

# Fungal denitrification contributes significantly to N<sub>2</sub>O production in a highly acidic tea soil

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## Abstract

**Purpose** Previous studies have indicated fungi might contribute substantially to soil N<sub>2</sub>O production under the acidic conditions. Yet, the fungal contribution to N<sub>2</sub>O emission in an acidic tea soil has been rarely reported. The objectives of the present research were to determine the fungal and bacterial contribution to N<sub>2</sub>O emission and identify, characterize N<sub>2</sub>O-producing fungal isolates, and testify their ability in producing N<sub>2</sub>O via inoculating strains to the sterilized acidic tea soil.

**Materials and methods** Two antibiotics (cycloheximide and streptomycin) combined with substrate-induced respiration (SIR) method were used in a microcosm experiment. Rose-Bengal medium was used to isolate the N<sub>2</sub>O-producing fungi. The active fungal strains were identified on the basis of 18S ribosomal DNA (rDNA) gene sequencing and then were inoculated into sterile soil and nonsterile soil in an incubation experiment.

**Results and discussion** Fungal denitrification comprised 70% of N<sub>2</sub>O production in this tea soil. Two fungi, namely *Penicillium* and *Hypocrea*, were isolated from tea soils at the pH of 3.8. N<sub>2</sub>O emission from *Penicillium* strain was 328 μg g<sup>-1</sup> biomass,

showing at least two orders of magnitude greater than that of *Hypocrea* strain. The inoculation of *Penicillium* strain into sterile or nonsterile soils suggested a significant contribution of fungal denitrification to N<sub>2</sub>O production in the tea soil. The relative contribution of fungi in this study was more intensified than the reported studies, suggesting that denitrifying fungi might turn to be more tolerant and dominant in this acidic soil, thus resulting to greater fungal contribution to N<sub>2</sub>O production.

**Conclusions** Fungal denitrification might play an important role in N<sub>2</sub>O emission in this highly acidic tea soil. Our finding might help to predict N<sub>2</sub>O emissions from acidic soils and provoke practicable methods to mitigate soil N<sub>2</sub>O emissions.

**Keywords** Fungal denitrification · N<sub>2</sub>O production · *Penicillium* strain · Tea soil

## 1 Introduction

Nitrous oxide (N<sub>2</sub>O) is one of the main greenhouse gases that strongly influence the global climate change. As the major source of N<sub>2</sub>O emission, soils contribute approximately 65% of N<sub>2</sub>O emission in the atmosphere (Smith and Conen 2004). Denitrification is an important microbial process of N<sub>2</sub>O production, especially in soils under O<sub>2</sub> limited conditions or with large amounts of NO<sub>3</sub><sup>-</sup> (Hefting et al. 2003). Nitrous oxide can be produced as the intermediate or final product of denitrification in the reduction of NO<sub>3</sub><sup>-</sup> to N<sub>2</sub>.

Tea is one of the important economic crops, planting on acid red soils of South China (Xue et al. 2010). The planting area of tea in China was 2.74 million hm<sup>2</sup> in 2014, accounting for 74.8% at the global scale (FAO 2014). Tea orchards have generally developed as a monoculture and were applied with increasing N fertilization to obtain higher yield and quality. Therefore, the soil pH decreases gradually and high amounts of N<sub>2</sub>O release

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into the atmosphere. Tokuda and Hayatsu (2004) showed that  $\text{N}_2\text{O}$  flux in the tea soil could be possibly strengthened by long-term heavy application of N fertilizer and soil acidification. The mean flux of  $\text{N}_2\text{O}$  in tea soils and rice paddy soils were 24.3 and 0.36  $\text{kg N ha}^{-1}$ , respectively (Akiyama et al. 2006). These studies indicated that the application of N fertilizer as well as soil acidification has an important effect on the nitrogen cycle in the tea garden soil.

