Fungal Ecology 17 (2015) 155–163

Contents lists available at ScienceDirect

Fungal Ecology

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

Nitrous oxide productivity of soil fungi along a gradient of cattle impact

Jiří Jirout

Institute of Soil Biology, Biology Centre CAS, v. v. i., Na Sádkách 7, CZ 37005 České Budějovice, Czech Republic

ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form
19 June 2015
Accepted 14 July 2015
Available online 8 August 2015

Corresponding editor: Björn Lindahl

Keywords:
Soil
Fungi
Nitrous oxide
Selective inhibition
Cattle overwintering
Oxygen availability

ABSTRACT

The objective of the study was to identify N_2O-producing fungi isolated from six qualitatively different sections of an overwintering pasture with substantial cattle impact. 80 out of 164 fungal isolates were considered as N_2O-producers in nitrite-containing medium, representing 33 fungal species of 23 different genera. Ability to produce N_2O was newly reported in eight genera: *Arthrinium*, *Gibellulopsis*, *Ilyonectria*, *Lichtheimia*, *Paraphaeosphaeria*, *Purpureocillium*, *Tolypocladium* and *Westerdykella*. Three levels of fungal N_2O-productivity were assigned according to the fraction of nitrite-N transformed into N_2O–N: < 1%, 1–10%, over 10%. Fungi capable of high and moderate transformation rates were predominantly isolated from sections under current or past cattle impact, where they contributed with a maximum of 65% of the total N_2O emissions. There was no significant effect of cultivation conditions on the fraction of N_2O-producing fungi. The results demonstrate that N_2O-producing fungi are a common constituent of fungal communities in soils impacted by overwintering cattle.

1. Introduction

Nitrous oxide (N_2O) contributes to global climate change as a very potent greenhouse gas, being about 300 times more effective at trapping heat than CO_2 (Durandeau et al., 2010). It is estimated that 65% of worldwide nitrous oxide (N_2O) emissions arise from soil during nitrification and denitrification processes (Smith and Conen, 2004). The rapidly increasing rate (up to 0.3% yr^{-1}) of atmospheric N_2O concentration (Khalil and Rasmussen, 1988; Hall et al., 2007) requires an improved understanding of soil microorganisms that facilitate N_2O-producing processes (Mothapo et al., 2013). Besides prokaryotic nitrifiers and denitrifiers (Werber and Mevarech, 1978; Smith and Zimmerman, 1981; Zumft, 1997), the ability to release N_2O has also been reported in eukaryotes, especially filamentous fungi and yeasts (Yoshida and Alexander, 1970; Bollag and Tung, 1972).

Fungi possess a multimodal type of respiration, which is a survival strategy in reaction to rapid and dynamic changes in the oxygen supply (Manohar and Raghukumar, 2009). Fungi are able to switch their metabolism from oxygen respiration through denitrification to ammonia fermentation under aerobic, suboxic and anoxic conditions respectively (Takaya, 2002). Fungal denitrification has been observed randomly across a wide range of different taxa (Shoun and Tanimoto, 1991; Kurakov et al., 1997; Zhang et al., 2001; Shoun and Takaya, 2002; Yanai et al., 2007; Jirout et al., 2013; Mothapo et al., 2013; Wei et al., 2014). Nevertheless, data concerning the distribution and significance of fungal-related N_2O emission from different environments are still limited.

Pasture soils are considered substantial contributors to global emissions of N_2O (Mosier et al., 1998; Oenema et al., 2005). Previous studies conducted on pastures for cattle overwintering showed that urine and dung excreted by grazing animals led to changes in the composition and biomass of microbial communities (Chroňáková et al., 2009; Jirout et al., 2011), as well as in the pattern of N_2O emissions (Šimek et al., 2006; Brůček et al., 2009; Jirout et al., 2013). An extensive study is therefore needed to examine the distribution of denitrification potential among soil fungi isolated at cattle overwintering pastures with high inputs of nitrogen into the soil.

