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New finding of *Trichoderma asperellum* in decreasing soil N_2O emission

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Abstract

Background: Global warming caused by greenhouse gas emissions affects sustainable human development. Agricultural practices are important source of greenhouse gases (GHG). Nitrous oxide (N_2O) contributes greatly to farming GHG. It is important to find a potential and practical biological technique that mitigate N_2O emissions in an environment friendly way.

Methods: N_2O-inhibiting fungi were isolated and identified in the lab. The fungi were added into the soil and placed in the incubator and interval gas sampling was analyzed by gas chromatograph.

Results: Fungus coding Z17 was identified molecularly with the same evolutionary branch on the phylogenetic tree with *Trichoderma asperellum* by BLAST comparison on NCBI GenBank. In the lab simulation, the N_2O emission flux was decreased by 28.18–47.16% by inoculating *Trichoderma asperellum* with 10^6 cfu·g^{-1}, 5×10^6 cfu·g^{-1} and 10^7 cfu·g^{-1} fungal spores in the soil compared to the control.

Conclusions: The N_2O-inhibiting fungus Z17 was identified as *Trichoderma asperellum*, capable of suppressing N_2O emissions from soil with at least 10^6 CFU·g^{-1} soil. The best N_2O-inhibiting effect was on day 9 of inoculation into soil because most of the fungal numbers were present in soil.

Keywords: Fungal nitrification inhibitor, Greenhouse gases, Carbon neutralization, *Trichoderma asperellum*, 18S rDNA

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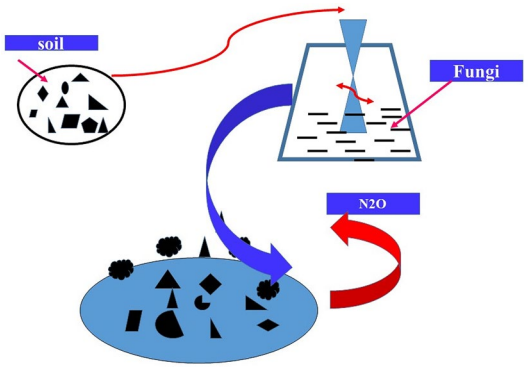
Graphical Abstract



Background

An increase in the world's population requires many more food and other products, resulting in much pressure on the agricultural sector and a rise in the utilization of chemical fertilizers and pesticides that are responsible for increasing environmental pollution and global warming. In recent years, food security has been threatened by population explosion due to lack of agricultural land [1]. Therefore, it has become a challenge to enhance the crop productivity to feed the population. Intensive use of chemical fertilizers resulted in the deterioration of both human health and environment. Consequently, to overcome the adverse effects of agrochemicals on our environment, there has been a shift towards organic fertilizers or other substitutes, which are eco-friendly and help to maintain a sustainable environment [2]. Global warming is one of the major threats to human survival and social development. Agriculture is an important source of GHG emissions globally due to its direct contribution to methane (CH_4) and nitrous oxide (N_2O) emissions [3], the largest source of atmospheric N_2O with about 298–300 times more global warming potential than CO_2, and are the most significant ozone-depleting substance of the twenty-first century [4], contributing approximately 6% to the observed climate change [5, 6], agricultural soils accounting for 60% of human-derived N_2O emissions [7].

Nitrogen (N) fertilizer is the major driver of N_2O emissions in agricultural soil [8]. Agricultural N_2O emissions are strongly correlated with the amount of chemical N fertilizer applied, that globally has increased eightfold over the last century, including a doubling in South Asia since 1990 [9]. Increased N_2O increases risks to global warming [10], the largest remaining threat to the stratospheric ozone layer [4]. N_2O emissions are rising at a rate of 0.2–0.3% annually [10, 11]. Agricultural soil microbial activity is driving N_2O production [12], that is widely recognized as a major source of N_2O, while annual 4 Tg of the total N_2O emission from N fertilizer in croplands was reported [13].

Traditionally, reduction of N fertilizer, water management, chemical urease inhibitors (N-serve (nitrapyrin), dicyandiamide (DCD), AM (2-amino-4 chloro-6 methylpyrimidine), sodium chlorate, sodium azide, and benzene hexachloride [14] were used to reduce the N_2O emissions. However, farmers would not like to reduce N fertilizer because they feared loss of grain yield. Soil sustainability is vital for enhanced nutrient turnover and optimal agricultural productivity. Application of chemical urease inhibitors would lead to potential environmental pollution. It is necessary to mitigate N_2O emissions from agricultural activities and agricultural ecosystems by developing practical N_2O mitigation strategies that can combat global climate changes [6, 15]. The development and verification of practical N_2O mitigation strategies, for example, enhancing N use efficiency and novel technologies (environmental effect factors (EEFs), precision agriculture, biological nitrification inhibition (BNI), N_2O reducing soil bacteria) [6], are required. Reducing N use [16, 17], application of biochar amendments [8, 17–20], co-application of biochar and electric potential [21], pelleted poultry manure with *Azoarcus, Niastella*, and *Burkholderia* [22], some plants such as cruciferous species[20], *Hyparrhenia diplandra* [23], *Megathyrsus maximus* [24], *Picea abies* and *Abies nordmanniana* [25], nitrification and urease inhibitors [26], nitrification inhibitor chlorinated pyridine (CP), 2-chloro-6-(trichloromethyl)-pyridine (Nitrapyrin, NP) and DMPSA and drip-fertigation [27–29] were used to mitigate N_2O emissions from agricultural soil.