Soil pH, the available nitrogen substrates, oxygen limitation, and organic carbon availability are the key factors that affect denitrification. The proportion of  $\text{N}_2\text{O}$  in denitrification processes generally increased with decreasing soil pH (Barton et al. 2013). Previous studies had showed that soil fungi rather than bacteria dominated the  $\text{N}_2\text{O}$  production in acidic soil (Ma et al. 2008; Rütting et al. 2013; Chen et al. 2014). The greater contribution of fungi to  $\text{N}_2\text{O}$  production was probably owing to the domination of fungal community under low pH conditions (Strickland and Rousk 2010) while soil bacteria are more sensitive to acidic conditions (Fierer and Jackson 2006). Denitrification can even occur under the presence of  $\text{O}_2$  (Tokuda and Hayatsu 2000; Takaya et al. 2003), implying the potential significance of aerobic denitrification (Bateman and Baggs 2005). Unlike bacterial denitrification that needs strict anaerobic condition for nitrate or nitrite reduction, fungal denitrification even requires small amounts of  $\text{O}_2$  (Zhou et al. 2001). The dominant microbial process of denitrification has been attributed to bacteria for over a century, recently fungal denitrification has been proposed as one of such domination in specific environments, e.g., acidic soils (Zumft 1997). A large number of studies have demonstrated that higher fungal dominance in acid soil leads to higher  $\text{N}_2\text{O}$  emission (Laughlin and Stevens 2002; Strickland and Rousk 2010; Rütting et al. 2013). Our earlier research also showed an important role of acidophilic denitrifiers in  $\text{N}_2\text{O}$  production in this acid tea soil (Huang et al. 2015). However, the mechanisms of dominant contributor (fungi or bacteria) in producing  $\text{N}_2\text{O}$  are not fully understood. Therefore, the objectives were to (i) detect the contribution to  $\text{N}_2\text{O}$  emission from fungi and bacteria and (ii) identify, characterize  $\text{N}_2\text{O}$ -producing fungal isolates, and testify their ability in producing  $\text{N}_2\text{O}$  by the inoculation of strains to the acidic tea soil.

## 2 Materials and methods

### 2.1 Soil sampling and description

Soil sampling was performed in a 100-year-old tea garden (constructed on a landfill in 1914) in the West Lake District of Hangzhou, China. The soil was classified as red soil by the China Classification System and was derived from quartzose sandstone interbedded with shales. Triplicate samples, each including eight soil cores (5 cm  $\times$  10 cm), were collected randomly from four sampling plots. The collected soils were mixed and

sieved (<2 mm). Then one part was air dried for chemical analysis and the other part was stored at 4 °C for about a week prior to microcosm experiment. Information on fertilization, farming management has been described in our previous studies (Huang et al. 2015) and selected soil properties were organic carbon (C) at 95.1  $\text{g kg}^{-1}$ , total N at 5.1  $\text{g kg}^{-1}$ , pH (1:2.5  $\text{H}_2\text{O}$ ) at 3.8, Bray 1-P at 79.1  $\text{mg kg}^{-1}$  soil, and microbial biomass C at 311.4  $\mu\text{g g}^{-1}$ .

### 2.2 Optimal concentrations of two antibiotics

To evaluate the contribution to soil  $\text{N}_2\text{O}$  production from fungi and bacteria, cycloheximide and streptomycin were used to inhibit fungal and bacterial activities in soils, respectively. To avoid overlapping inhibition of cycloheximide or streptomycin on bacteria and fungi, respectively, the SIR inhibition method was used to optimize concentrations of cycloheximide and streptomycin (Anderson and Domsch 1973). Soil  $\text{CO}_2$  flux rate was determined in control soil (no antibiotic); soils treated with cycloheximide at 2.0, 6.0, and 10.0  $\text{mg g}^{-1}$  dry soil; soils treated with streptomycin at 2.0, 6.0, and 12.0  $\text{mg g}^{-1}$  dry soil; and soils treated with both the combination of two antibiotics. We used inhibitor additivity ratio (IAR) to evaluate nontarget inhibition of cycloheximide and streptomycin (BeareMH et al. 1990; Seo and Delaune 2010). IAR value was calculated by:  $(A - B) + (A - C)/A - D$ , where  $A$ ,  $B$ ,  $C$ , and  $D$  represents  $\text{CO}_2$  flux rates from control soil, streptomycin-treated soil, cycloheximide-treated soil, and both antibiotics-treated soils, successively. The IAR value at 1.0 implies the usable concentrations of both antibiotics that have no nontarget inhibition effect. The minimum available concentrations for two antibiotics were streptomycin at 6.0  $\text{mg g}^{-1}$  dry soil and cycloheximide at 10.0  $\text{mg g}^{-1}$  dry soil, respectively.

