease of transportation, storage and handling. However, the application of organic fertilizers contributes to higher N_2O emission from soils compared with chemical fertilizers (Akiyama and Tsuruta, 2003; Jones et al., 2007; Hayakawa et al., 2009; Toyoda et al., 2011).

N_2O is produced via nitrification and denitrification processes in soils (Butterbach-Bahl et al., 2013). A wide phylogenetic range of bacteria are involved in denitrification, in which nitrate and nitrite are reduced to gaseous N_2O. Ammonia-oxidizing bacteria (AOB) and archaea (AOA) produce N_2O as a byproduct through the oxidation of ammonia to nitrite via the nitrification process (Arp and Stein, 2003; Santoro et al., 2011). Because bacterial nitrification and denitrification process are well known, the emission of N_2O from agricultural soils has been studied mainly by focusing on

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http://dx.doi.org/10.1016/j.soilbio.2013.10.044

these contributions (Conrad, 1996; Miller et al., 2008; Hamonts et al., 2013). However, many fungal species (e.g., *Trichoderma hamatum*, *Chaetomium funicola*, *Neocosmospora vasinfecta*, *Paxillus involutus* and *Penicillium digitatum*) can produce N_2O (Shoun et al., 1992; Yanai et al., 2007; Prendergast-Miller et al., 2011; Jirout et al., 2013); this finding was elucidated after the initial discovery that a hyphomycetes fungus, *Fusarium oxysporum*, has the distinct ability to produce N_2O (Shoun and Tanimoto, 1991). Moreover, some recent studies have demonstrated that fungal contributions to N_2O emissions in terrestrial environments such as grassland fields and forest soils are larger than the contributions of bacterial denitrification and/or nitrification (Laughlin and Stevens, 2002; Spokas et al., 2006; Laughlin et al., 2009; Blagodatskaya et al., 2010). However, despite the importance of agricultural soils as a large N_2O emission source, the extent of fungal contributions to such emissions and the fungal communities that produce this N_2O in cropland soils have not been well characterized.

As soil nutrients are rapidly consumed during crop growth, fertilizer is usually applied several times per growing season. The first (basal) fertilization is performed by incorporating fertilizer into the plowed layer, and several top-dressings are applied later. The rate of N_2O emission can drastically increase after the basal fertilization, particularly in the wake of rains (Li et al., 2002; Hayakawa et al., 2009). In our radish-growing field located in Niigata, Japan (described below), a large quantity of N_2O was emitted after the basal fertilization, as observed. However, substantial quantities of N_2O were also emitted after later surface applications. The fertilizers applied onto the field surface were obviously covered by fungal mycelia. Based on these observations, we hypothesized that fungi rather than bacteria were responsible for the N_2O emission after the application of organic fertilizers as top-dressing in the cropland field.

To test this hypothesis, we performed experiments (1) to assess the relative contribution of bacteria and fungi to N_2O production using antibiotics in a laboratory-scale soil microcosm system that imitated the field conditions, (2) to examine the difference in fungal population density and community composition between surface-fertilized and non-fertilized soil in the field, and (3) to isolate the abundant fungi in the surface-fertilized soil and analyze their N_2O and N_2 producing activities.

2. Materials and methods

2.1. Study site and field management

The study field is located at the Niigata Agricultural Research Institute (N37°26′, E138°52′, Nagaoka, Niigata, Japan). Radish (*Raphanus sativus* var. *longipinnatus*) was cultivated in the field from Sep. 8 to Nov. 29 in 2011. The total precipitation and mean daily air temperature during the cultivation period were 672.1 mm and 15.5 °C, respectively. The soil is of an Andisol type, which is widespread in Japan. The physicochemical properties of the soil are as follows: total carbon, 39 g-C/kg-soil; total nitrogen, 2.6 g-N/kg-soil; bulk density, 0.81 g cm^{-3}; solid phase rate, 34.5%; and pH, 6.5. The field experiment was arranged in a randomized block design with three replicate plots per treatment. Each block was 25 m^2 (5 m × 5 m) and comprised two 5-m × 2.5-m plots: one with applied organic fertilizer and one without fertilizer application. We used a commercially available granulated organic fertilizer that is a mixture of food manufacturing residues such as feather meal, fish meal, rapeseed meal, rice bran, oil palm ash and poultry litter ash (Total N: 6%, P_2O_5: 6%, K_2O: 6%). A basal fertilization of granular organic fertilizer at 21 g N m^{-2} was performed on Sep. 7 in 2011 by incorporating the fertilizer into the plowed layer. Seeds were sown in all the plots on Sep. 8. Supplemental top-dressings of granular organic fertilizer at 3 g N m^{-2} were performed on Oct. 7 and 31, respectively. All radishes were harvested on Nov. 29.