We have conducted many investigations on antagonistic microbe–pathogen interactions over 20 years. Over 60 species of antagonistic microbes against watermelon, cucumber and other vegetable and melons' disease have been isolated and identified and stored at our

lab. Microbe-based biofertilizers may contribute substantially to the soil health, fertility status and microbial biomass. These microbe-based fertilizers help to increase the nutrient solubilization/mobilization (N, P, and K), hydrogen cyanide (HCN) production, pathogen control and induced systematic resistance, etc. Moreover, the application of compost-based biofertilizers also helps to increase the production of phytohormones, siderophore, vitamins, protective enzymes, antibiotics, etc. We were enlightened by such microbial antagonism and imagined whether there will be some inhibitory effect of these antagonistic microbes on nitrifier or denitrifier consequently leading to a reduction of N_2O emission from soil. So we began the investigation of microbial inhibition of N_2O emission from soil. We proposed the hypothesis as follows: (1) fungi can suppress N_2O emissions from the soil by inhibiting nitrifier activities, and (2) fungi can inhibit the growth of crop pathogens in the soil to protect the crop from disease to decrease the production and application of chemical fertilizers and pesticides. The aims were to explore the possibility of microbes inhibiting N_2O emissions from the soil. Soil microbes were isolated and identified in the present work to screen and develop some potential N_2O-inhibiting fungi for application in agricultural carbon neutralization and sustainability.

Materials and methods

Microbial sources

Fungi numbered Z3, Z6, Z8, Z9, Z11, Z13, Z14, Z15, Z16, and Z17 were isolated from different watermelon field soils (different species of watermelon, region and greenhouse cultivation and traditional cultivation) while screening antagonist microbes and stored in our lab 8 years ago.

Soil collected and processed

The soil for this experiment was collected from an agricultural field at a depth of 20 cm from the Agricultural Meteorological Experimental Station, Nanjing University of Information Science and Technology China, June 2015. The soil's physical–chemical properties are listed in Table 1. The soil microbial composition was 2.6×10^8 CFU·g^{-1} of bacteria, 4.1×10^6 CFU·g^{-1} of actinomycetes and 3.7×10^4 CFU·g^{-1} of fungi.

The soil was wind-dried for 20 days and ground to pass through a 60-mesh sieve. A total of 2.5 kg of 60 mess soil was added to 5 L of a glass bottle (soil culture apparatus made by ourselves) to investigate N_2O emission and the fungal inhibiting effect on N_2O.

Table 1 Physical–chemical properties of tested soil

Organic matter (g·kg^{-1})	Total N (g·kg^{-1})	Fast available P (mg·kg^{-1})	Fast available K (mg·kg^{-1})	Basic hydrolysis N (mg·kg^{-1})	pH
12.01	0.75	15.08	87.65	80.27	7.2

Preparation of microbial culture media

LB (Luria broth) culture medium: casein peptone 1.0 g, yeast extract 0.5 g and NaCl 1.0 g were added in 100 mL distilled water to maintain pH 7.4

PDA (potato dextrose agar) medium: potato chip 2.0 g, dextrin 0.2 g were added in 100 mL distilled water while maintaining natural pH value. The potato was peeled and cut into small chips to boil for 30 min. The boiled potato chips were filtered with cotton gauze. Add distilled water to 100 mL. Agar (1.8 g) was added to the potato filtrate, sterilized for 30 min at 114 °C and cooled for storage.

Preparation of fungal suspension

The fungi numbered Z3, Z6, Z8, Z9, Z11, Z13, Z14, Z15, Z16, Z17 were inoculated in a culture dish to activate at 28 °C in an incubator for 6 days. After 6th day of culture in the dish, a fungal colony plug with a diameter of 5 mm was taken from the plates to inoculate the fungal liquid media in a conical flask, which was cultured at 28 °C and 170 rpm in a shaking flask incubator for 4 days. The culture broth was filtered with 4 layers of cotton gauze, and the liquid was collected after 96 h of culture. The filtrate was centrifuged at 8000 r·min^{-1} for 10 min to collect fungal mycelium and spores. The collected fungal mycelium and spores were re-suspended in sterile water. The re-suspended spores were counted with a hemocytometer to prepare a series of concentrations of spore suspension.

N_2O-inhibiting fungal screening, identification, and confirmation

We prepared the screening equipment ourselves (Fig. 1). A total of 2.5 kg of soil was added to the soil incubator to culture fungi at 28 °C for 7 days in the lab. Fungi were inoculated into the soil to check whether N_2O formation and emission from the apparatus were inhibited by fungi. The screening experimental design is shown in Table 2.

The potential N_2O-inhibiting fungi in the first-round experiment was further verified a second time to examine the actual capability of the preliminary N_2O-inhibiting fungi as indicated in Table 3. The apparatus was incubated at 28 °C in the lab with 3 replicates for each treatment.