### 2.3 The contribution of soil fungi and bacteria to $\text{N}_2\text{O}$ emissions

A microcosm experiment was replicated three times with the following treatments: (1) soil with no antibiotics, (2) soil with streptomycin at 6.0  $\text{mg g}^{-1}$  dry soil, (3) soil with cycloheximide at 10.0  $\text{mg g}^{-1}$  dry soil, and (4) soil with streptomycin at 6.0  $\text{mg g}^{-1}$  dry soil and cycloheximide at 10.0  $\text{mg g}^{-1}$  dry soil. Firstly, soils (15 g dry weight) were collected into 120-ml flasks and preincubated for 2 days in the dark at 25 °C. Glucose was chosen as a substrate in this method because most known soil bacteria and fungi can use it as a source for energy and growth. Acetylene (10% v/v) was often used to inhibit bacterial  $\text{N}_2\text{O}$  reduction to  $\text{N}_2$  and avoid underestimation of bacterial contribution. Therefore, soils from the above four treatments were added with 2 ml of glucose (5  $\text{mg g}^{-1}$ ),  $\text{KNO}_3$  (0.1  $\text{mg N g}^{-1}$ ) to sustain soil water content to 75% of maximum water holding capacity (MWHC). Secondly, the jars were sealed with rubber stoppers and aluminum covers after the addition of substrate solution. The

slurries of soil were injected with or without 10% v/v C<sub>2</sub>H<sub>2</sub> for the determination of N<sub>2</sub>O production rate. Thirdly, after 1 and 7 h, 3-ml gas samples were collected and N<sub>2</sub>O was instantly determined using robotized incubation system (Molstad et al. 2007). Soil N<sub>2</sub>O flux (mg h<sup>-1</sup> kg<sup>-1</sup> dry soil) in the headspace was calculated using the modified equation based on previous studies (Chen et al. 2014):

$$\frac{44 \times C_{\text{sample}} \times V}{r \times m \times t}$$

where 44 is the molar mass of N<sub>2</sub>O (g mol<sup>-1</sup>), C<sub>sample</sub> is the calculated N<sub>2</sub>O concentration of the gas sample (ppm) based on standard curve of N<sub>2</sub>O obtained by robotized incubation system, V is the volume of headspace (L), r is molar volume at 25 °C and 1 atm (24.436 L mol<sup>-1</sup>), m is the dry weight of soil (g), and t is the incubation time (h).

#### 2.4 Isolation of N<sub>2</sub>O-producing fungal strain

Using a dilution plating method, fungal strains were isolated from the tea soil. Firstly, liquid Rose-Bengal medium (10 g glucose, 5 g peptone, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g KH<sub>2</sub>PO<sub>4</sub>, 3.3 mL 1% Rose-Bengal solution and in 1 L distilled H<sub>2</sub>O) was prepared. The solution was added with appropriate 0.5 M H<sub>2</sub>SO<sub>4</sub> to adjust the pH of medium to 3.8 and 20 g agar including 100 mg mL<sup>-1</sup> streptomycin sulfate to provide a bacteria-free microcosm (Yanai et al. 2007). Secondly, soil slurry composing of 10:1 buffer (mL) to soil (g) ratio was successively diluted up to the 10<sup>-5</sup> level and 0.2 mL of each diluent was inoculated into the solid culture medium. Thirdly, inoculated mediums were incubated aerobically at 30 °C for up to a week. Fungal colonies based on different colony morphology were selected and repeated plate streaking to obtain pure cultures. After that, hyphae were transferred from individual colonies into new mediums. Additional four transfers were also carried out to obtain pure colonies that were kept in Rose-Bengal medium and placed at 4 °C.

To prevent the bacterial contribution to N<sub>2</sub>O production, purified fungal isolates were detected for N<sub>2</sub>O-emitting ability in a liquid Rose-Bengal medium including streptomycin to avoid bacterial contamination. In addition, four treatments were used to evaluate N<sub>2</sub>O emission from the liquid medium was either a fungal or chemical reaction: the medium alone, the medium containing 2 g L<sup>-1</sup> KNO<sub>3</sub>, the medium containing isolated strain, the medium containing isolated strain, and 2 g L<sup>-1</sup> KNO<sub>3</sub>. Each colony was inoculated into 120-mL amber jars with 25 mL liquid medium. The jar was first sealed with cotton wool and kept in an incubator at 37 °C for 72 h. The cotton wool was then removed and each jar was closed with a rubber stopper. After the certain incubation time, 3-mL gas samples were collected and N<sub>2</sub>O was instantly determined as previously described. To determine the biomass of each fungal isolate (g<sup>-1</sup> dry mycelium), the Rose-

Bengal medium solution was filtered through a 0.45-mm filter paper, dried for 48 h at 80 °C, and then was weighed by an electronic balance.