The objective of this study was to identify and test N_2O-producing fungi isolated from soils of different sections of an overwintering pasture with substantial cattle impact. The first hypothesis tested was that N_2O-producing activity can be found in taxonomically diverse species of fungi. A second hypothesis was whether fungal species with a greater activity of N_2O production were more frequent at sites with high inputs of animal excrements.

E-mail address: jirout.jiri@email.cz.

http://dx.doi.org/10.1016/j.funeco.2015.07.003
1754-5048/

2. Materials and methods

2.1. Study area

Soil samples were collected at an upland pasture used for overwintering cattle, located on the Borová farm in South Bohemia, Czech Republic (latitude 48°52′41″ N, longitude 14°13′14″ E), which has been thoroughly described in previous studies (Šimek et al., 2006; Hynšt et al., 2007; Chroňáková et al., 2009; Jirout et al., 2011). The total area of the overwintering pasture is approximately 4 ha. Soil samples were collected from six sections of the overwintering pasture, differing in area, management history and intensity of animal impact: (1) SI – severe impact lasting over 15 yr, 750 m^2; (2) SR – severe impact, regenerating for 5 yr, 2500 m^2; (3) BI – beginning (5 yr) impact, formerly inaccessible for cattle, 6000 m^2; (4) MR – moderate impact, regenerating for 5 yr, 4000 m^2; (5) NI – minimal impact, 2000 m^2; and (6) CO – control, no impact by cattle, 1500 m^2. Each sampling site was represented by three composite samples, each consisting of three random single samples of thoroughly homogenised soil from the top 15 cm soil layer. These composite samples were, however, used only for the selective inhibition experiment. For isolation-based experiments, fungal isolates were collected as combined output of all composite samples from each section of the overwintering pasture. Soil samples were collected in May 2013, several days after cattle had left the winter pasture.

2.2. Isolation and identification of fungi

Soil fungi were isolated from 2 g of fresh soil using the plate dilution method with Beer wort agar (Merck, Germany) enriched with rose Bengal to prevent the growth of bacteria (Smith and Dawson, 1944). After 7 d of incubation at 28 °C, all morphologically different colonies from each soil were transferred onto fresh Beer wort agar plates (without rose Bengal) to obtain pure fungal isolates. Pure fungal isolates (maximum 2 weeks old) were stored using the silica gel storage method (Nakasone et al., 2004) at room temperature prior to subsequent analysis.

Identification of isolates was provided by sequencing of the ITS region of the ribosome encoding operon (Nilsson et al., 2009). DNA from fungal mycelium was extracted using the NucleoSpin® Soil kit (Macherey–Nagel, Germany) (standard protocol with SL1 buffer and SX enhancer). PCR was performed with the primer pair ITS1F-ITS4 (White et al., 1990; Gardes and Bruns, 1993) in a total volume of 50 μl containing: 1 μl of template DNA, 25 μl of 2x Master Mix (Thermo Scientific, USA), 2 μl of each 10 μM primer (East Port Praha s.r.o, Czech Republic) and 20 μl of MilliQ water. PCR products were checked by agarose gel electrophoresis (1.0% w/v agarose; 110 V, 45 min) with GelRed™ staining. After purification (Diffinity RapidTip®2, Diffinity Genomics, Inc., USA), PCR products were directly sequenced with ITS1F as the sequencing primer (SEQme s.r.o., Czech Republic). A sequence similarity search was performed using the BLASTN 2.2.23 + program (Zhang et al., 2000) on the NCBI web interface (http://blast.ncbi.nlm.nih.gov/) to compare nucleotide sequences to sequence databases (Altschul et al., 1990). A species name was assigned to a specimen according to the match with the highest query coverage and maximum identities of nucleotides (Supplementary Table S1).