The fungi from the second screening were verified a third time, as listed in Table 4. A total of 10^6 cfu·g^{-1}, 5×10^6 cfu·g^{-1}, and 10^7 cfu·g^{-1} soil fungal spores were

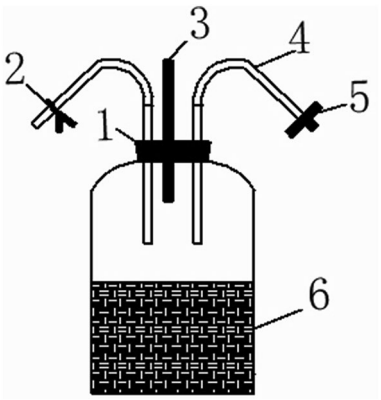


Fig. 1 Lab soil culture apparatus. **1** Rubber stopper, **2** seal clamp, **3** thermometer, **4** rubber tube, **5** T-type valve, **6** soil

inoculated into the soil for further culture to select the best N_2O-inhibiting fungus. The culture apparatus was incubated at 28C in the lab with 3 replicates for each treatment.

The best N_2O-inhibiting fungi were cultured on plates for morphological and molecular identification. The fungus was grown on an agar plate for 7 days. The fungal colony and mycelial morphology were observed microscopically. Fungal mycelium and spores were scraped and added to a 1.5-mL centrifuge tube. The cell lysis digestion liquid was added to the tube to dissociate the cell wall and nuclear envelope to release DNA.

The total DNA of the fungus was extracted with an OMEGA soil DNA extraction kit (E.A.N.A. Fungal DNA Mini kit).

The fungal PCR 18S rDNA was conducted with primers ITS4: 5′TCCTCCGCTTATTGATATGC3′, ITS: 5′-GGA AGTAAAAGTCGTAACAAGG3′. PCR reaction system consisted of 0.5 μL of ITS4 primer, 0.5 μL of ITS5 primer, 10 × PCR Buffer (Mg^{2+}) 2.0 μL, Mg^{2+} 1.5 μL, DNA template 2.0 μL, *Taq* polymerase (2.5 U. μL^{-1}) 0.5 μL, ddH_2O 12.0 μL. Total volume 20.0 μL, PCR reaction conditions: 94 ℃ pre-denaturing 2 min, 94 ℃ denaturing 30 s, 52℃ annealing 30 s, 72 ℃ elongation 1 min, 30 cycles, 72 ℃ elongation 10 min. 10 storage. A 1% agarose gel electrophoresis was used to check the purity of PCR products.

The PCR products were purified with a DNA purification kit (AxyPrep PCR Cleanup kit, Axygen) based on the manufacturer's instructions.

Ten microliters of purified PCR product was sent to Nanjing Jinsirui Biotechnology Company Ltd. for sequencing.

Table 2 Different treatments of preliminary screening of N_2O-inhibiting fungi

Number	Fungal code	Treatment
1	CKZ	2.5 kg soil + 800 ml water + 100 ml fungal medium
2	Z3	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z3
3	Z6	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z6
4	Z8	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z8
5	Z9	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z9
6	Z11	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z11
7	Z13	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z13
8	Z14	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z14
9	Z15	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z15
10	Z16	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z16
11	Z17	2.5 kg soil + 800 ml water + 100 ml fungal medium + Z17

CKZ indicating check, fungal inoculation volume 0 cfu·g^{-1}, Z3–Z17 indicating fungal code, inoculation volume 50 mL/bottle

Table 3 Experimental design of second screening of N_2O-inhibiting fungi

Number	Fungal code	Treatment
1	CKZ	2.5 kg soil + 800 ml water + 100 ml PDA medium
2	Z3	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z3 inoculant
3	Z8	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z8 inoculant
4	Z11	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z11 inoculant
5	Z17	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z17 inoculant

CK indicating check, fungal inoculation volume 0 cfu·g^{-1}, Z3–Z18 indicating fungal code, inoculation volume 50 mL/bottle to make final soil fungus 10^6 cfu·g^{-1}

Table 4 Experimental design of the third screening of N_2O-inhibiting fungi

Number	Bacterial code	Treatment
1	CKZ	2.5 kg soil + 800 ml water + 100 ml PDA medium
2	Z17-I	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z3 inoculant 10^6 cfu·g^{-1}
3	Z17-II	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z3 inoculant 5×10^6 cfu·g^{-1}
4	Z17-III	2.5 kg soil + 800 ml water + 100 ml PDA medium + Z3 inoculant 10^7 cfu·g^{-1}

CKZ indicating check, fungal inoculation volume 0 cfu·g^{-1}, 3 different inoculants of Z17 was 50 mL/bottle to make final soil fungus 10^6 cfu·g^{-1}

The sequence was submitted to NCBI GenBank online to BLAST and compare 99% of the sequence homology with other known microbes in GenBank. Subsequently, the best N_2O-inhibiting fungus was identified based on DNA sequence homology. The fungal phylogenetic tree was constructed with Mega 3.0.

Real-time quantitative PCR (qPCR) qualifying the number of N_2O-inhibiting fungus in soil after inoculation

The best N_2O-inhibiting fungus Z17 was cultured on plates for qPCR to identify the number of soil [30]. The fungus was grown on an agar plate for 7 days. Fungal mycelium and spores were scraped and added to a 1.5-mL centrifuge tube. The cell lysis digestion liquid was added to the tube to dissociate the cell wall and nuclear envelope to release DNA.

The total DNA of the fungus was extracted with an OMEGA soil DNA extraction kit (E.A.N.A. Fungal DNA Mini kit) after day 9, 18, 24 and 30 inoculated in soil.