#### 2.5 Identification of N<sub>2</sub>O-emitting fungal strains

First, pure colonies of each fungal strain were sustained in the Rose-Bengal medium solution for 1 week at 37 °C. Then, the solution was gathered and transferred to a 1.5-mL centrifuge tubes. DNA was extracted from each culture by Bioteke new rapid plant DNA extraction kit (Bioteke Corporation) based on the instructions of the manufacturer. The primers NS1 (5'-GTAG TCATATGCTTGTCTC-3') and NS8 (5'-TCCG CAGGTTACCTACGGA-3') (Invitrogen Life Technologies, Shanghai, China) were used for amplifying the 18S ribosomal DNA (rDNA) from total DNA extracts (Martin and Rygielwicz 2005). PCR reaction included 2 μL of 5 ng DNA, 0.5 μL of 10 μM of each primer 12.5 μL Promega Gotaq DNA polymerase, added to a total volume of 25 μL with sterile Milli-Q water. Gene amplification was conducted in a GeneAmp PCR System 9700 Thermal Cycler consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR reactions of each sample were directly performed sequencing after purification. Translated gene sequences were aligned with reference fungal sequences using ClustalW implemented in BioEdit (Hall 1999). Phylogenetic trees were established with distance matrices and the neighbor-joining method in MEGA 5.0.

#### 2.6 Determination of the potential N<sub>2</sub>O production activities of isolated fungal strains in soil

Soils were divided into 72 microcosms of 50 g before sterilization by γ-ray radiation (35 kGy, Compton Irradiation Technology Co., Ltd, Fujian, China). In this soil microcosm experiment, four sterile treatments with three replicates were carried out: nitrate (100 mg N/kg dry soil) and *Penicillium* solution (SNP), *Penicillium* solution (SP), nitrate solution (SN), control (SC). Two nonsterilized treatments with three replicates were also carried out as control: nitrate and *Penicillium* solution (NP), nitrate solution (N). Soils (equivalent to 50 g dry weight) were mixed with *Penicillium* solution (mycelium spore suspension 2.7 × 10<sup>7</sup> per 1 mL) and/or nitrate solution and then packed into each sterilized 120-ml serum bottle. Then bottles were sealed with sterilized rubber stoppers and aluminum covers and then transferred to the room temperature (25 °C) incubator. We incubated soils for 15 days at 75% of MWHC by adding appropriate sterile purified water. After days 3, 4, 7 and 15, 3-mL gas samples were collected and N<sub>2</sub>O concentration was instantly determined as Section 2.3 described.

## 2.7 Statistical analysis

An ANOVA was performed to determine the least significant difference (LSD) between samples using SPSS 19.0 and the  $P$  values  $<0.05$  was considered significant. LSD and Dunnett's T3 tests were performed respectively based on the test of homogeneity of variances. The frequency and distribution of the denitrifier genotypes using 1000 permutations were evaluated. Based upon the evolutionary distance (maximum likelihood algorithm with Jukes–Cantor model, 1000 bootstrap repetitions performed), the phylogenetic trees of the 18S rRNA gene were established using MEGA 5.0.

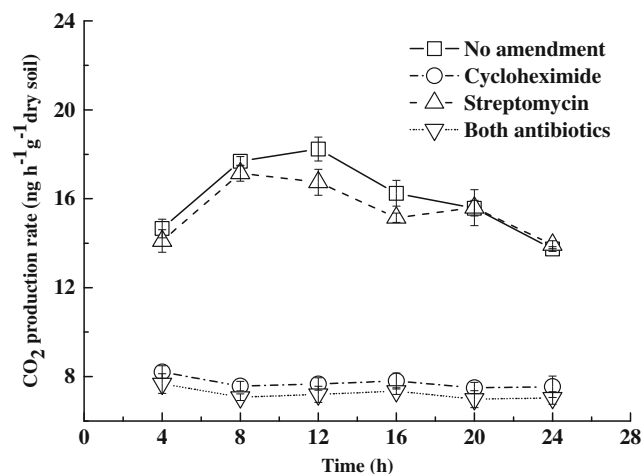
## 3 Results

### 3.1 The optimal concentration of antibiotics we used

Minimum concentrations of detected antibiotics were streptomycin at  $6.0 \text{ mg g}^{-1}$  dry soil and cycloheximide at  $10.0 \text{ mg g}^{-1}$  dry soil, respectively. Below or above these concentrations, the IAR value was greater or less than 1.0, suggesting noticeable overlapping inhibitory effects. Figure 1 showed the curves that used for the calculation of the ratio of bacterial to fungal respiration in the tea soils. Half of soil  $\text{CO}_2$  flux rates were reduced in both streptomycin and cycloheximide-treated soils. The respiration reduction was higher in cycloheximide-treated soils than that in streptomycin-treated soils, regardless of incubation hours.