2.3. Incubation experiment and N_2O measurements

The following modifications were introduced to the method of air-tight submerged cultivation, originally described by Kurakov et al. (2000). Briefly, instead of pre-grown mycelium, 20 silica gel beads with fungal propagules were directly inoculated into 100 ml flasks filled with 20 ml of sterile liquid medium containing glucose, salts and trace elements, pH 6.7 (for details see Kurakov et al., 2000), and equipped with screw caps and butyl rubber stoppers. Since a majority of fungi possess nitrite reductase (Nir) rather than nitrate reductase (Nar) (Shoun et al., 2012), sodium nitrite ($NaNO_2$; 10 mM) was selected as the nitrogen source (Jirout et al., 2013). For each isolate, two treatments were established to screen fungal isolates for N_2O-producing ability under conditions of decreasing oxygen level: *initially aerobic* (IAE, headspace atmosphere unchanged) and *continuously anaerobic* (CAN, headspace atmosphere replaced with argon). Flasks (one replicate for each strain and treatment) were then incubated for 168 hr at 28 °C in a horizontal shaker (KS-15 control, Edmund Bühler GmBH, Germany). Thereafter, N_2O concentrations in the headspaces were measured using a gas chromatograph equipped with a 3 m, 0.318 cm i.d. stainless steel Porapak Q column and electron capture detector (GC-ECD, Agilent HP 5890, Agilent Corp., USA). The temperature of the column and detector were 80 °C and 300 °C respectively. Peak areas were estimated using an HP integrator and the results were computed from detector responses to N_2O standards (Hynšt et al., 2007). Control flasks (blanks) for both IAE and CAN conditions were filled with the same liquid medium, including sodium nitrite, but without fungal inoculation to deduct N_2O that evolved chemically. The rate of N_2O production in the flasks was expressed as nmol N_2O d^{-1} flask $^{-1}$. Diffusion of N_2O into the liquid medium was considered and included in the computing formula. As the incubation experiment was continued for the purpose of other analyses, the biomass of isolates as well as analyses of the growth media could not be assessed.

2.4. Selective inhibition

Bacterial and fungal contributions to the total emissions of N_2O from studied soils were estimated according to a modified selective inhibition technique (Anderson and Domsch (1975). Soil (25 g) from each composite sample was pre-weighed into 100 ml flasks. To keep laboratory conditions as close as possible to natural ones, no amendments of glucose or source of nitrogen were made. Four treatments were then established: (i) control without biocides (2 ml of distilled water were added); (ii) inhibition of fungi by the same volume of cycloheximide dissolved in water (2.0 mg g^{-1} of soil; Castaldi and Smith, 1998); (iii) inhibition of bacteria by the same volume of bronopol dissolved in water (2.0 mg g^{-1} of soil; Bailey et al., 2003); and (iv) inhibition by both biocides (2 ml of combined biocides of the dissolved in water, each 2.0 mg g^{-1} of soil). Flasks were tightly sealed with a butyl rubber stopper and screw cap. Acetylene (10% v/v) was added to each treatment to inhibit bacterial N_2O reductase which reduces N_2O to dinitrogen (N_2) (Knowles, 1982). The soils were then incubated for 1 week at 28 °C in the dark. N_2O concentrations in the headspaces were measured after 24, 48, 36 and 144 hr using the same equipment and conditions as above.

2.5. Statistical analyses

Significance of the relationship between species abundances and cattle impact intensity at the overwintering pasture was tested using canonical correspondence analysis (CCA) with a MonteCarlo test (number of permutations = 499) (CANOCO for Windows, ver.4.5, ter Braak and Šmilauer, 2002). Cattle impact intensity was assessed on the basis of soil pH – a factor which is directly affected by cattle excretion and incorporation of excrements into soil by trampling (Whalen et al., 2000). Correspondence analysis (CA) was performed to show the distribution of fungi along the cattle impact intensity gradient. The scores of fungal species on the second axis of