The fungal PCR 18S rDNA was conducted with primers ITS1F: 5′CCAAACTCTTTCTG-3′, ITS1R: 5′-GCATTTCGCTGCGTTCTT3′. qPCR reaction system consisted of 1 μL of ITS1F primer (10 μmol/L), 1μL of ITS1R primer, SYBR Premix Ex Taq (2×)12. 5 μL, DNA template 1.0 μL, ddH_2O 9.5 μL. Total volume 25.0 μL, qPCR reaction conditions: 95 ℃ predenaturing 10 s, 95℃ denaturing 15 s, 60 ℃ annealing 15 s, 72 ℃ elongation 30 s, 45 cycles, 55 ℃ elongation 10 s. The reaction temperature was increased from 55 ℃ to 95 ℃ at a speed of 0.1 ℃/s. Fluorescence signal was detected one time every temperature-rising of 0.5 ℃ to conduct melting curve analysis. Standard curve was drawn on the basis of X-axis of logarithm of standard plasmid DNA VS Y-axis of reaction cycle (Ct). Sample concentration and gene copy were computed as formula: detected sample DNA concentration (ng/μL) = OD260 × 50 × dilution times. Sample DNA molecular weight = base number × 324. Detected sample DNA copy number = (detected sample DNA concentration/sample DNA molecular weight) × 6 × 10^{14}.

Preparation of the apparatus for soil greenhouse gas generation and collection

The soil culture apparatus was designed and made with a large glass bottle of 5 L. The apparatus shown in Fig. 1 comprises gas emission, pumping, and collecting. The soil was added to the bottle, and the fungal spore suspensions were inoculated into the soil to monitor the N_2O-inhibiting effect. A plug (diameter of 5 mm) of the best N_2O-inhibiting fungal colony was inoculated into 5 mL of liquid PDA medium at 28 °C and 170 rpm in a shaking flask at logarithmic stage. The broth was centrifuged to collect fungal spores. The centrifugal sediments were added to distilled water to dilute a series of concentrations of fungal spore suspensions. The spore suspension was quantified and added to the soil and mixed. The apparatus was placed in a constant temperature room. Gas was sampled from the sampling valve to determine the N_2O concentration by gas chromatograph (GC) (Agilent 7890A).

Soil, water, and fungal suspensions were added to the bottles and blended thoroughly when the experiment was started. The airproof of the rubber stopper of the bottle was checked with water. The apparatus was put in the lab to culture. All valves of the tubes were opened to freely maintain air ventilation. The valves of the hoses and tubes were closed to enrich the N_2O concentration before 20 min of sampling gas.

Sampling gases began from the third day after the soil and fungi were blended in the bottle. The gas sample was collected from 9:00 in the morning and 15:00 in the afternoon once after every 3 days. Valves were switched off to concentrate N_2O inside the bottle before 20 min of sampling. A 20-mL injector was connected to a T-type valve to make the injector connect to the bottle. Initially, the bottles were pumped in and out 4 times to mix the bottle gases fully, and then the gas sample was collected. One gas sample was collected after every 80 min. Consequently, 3 samplings were conducted for one bottle at one time. After finishing gas sampling, the valves were opened. The air temperature inside the bottle was recorded.

Gas N_2O was analyzed by GC (Agilent 7890A). The GC analysis parameters included an ECD

detector, 300 °C detection temperature, a chromatography column of SS-1 m × 2 mm × PorapakQ (80/100), SS-3 m × 2 mm × PorapakQ (80/100), a chromatography column temperature of 50 °C, a carrier gas of pure dinitrogen gas, a flow rate of 25 $cm^3 \cdot min^{-1}$, a retention time of 3.35 min, and a standard N_2O gas of 320 ppb made by the Nanjing Shangyuan industrial gas factory.

N_2O emission flux calculation

N_2O emission flux was calculated by the formula: $F = 60 \times \rho \cdot (V/m) \cdot (dc/dt) \cdot 273/(273 + T)$, where F indicates N_2O flux emitted from soil ($\mu g \cdot kg^{-1} \cdot h^{-1}$), ρ indicates the density of N_2O at standard state (1.25 $kg \cdot m^{-3}$), V shows effective space volume of culture bottle (m^3), m indicates soil quality inside the bottle (kg), dc/dt demonstrates N_2O emission rate ($ppb \cdot min^{-1}$), and T indicates the average air temperature inside the bottle during gas sampling (℃) [31].

N_2O emission flux was expressed as the means of three replicates plus the standard deviation for each treatment.

Data processing and analysis

Microsoft Excel 2010 was used to calculate the data. Origin2018 was used to draw data figures and AUTOCAD2010 was used to draw apparatus picture. SPSS 19.0 was used to carry out significance difference and correlation analyses. Analysis of variance (ANOVA) was used to conduct variance analysis at a 95% confidence level, and least significant difference (LSD) was used for multi-comparison among treatments.

Results

Preliminary identification of N_2O-inhibiting fungi

In the present experiment, several fungi were found to have the capacity to suppress N_2O generation and emission. The N_2O flux was reduced in the Z3, Z8, Z11, Z14, and Z17 treatments relative to the CK treatment during the sampling duration. Compared with CK, the N_2O emissions in the Z3, Z8, Z11, Z14, and Z17 treatments decreased by 18.54%, 19.15%, 9.53%, 28.0%, and 38.22%, respectively, among which the greatest reduction was 38.22% for Z17. Initially, a decreasing tendency of N_2O emissions was observed. It was determined that nitrification inhibitors in the soil may grow quickly due to PDA culture, possibly resulting in the decrease in N_2O emissions in all treatments. This result indicated that Z17 was the best N_2O-inhibiting fungus (Fig. 2). On day 9, N_2O emission was the lowest, while it was gradually higher after day 9. Of course, the N_2O emission for all treatments was lower than CK. It was suggested that the best effect of N_2O-suppression was observed after inoculation on day 9 that indicate optimal conditions suitable for the growth of fungus.