### 3.2 Soil $\text{N}_2\text{O}$ emissions from fungi and bacteria

$\text{N}_2\text{O}$  fluxes from control, cycloheximide-amended, and streptomycin-amended soils were  $10.1$ ,  $3.4$ , and  $7.8 \text{ mg kg}^{-1} \text{ h}^{-1}$ , respectively. Therefore, soil  $\text{N}_2\text{O}$  flux decreased approximately



**Fig. 1**  $\text{CO}_2$  production rate in soils supplemented with cycloheximide ( $10 \text{ mg g}^{-1}$ ) and streptomycin ( $6 \text{ mg g}^{-1}$ ). Mean values of the three replicates are shown ( $n = 24$ )

$70\%$  in cycloheximide-amended soils while soil  $\text{N}_2\text{O}$  flux decreased by  $23\%$  in streptomycin-amended treatments. Though both cycloheximide and streptomycin addition significantly ( $P < 0.05$ ) reduced soil  $\text{N}_2\text{O}$  production rate, it showed no significant difference with that in cycloheximide-amended soils.

### 3.3 Isolated fungal strains

Two fungal species were obtained from the tea soil and were grown in a Rose-Bengal medium at pH 3.8. According to the morphological characteristics and the 18S rDNA phylogenetic analysis, the two strains were identified to be *Penicillium* and *Hypocrea* (Fig. S1, Electronic Supplementary Material), respectively. Nucleic acid sequence accession numbers in GenBank for *Penicillium* and *Hypocrea* are KJ680324 and KJ680323, respectively. Based on the partial 18S rRNA gene (Fig. S2, Electronic Supplementary Material), the phylogenetic tree suggested that two fungal strains isolated belonging to one cluster representing phyla Ascomycota. Isolate *Penicillium* strain (KJ680324) shared highly similar sequences with the  $\text{N}_2\text{O}$ -producing *Penicillium citrinum* DAOM 221147 while *Hypocrea* strain (KJ680323) shared similar sequences with *Bionectria ochroleuca* WY-1 and *Neocosmospora vasinfecta* RSA 1942.

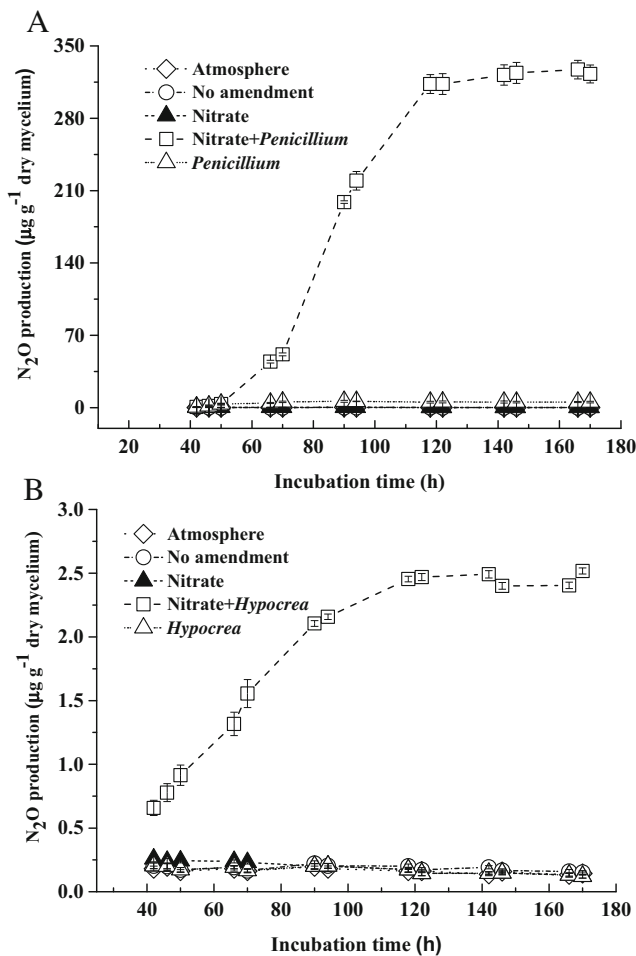
### 3.4 Denitrification activities of fungal strain