2.2. Measurements of N_2O flux and soil N concentrations during the cultivation period

N_2O flux in the field was measured every week during the cultivation period using the chamber method. Chambers were set at three locations in each plot. Gas samples (500 ml) were taken from the chambers at 0, 15, and 30 min after closure. The N_2O concentration in the samples was measured using a gas chromatograph equipped with an electron capture detector (GC-ECD; GC-14B, Shimadzu, Kyoto, Japan). The N_2O flux was calculated from the increase in the N_2O concentration of the sample. Soil samples were collected at 0–5 cm depth at three locations in each plot on the same day to measure the nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in the soils. Ten-gram soil samples were extracted with 100 ml of 2 M KCl solution. The NH_4^+ and NO_3^- concentrations in the extract were measured colorimetrically (Akiyama and Tsuruta, 2003).

2.3. Establishment of laboratory-scale soil microcosm

A laboratory-scale soil microcosm system was established to imitate the field fertilization conditions. Twenty grams of non-fertilized soil were placed in 80-ml glass bottles and mixed with 0.25 g of granular organic fertilizers as the basal fertilization. The soil water content was adjusted to 50% of maximum water holding capacity (MWHC) by adding distilled water. All bottles were covered with aluminum foil and incubated at 27 °C for 46 days. Soil water content was increased to 70% of MWHC on the 4th day of incubation to imitate a field rain event. A 0.25-g top-dressing of granular organic fertilizer was applied on the 18th day of incubation. Soil water content was again increased to 70% of MWHC on the 24th day of incubation. The N_2O flux was measured every 2 days after sealing and incubating the bottles for 60 min. The N_2O concentration was measured using a GC-ECD instrument (GC-2014, Shimadzu, Kyoto, Japan).

2.4. Substrate-induced respiration (SIR) inhibition experiment

The relative contributions of fungal and bacterial activity to N_2O emission were evaluated through the substrate-induced respiration (SIR) inhibition method (Anderson and Domsch, 1975) using the soil of the soil microcosm systems on the 8th and 34th incubation days when N_2O flux peaks were observed after the basal and additional fertilizations, respectively. Optimal inhibitor concentrations (5 mg g^{-1} soil of cycloheximide and 8 mg g^{-1} soil of streptomycin) were determined through preliminary experiments, in which glucose (5.0 mg g^{-1} soil) as a C source, cycloheximide (0, 2.0, 5.0, and 10.0 mg g^{-1} soil) as a fungal inhibitor and streptomycin sulfate (0, 2.0, 5.0, 8.0, and 12.0 mg g^{-1} soil) as a bacterial inhibitor were used according to Laughlin and Stevens (2002). The inhibitors and glucose were dissolved in 5 ml of distilled water and applied to the duplicate soil microcosm system containing 20 g of the soils as described above. The bottles containing soil, glucose, and antibiotic solutions were incubated at 27 °C under aerobic conditions for 2 h on a rotary shaker (150 rpm). The bottles were then sealed and incubated for 4 h under the same conditions, and gaseous N_2O and CO_2 concentrations were measured every 2 h using a GC-TCD instrument (GC-14, Shimadzu, Kyoto, Japan).

The contributions of nitrifiers to N_2O emission were determined using 0.01% acetylene (C_2H_2) as a nitrification inhibitor (Schimel et al., 1984). The C_2H_2 was added in the headspace of the soil samples on the 8th and 34th incubation days after sealing the