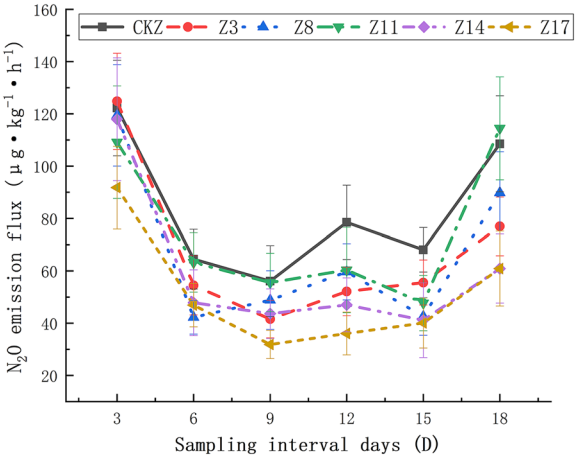


Fig. 2 Preliminary screening of N_2O-inhibiting fungi and N_2O emission. CKZ-check, without fungi, Z3, Z8, Z11, Z14, Z17-addition of 50 mL fungal suspension of Z3, Z8, Z11, Z14, Z17, respectively

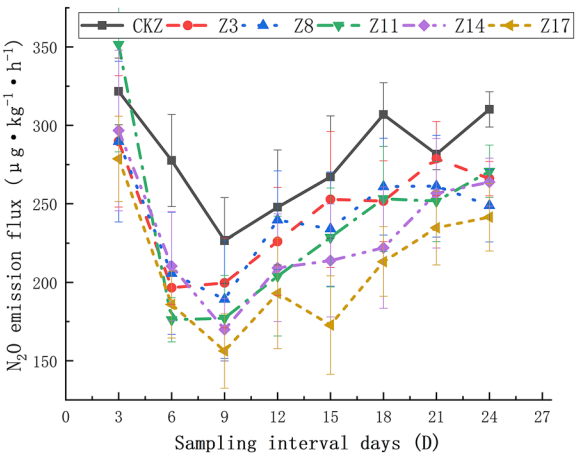


Fig. 3 Second screening of N_2O-inhibiting fungi and N_2O. CKZ-check, 0 $cfu \cdot g^{-1}$ of inoculum, the inoculum amount of Z3, Z8, Z11, Z14, Z17 was 10^6 $cfu \cdot g^{-1}$

Second screening of N_2O-inhibiting fungi

N_2O emissions in the Z3, Z8, Z11, Z14, and Z17 treatments were significantly suppressed, and the Z17 treatment had the best inhibitory effect on N_2O emissions (Fig. 3). The N_2O emission flux declined by 14.58%, 13.86%, 12.41%, 17.71%, and 25.16% compared to CK. This tendency complied with the preliminary experimental result. The greatest decrease in N_2O by the fungus was observed for Z17, demonstrating that Z17 was the best N_2O-inhibiting fungus. On day 9, the N_2O emission was the lowest, while it was gradually higher after day 9. Of course, the N_2O emission for all treatments was lower than CK. It was suggested that the best effect of

N_2O-suppressing was on day 9 of inoculation when there would be optimal conditions suitable for the fungus.

Third time identification and confirmation of the best N_2O-inhibiting fungus, Z17

To investigate the actual capacity of N_2O-inhibiting fungus Z17, 3 inoculation concentrations of 10^6 cfu·g^{-1}, 5×10^6 cfu·g^{-1}, and 10^7 cfu·g^{-1} fungal spores were used to incubate precisely in the lab based on the second time test. The N_2O emissions from the Z17 treatment in the whole test period were much lower than those from the CKZ treatment (control). Initially, the difference was not as significant among the different inoculating concentrations of Z17, while a significant difference was found with increasing culture duration. The N_2O emissions in treatments Z17-1, Z17-2, and Z17-3 were reduced by 28.18%, 32.79%, and 47.16%, respectively, compared with CKZ (control) (Fig. 4). Z17 significantly suppressed N_2O emissions from the soil. The greater the Z17 addition, the lower the N_2O emissions. The inhibitory effect of Z17 on N_2O emission was the best. When the inoculant of Z17 reached 10^7 cfu.g^{-1}, the best N_2O-inhibition effect was obtained. On day 9, the N_2O emission was the lowest, while it was gradually higher after day 9. Of course, the N_2O emission for all treatments was lower than CK. It contributed to a decrease of Z17 number of DNA copy by qPCR colonized in soil gradually depending on time. The gene copies in soil on day 9 were 3.724×10^3 ng/g, while the DNA copies on days 18, 24, 30 were 3.026×10^3 ng/g, 2.583×10^3 ng/g, 2.149×10^3 ng/g (Fig. 5). It was suggested that the best effect of N_2O-suppressing was on day 9 of inoculation where the number of soil Z17 was found the most.