As shown in Section 2,  $\text{N}_2\text{O}$ -emitting abilities of fungal strains were detected in a liquid nutrient medium at pH 3.8. The result suggested that  $\text{N}_2\text{O}$  production significantly increased between 48 and 172 h in the medium treated with nitrate after *Penicillium* addition. The highest rate rose to  $328 \mu\text{g g}^{-1}$  dry mycelium from *Penicillium* at 174 h (Fig. 2a). In comparison, no significant difference was observed in the  $\text{N}_2\text{O}$  emission rate from *Hypocrea* and control, ranging from  $0.6$  to  $2.5 \mu\text{g g}^{-1}$  dry mycelium (Fig. 2b).

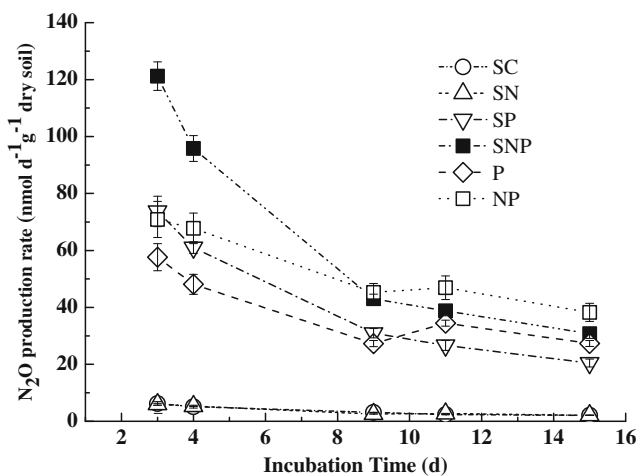
### 3.5 The fungal strain with $\text{N}_2\text{O}$ -producing ability in the sterilized tea soil

After inoculated the  $\text{N}_2\text{O}$ -producing *Penicillium* strain into  $\gamma$ -sterile soil, the  $\text{N}_2\text{O}$  production rate varied from  $20.4$  to  $73.7 \text{ nmol day}^{-1} \text{ g}^{-1}$  dry soil, which was significantly higher than SC and SN treatment (Fig. 3). In the SNP treatment, the rate ranging from  $30.7$  to  $121.2 \text{ nmol day}^{-1} \text{ g}^{-1}$  dry soil was higher than that from NP treatment. However, the  $\text{N}_2\text{O}$  production rate from NP treatment increased to a higher degree from the 9th day of incubation. No significant difference was observed in  $\text{N}_2\text{O}$  emission rate from *Hypocrea*-inoculating incubation (data not shown).

With *Penicillium* strain inoculation, cumulative fluxes of  $\text{N}_2\text{O}$  over the experimental period (15 days) were  $0.41$  and  $0.31 \text{ kg kg}^{-1}$  dry soil from sterile and nonsterile soils, respectively (Fig. 4). Meanwhile, the addition of nitrate increased the activity



**Fig. 2** N<sub>2</sub>O production in *Penicillium* (a) and *Hypocrea* (b) isolates inoculated into nutrient media



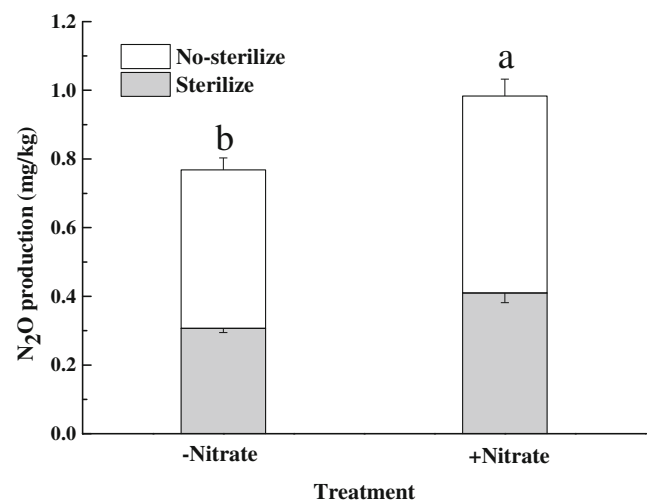
**Fig. 3** N<sub>2</sub>O production rate in *Penicillium* isolates inoculated into soils with or without  $\gamma$ -sterilization. *SNP* sterile soils treated with nitrate and *Penicillium* solution, *SP* sterile soils treated with *Penicillium* solution, *SN* sterile soils treated with nitrate, *SC* control, *NP* nonsterile soils treated with nitrate *Penicillium* and solution, *N* nonsterile soils treated with nitrate solution