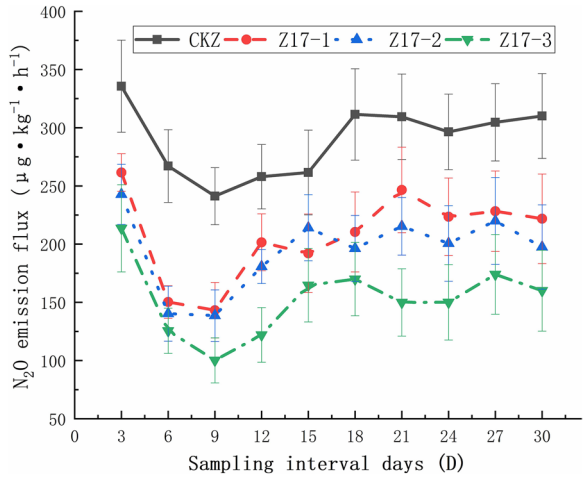


Fig. 4 Third screening of N_2O-inhibiting fungi Z17 at different inoculant gradients and N_2O. Note: CKZ-check, inoculation of 0 cfu·g^{-1}, Z3, Z8, Z11, Z14, Z17 inoculation of 10^6 cfu·g^{-1}, 5×10^6 cfu·g^{-1}, 10^7 cfu·g^{-1}

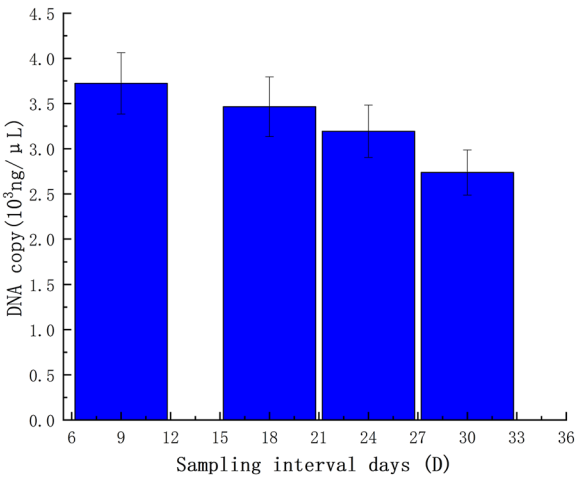


Fig. 5 The fungal DNA copies in the soil in the third time screening of N_2O-inhibiting fungus Z17 at different inoculant gradient and N_2O

Morphological and molecular identification of N_2O-inhibiting Z17

The N_2O-inhibiting fungus Z17 was grown on PDA slowly in the dark. The mycelium was initially white and gradually became gray-green, while green spores stretched from the center to the dish margin to form layers of concentric circles. Z17 colonies on plates are shown in Fig. 6. Conidiophores had a much-branched hierarchy, with many swollen peduncles in the middle affiliated with the ends of branches, namely, spore-producing cells. The view of plate colony morphology (up Fig. 6), spore (middle Fig. 6), and microscopic vision of conidiophore, mycelium, and spores (down Fig. 6).

Approximately 700 bp of the DNA fragment of 18S rDNA (ITS4/ITS5) PCR product of Z17 was obtained after 1% agarose gel imaging. Based on the DNA sequence, homologically identical partner was searched in NCBI GenBank. For this purpose, Blastn was used to identify the closely related organism using 18S rDNA gene sequence. In the GenBank database the 18S rDNA gene sequence was deposited under accession number and a phylogenetic tree was constructed through neighbor-joining (Fig. 7). Strain Z17 was placed on the same evolutionary branch as the fungus *Trichoderma* asperellum (No. KM456217.1) on the phylogenetic tree with 99% homology. Finally, Z17 was identified as *Trichoderma asperellum.*

Fig. 6 Morphological characteristics of Z17 colonies on a plate and microscopic observation of conidiophores, mycelia and spores (× 800)

Discussion

Nitrous oxide, global environmental change, and agriculture

Agriculture plays a major role in production of nitrous N_2O. More than two-thirds of global N_2O emissions originate from soil, and the main sources of N_2O are N fertilizers, urine and dung deposition during grazing, and manure application onto grasslands [26]. Nowadays, huge amount of organic waste is being generated by farm operations due to the disposal difficulties posing serious threats to the environment, human population and soil fertility. Globally, 3.3 Tg N_2O-N per year is emitted from fertilized croplands due to the use of N fertilizer and animal manure [26].

The global nitrogen fertilizer application rate is getting double at a rate of 3.0×10^8 t per year [32], and the annual nitrogen loss is 6.15×10^7 t due to the loss of nitrate-nitrogen from agricultural systems [33]. An increase in the risk of nitrogen loss from agricultural ecosystems and environmental pollution problems will be warned [17]. Much more N_2O will be emitted from the soil. Agricultural cleaner production and carbon neutralization are urgently required. Hence, there is a need to develop a sustainable agriculture system with enhanced soil fertility and least ecological threat. As substitute fertilizers, microbe-based biofertilizers are sustainable, cost-effective and eco-friendly.

N_2O inhibition and cleaner production approaches in agriculture

Biochar has attracted many scientists focusing on mitigating N_2O emissions from the soil since the twenty-first century. Biochar not only mitigates soil N_2O emissions, but also ameliorates soil and stimulates crop growth. Presently, biochar has been a research hot spot. Biochar amendment has been recommended as a potential strategy to mitigate N_2O and NO emissions for crop production [27, 34].

However, the cost of biochar production and application is so expensive that it is difficult for use in agriculture. The second pollution during the production and application of these chemical inhibitors was given little consideration. N_2O emission mitigation was obtained by modifying soil conditions, such as improving drainage and reducing compaction, which are adverse to denitrification [35].