of N<sub>2</sub>O production and reached 0.46 and 0.57 mg kg<sup>-1</sup> dry soil from sterile and nonsterile soils, respectively. The calculated contribution of fungi to denitrification processes in soils may reach 80% which is in agreement with the results obtained in the experiments with antibiotic selective inhibition.

## 4 Discussion

### 4.1 The contribution of fungal denitrification to soil N<sub>2</sub>O

Our earlier research indicated that bacterial denitrifiers dominated in this tea soils, suggesting an important role of acidophilic denitrifiers in N<sub>2</sub>O production (Huang et al. 2015). However, lack of relationship between N<sub>2</sub>O emission and denitrifying bacterial gene abundance implied that other unexamined denitrifying community, e.g., fungi, may also contribute to N<sub>2</sub>O emission in the tea soils. Therefore, both antibiotic and culture-dependent approaches were used to detect the relative contribution to N<sub>2</sub>O production from fungi and bacteria in this study. By using antibiotic inhibition method, the result suggested that fungal denitrification accounted for a higher percentage of N<sub>2</sub>O productions. Despite the highly acidic soil pH, soil fungi made a contribution of higher than 70% of N<sub>2</sub>O production. Large numbers of studies have demonstrated that the denitrification rate is strongly regulated by soil pH (Van Den Klemmedtsson et al. 2010; Van Den Heuvel et al. 2011; Hu et al. 2013). Rousk et al. (2010) also showed that soil fungi are capable to grow over a wider range of pH and fungal community may be rarely affected by acidic pH. Under acidic conditions, the fungal dominance of N<sub>2</sub>O production was attributed to the prevailing growth of fungi (Chen et al. 2015a). The relative contribution of fungi in this study was



**Fig. 4** N<sub>2</sub>O production from sterile and nonsterile soils inoculated with *Penicillium* strain. The different letters indicate significant differences observed in various comparison groups ( $P < 0.05$ )

more intensified than the reported studies (Laughlin et al. 2009; Herold et al. 2012; Chen et al. 2014), suggesting that denitrifying fungi turned to be more tolerant and dominant in this acidic soil (Baggs et al. 2010), thus resulting to greater fungal contribution to  $N_2O$  production in the tea soil. By inoculating active fungal strain *Fusarium oxysporum* 11dn1 into sterile soil, previous studies showed a significant contribution of fungi to  $N_2O$  emission under optimal conditions (Lavrent'ev et al. 2008). In this study, the fungal contribution to denitrification processes in soils can be evaluated via comparing our data in the intensity of  $N_2O$  production by active strain *Penicillium* inoculated into the sterile nitrate-treated soil with that in the nonsterile nitrate-treated soil. The contribution of *Penicillium* strain to denitrification processes may reach 80% which is in agreement with the results obtained in the experiments with antibiotic selective inhibition.

#### 4.2 Factors controlling fungal contribution to soil $N_2O$ flux

The dominance of fungal contribution to soil  $N_2O$  flux has been demonstrated in the range of pH 0.5–4.0 in studies of woodlands, grasslands, and peatland (Laughlin and Stevens 2002; Laughlin et al. 2009; Yanai et al. 2007). Although under aerobic conditions, the results of our study were consistent with these studies. It should be noted that in our study Rose-Bengal medium around pH 3.8 was available for isolating soil fungi with  $N_2O$ -producing ability. Two fungal species were identified from tea soil and *Penicillium* strain showed greater  $N_2O$  production than *Hypera* strain (Fig. 2a). The strains of denitrifying fungi retrieved in this study were lower than those of the previous studies (Lavrent'ev et al. 2008; Mothapo et al. 2013), suggesting that specific species dominated the fungal  $N_2O$  production in this tea soil. Oxygen limitation is another critical factor that control fungal denitrification in the sense that fungal denitrification required oxygen owing to the coexistence of oxic respiration system. Lavrent'ev et al. (2008) showed that fungal  $N_2O$  production was generally greater under microaerobic conditions by inoculating *F. oxysporum* and *Fusarium solani* into sterile soils. In comparison, Chen et al. (2015b) found that fungi and bacteria enjoyed the same sub-anoxic environment for the greatest  $N_2O$  production and fungal contribution declined if soil water content were higher than 65%. Our previous studies also demonstrated that denitrification is the major source of  $N_2O$  production under aerobic conditions in this tea soil (Huang et al. 2014). In this study, significant  $N_2O$  production from *Penicillium* strain after inoculated into nonsterile soil suggested that fungal denitrification could be satisfied fully at 75% WMHC. Therefore, results of this study indicated that fungal denitrification contributes greater  $N_2O$  production under aerobic conditions in this acidic tea garden soil.