Most considerations of chemical inhibitors to inhibit N_2O-related enzymes were given. Less attention has been paid to plants modifying nitrification in situ, such as *Brachiaria humidicola*, a tropical grass species, inhibiting the *Nitrosomonas europaea* strain (nitrifying bacteria) by exuding active chemicals during nitrification. However, how and what triggers or molecularly controls biological nitrous oxide inhibitor production are unknown [33].

Some plants capable of suppressing nitrification, such as *Hyparrhenia diplandra* [23], *Picea abies* and *Abies nordmanniana* [18], *Megathyrsus maximus* [24], *Lolium perenne*, *Melinis minutiflora*, *Arachis hypogaea*, *Panicum maximum* [32], *Andropogon gayanus* [32, 36], *Leymus racemosus* [37], *Periandra mediterranea* [38], and *Moringa oleifera* [39], have been reported [17]. Some chemically organic active substances excreted from plants are capable of suppressing nitrification [17], for example, kuanjin extracted from the seeds of *Pongamia glabra* [40], oil from *Mentha spicata* [41–43], *nimin oil* from the seeds of *Azadirachta indica* [44], gallocatechin and catechin from the degradation of *Arbutus unedo* leaves [45]. Among them, phenolic compounds and terpenoids have been investigated with the capability of nitrification inhibition [17]. These active chemicals from plant secretion or degradation were referred to as the BNI concept by Japanese scientists [37], which is also called a "natural nitrification inhibitor" [14].

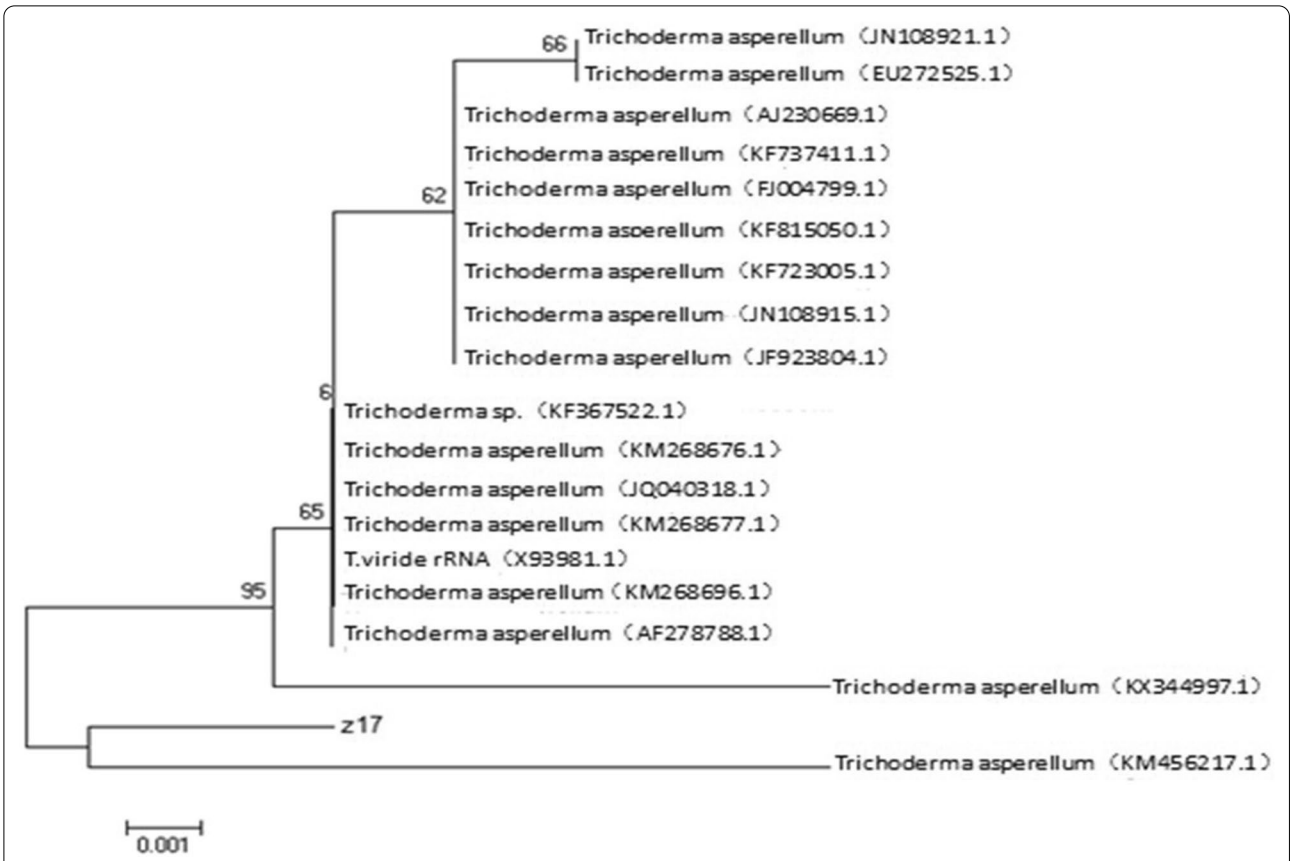


Fig. 7 Phylogenetic tree of strain Z17 related to *Trichoderma* sp

N_2O-inhibiting microbes

N_2O-inhibiting microbes could be used to mitigate soil N_2O emissions to reach agricultural carbon neutralization. In the current study, Z17 was identified as a fungus that effectively inhibited N_2O emissions from agricultural soil. Approximately half of N_2O was reduced by this magic tiny organism compared to that in a 2-month lab simulation; in other words, a reduction of 1.6 Tg N_2O per year is expected globally if the fungus is applied in agriculture worldwide. This will provide a novel thought and technological tool for agricultural carbon neutralization. However, based on results, on day 9, the N_2O emission was the lowest, while it was gradually higher after day 9. Of course, the N_2O emission for all treatments was lower than CK. It was ascribed to the decreased fungal number in soil. It suggested that the best effect of N_2O-suppressing was on day 9 of inoculation when there would be optimal conditions suitable for the fungus. Surely, many questions have to be further investigated whether this potential fungus could be produced and applied to agricultural carbon neutralization in the next step.

In the present work, Z17 was identified as *Trichoderma asperellum*, with reproductive mycelium, spore, and green colonies on PDA. The mycelium and spores tolerated stress and could survive in the soil for a long time. *Trichoderma asperellum* modulates defense genes and potentiates gas exchanges in upland rice plants and is a stable combination for a multiple function biological agent [46]. *Trichoderma* spp. is a universal saprotrophic fungus in terrestrial ecosystems, and as rhizosphere inhabitants, they mediate interactions with other soil microorganisms, plants, and arthropods at multiple trophic levels. In the rhizosphere, *Trichoderma* can reduce the abundance of phytopathogenic microorganisms, which involves the action of potent inhibitory molecules, such as gliovirin and siderophores, whereas endophytic associations between *Trichoderma* and the seeds and roots of host plants can result in enhanced plant growth and crop productivity, as well as the alleviation of abiotic stress. Such beneficial effects are mediated via the activation of endogenous mechanisms controlled by phytohormones such as auxins and abscisic acid, as well as by alterations in host plant metabolism [47].

Trichoderma can be fermented to make organic fertilizer with animal dung to apply to the soil, where the bio-organic fertilizer not only supports nutrients for crop growth and protects plants from the pathogen, as a well-known effective antagonist agent, but also improves soil quality and further mitigates N_2O emission from soil [48]. The ideal mixture of soil and organic compounds with *Bacillus* sp. and *Trichoderma asperellum* inoculations stimulated optimal growth and provided nutrient content to banana seedlings [49, 50]. *Trichoderma*-enriched biofertilizer reduces the application of chemical fertilizers and therefore can be considered a noble practice in sustainable agriculture. It is helpful for cleaner agricultural production [48]. These fertilizers are developed by adding the characterized plant growth-promoting bacterial or fungal strains in compost produced from agricultural waste. The microbial strains with known plant-beneficial role and ability to survive and thrive in compost can be used to produce such biofertilizers.

Further investigation of the effects of environmental factors on *Trichoderma asperellum* growth, reproduction, and best application conditions should be conducted. Thoroughly exploring the N_2O-inhibiting mechanism of *Trichoderma asperellum*, physiological active components, and regulatory functional genes will be very beneficial to agricultural carbon emission reduction and sustainable development. Hopefully, the N_2O-inhibiting functional gene of *Trichoderma asperellum* will be cloned and sequenced for recombination and transfer to plants to mitigate soil N_2O emissions.

Comparison of present nitrification inhibitors

In comparison with present reputed nitrification inhibitors, such as 2-urease inhibitor [43, 51], coated urea [52], nitrapyrin [53], dicyandiamide (DCD), and N-(n-butyl) thiophosphoric triamide (NBPT) [26], DCD and (3,4-dimethyl-1H-pyrazol-1-yl), succinic acid isomeric mixture (DMPSA) [29], fungal nitrification inhibitor not only effectively mitigated N_2O emission from agricultural soil but little second pollution and energy-consumption different from chemical inhibitors as well. Meanwhile, fungal N_2O inhibitors, such as *Trichoderma asperellum,* protected plants from disease due to their antagonism and stimulated crop growth when mixed with animal dung to produce bio-organic fertilizer to reduce environmental pollution of the production and application of the chemical fertilizer and pesticides, leading to cleaner husbandry production.

Although plant chemical secretion substances inhibit soil nitrification and N_2O emissions, plants grow long and occupy much land to decrease the food supply and economic harvest, so farmers are reluctant to do so. It is difficult to practice. Relatively, fungal nitrification inhibitors such as *Trichoderma asperellum* in the present work can be fermented and produced in a shorter time to blend with animal dung to produce organic fertilizer, both providing plant nutrients, defending plant disease, stimulating plant growth, and reducing N_2O emissions, instead of chemical fertilizer and pesticides, promoting agricultural carbon neutralization and decreasing environmental pollution. *Trichoderma asperellum* could be further investigated and developed into products that can be applied in agricultural carbon emission reduction and sustainable development.

Conclusions

The N_2O-inhibiting fungus Z17 was identified as *Trichoderma asperellum*, capable of suppressing N_2O emissions from soil with at least 10^6 CFU.g^{-1} soil. The best N_2O-inhibiting effect was on day 9 of inoculation into soil because most of fungal numbers were present in soil.

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Author contributions

HSW: experimental design, writing and instruction, supervision; SYC: conducting experiment, determination and data process and analysis; JD: assisted data analysis; LDS: data analysis; WT: experimental design; TJW: data process and graphics; YHL: preview of manuscript and corrections; ZL: preview of manuscript and corrections; JL: part of experimental work. All the authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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