#### 4.3 The specificity of the antibiotics method

The SIR inhibition method was first posed to determine the fungal and bacterial contribution to soil respiration (Anderson and Domsch 1973). The main difficulties obtained by using antibiotics are that inhibitors are often inactivated by soil or degraded by the soil microbes. However, the specificity of the antibiotics used was tested by previous study using mixed bacterial and fungal communities (Anderson and Domsch 1973). The results have suggested that antibiotics can be used as a tool to determine the fungal and bacterial contributions to soil respiration. The antibiotics method could be safely and considerably shortened by elimination of the tests for antibiotics selectivity. In this study, hypothesis from degradation of the antibiotics could also be eliminated as the antibiotics experiment is kept short (within 8 h) (Anderson and Domsch 1973).

In the last decade, a large number of studies have distinguished fungal contribution to soil  $N_2O$  flux by using the antibiotics method (Laughlin and Stevens 2002; Yanai et al. 2007; Wei et al. 2014; Jirout 2015;) and the only caution they focused on was to determine the minimum antibiotics concentrations using SIR inhibition method as previous described (Anderson and Domsch 1973). The experiments necessary for the antibiotics as the previous study (Anderson and Domsch 1973) mentioned were the establishment of the concentration of antibiotics and quantification of fungal and bacterial contribution to soil respiration which has been described in this study. Our results suggested that minimum concentrations of detected antibiotics were streptomycin at  $6.0 \text{ mg g}^{-1}$  dry soil and cycloheximide at  $10.0 \text{ mg g}^{-1}$  dry soil, respectively. Below or above these concentrations, the IAR value was greater or less than 1.0, suggesting noticeable overlapping inhibitory effects. Regardless of this uncertainty, the relative fungal contribution to  $N_2O$  flux in this tea soil using inhibitors method should be a valuable result.

#### 4.4 Methodological considerations

While bacterial denitrifiers are able to promote the reduction of  $N_2O$ , fungi cannot reduce  $N_2O$  to  $N_2$  owing to the absence of  $N_2O$  reductase. Therefore, wide concerns about reliable determination of soil  $N_2O$  from fungi and bacteria were raised due to this physiological difference (Chen et al. 2014). In experiments with inoculated *Penicillium* strain into sterile or nonsterile nitrate-treated soils,  $N_2O$  production by fungi could be overestimated in sterile soils compared to that in nonsterile soils. On the other hand, glucose addition increased the electron donor and therefore bacterial  $N_2O$  reduction could also be enhanced. The underestimation of bacterial contribution to  $N_2O$  might be made more severe by substrate addition. Despite these uncertainties, the comparison between soils inoculated with *Penicillium* strain should be a valid result, representing the greater fungal contribution to  $N_2O$  production in this acidic tea orchard soil.

## 4.5 Conclusions

The present study supplies more particular evaluation of soil fungal contributions to N<sub>2</sub>O emission than previous studies. We showed greater fungal domination in producing N<sub>2</sub>O in an acidic tea soil via using selective antibiotic inhibition method. Meanwhile, two fungal strains were identified and *Penicillium* strain was demonstrated to have a strong N<sub>2</sub>O-producing ability. In addition, the inoculation of *Penicillium* strain into sterile or nonsterile soils suggested a significant contribution of fungal denitrification to N<sub>2</sub>O production in the tea soil. Results obtained from this study suggest that fungal denitrification was unneglectable in N<sub>2</sub>O production of acid tea soil. Further studies are needed to confirm the relationship between the fungal N<sub>2</sub>O production and the N<sub>2</sub>O emissions at the field site and to explore the implication of shifts in AOA and AOB communities due to the fires for N management in forest ecosystems.

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