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N_2O production, a widespread trait in fungi

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N_2O is a powerful greenhouse gas contributing both to global warming and ozone depletion. While fungi have been identified as a putative source of N_2O, little is known about their production of this greenhouse gas. Here we investigated the N_2O-producing ability of a collection of 207 fungal isolates. Seventy strains producing N_2O in pure culture were identified. They were mostly species from the order *Hypocreales* order—particularly *Fusarium oxysporum* and *Trichoderma* spp.—and to a lesser extent species from the orders *Eurotiales, Sordariales, and Chaetosphaeriales.* The N_2O ^{15}N site preference (SP) values of the fungal strains ranged from 15.8‰ to 36.7‰, and we observed a significant taxa effect, with *Penicillium* strains displaying lower SP values than the other fungal genera. Inoculation of 15 N_2O-producing strains into pre-sterilized arable, forest and grassland soils confirmed the ability of the strains to produce N_2O in soil with a significant strain-by-soil effect. The copper-containing nitrite reductase gene (*nirK*) was amplified from 45 N_2O-producing strains, and its genetic variability showed a strong congruence with the *ITS* phylogeny, indicating vertical inheritance of this trait. Taken together, this comprehensive set of findings should enhance our knowledge of fungi as a source of N_2O in the environment.

Terrestrial ecosystems are a major source of nitrous oxide (N_2O)[1,2], a so-called greenhouse gas also commonly known as laughing gas. Although it has received much less attention than CO_2, the 100 year global warming potential of N_2O is 298 times greater than that of CO_2 due to the much longer half life of N_2O[3]. There is also growing concern over nitrous oxide concentrations because, following the reduction of chlorine- and bromine-containing halocarbons by the Montreal Protocol, N_2O has become the main ozone-depleting substance emitted to the stratosphere[4].

Nitrous oxide emissions are mostly due to two microbial processes: nitrification and denitrification. Nitrous oxide is a by-product of the first step of nitrification, the oxidation of ammonia to nitrite[5]. In contrast, N_2O is either an intermediate or the end product of the denitrification cascade, which consists in the reduction of nitrate or nitrite into nitric oxide, nitrous oxide and dinitrogen[6]. Sixty-two percent of the total global N_2O emissions are from natural and agricultural soils (6 and 4.2 Tg N yr^{-1}, respectively[7]), and denitrification is traditionally considered as the main source of these emissions[8].

It is well known that denitrification is widespread among prokaryotes—indeed, the ability to denitrify has been observed in more than 60 bacterial and archaeal genera[9]. Moreover, eukaryotes such as fungi in soils[10] or foraminifers in aquatic environments[11,12] are also capable of denitrification. Characterization of the fungal denitrification ability in *Fusarium oxysporum* and *Cylindrocarpon tonkinense* has shown that this reductive process was performed via a copper-containing nitrite reductase (NirK) and cytochrome P450 nitric oxide reductase[10]. However, no nitrous oxide reductase has been identified in fungi and N_2O is the end product of denitrification in the few characterized fungal strains[13,14]. By using fungal or bacterial inhibitors to distinguish the microbial origin of N_2O, previous studies have reported that fungi could contribute up to 18% of potential denitrification[15] and be significant N_2O producers in some terrestrial systems[16,17]. Despite the importance of fungi in several soil functions, such as organic matter decomposition and primary production through symbiotic or pathogenic relationships with plants[19], the production of N_2O by fungi has only been studied in a limited number of strains[14,20]. To what extent this trait is conserved amongst fungi remains unknown, but understanding the microbial sources of this greenhouse gas will be crucial for selecting mitigation strategies. Here, we screened a collection of 207 fungal strains belonging to 9 classes and 23 orders to determine the prevalence of the N_2O-

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producing capacity among fungi. We further characterized the initial and end-products of denitrification of the N_2O production-positive strains in pure culture and determined their N_2O isotopic signature. Positive fungal strains were also inoculated into pre-sterilized arable, forest and grassland soils in order to verify their ability to produce this greenhouse gas in soil. Finally, we studied the phylogeny of the *nirK* gene, which encodes the copper-containing nitrite reductase using newly developed primers, and investigated the relationships between the nuclear ribosomal internal transcribed spacer (ITS) region and *nirK* phylogeny, N_2O production rates and N_2O isotopic signatures.

Results and Discussion

To assess how the N_2O producing ability is distributed within fungi, 207 fungal strains comprising 23 orders and 54 genera were screened by incubating the strains in liquid culture under conditions that were previously reported to favour fungal denitrification[21]. The strains were selected to cover the largest possible fungal diversity within the Microorganisms of Interest for Agriculture and Environment collection (MIAE) (INRA, Dijon, France), which is dedicated to soil microbial diversity. At the end of the incubation, differences in the pH of the media were observed between strains. Since N_2O can also be produced by chemical denitrification at low pH[22], abiotic N_2O production from nitrite was evaluated in sterile media with a pH gradient, and strains were scored positive when the N_2O concentrations in the headspace were higher than those in the sterile flasks at the same pH (Fig. S1). When nitrite was used as an electron acceptor, more than a third of the strains were capable of producing N_2O, with activities ranging from 0.5 ± 0.1 to 60.0 ± 36.0 mg N_2O-N g^{-1} dry fungal biomass (Fig. 1). The N_2O-producing activities were much lower when nitrate was used as an electron acceptor (<0.1 mg N_2O-N g^{-1} fungal biomass; $F = 108.55$, $P < 0.0001$), supporting previous studies showing that nitrite rather than nitrate is preferable for fungal denitrification[23]. No difference was observed when incubating the positive strains with and without acetylene, indicating that the fungi does not reduce N_2O, which was also in accordance with previous studies[24]. Accordingly, amplification of the *nosZ* gene using various primer sets[25,26] was not successful (data not shown). The high proportion of N_2O-producing fungal strains observed in our study contrasts with previous studies in which only 1% to 10% of examined bacteria were capable of denitrification based either on culture-based, direct-molecular approaches or genome analyses[27–29]. However, the maximum percentage of nitrogen recovered as N_2O from nitrite in our work was about 38%, and most of the fungal strains reduced between 3% and 10% of the nitrite, while denitrifying bacteria are capable of reducing at least 80% of soluble nitrogen into gas[30]. Nonetheless, lower percentages were also reported for denitrifying bacteria such as *Bacillus* species, with ranges between 3.5% and 13.2% of the nitrate reduced to gaseous nitrogen after 48 h of growth[31]. Although we cannot rule out that growing the strains in different media or conditions may have resulted in different rates of nitrogen reduction, our incubation experiment using the standard media and conditions showed that N_2O production is common within the fungal kingdom.

Fifty out of the 70 positive strains belonged to the *Hypocreales* order, and *Fusarium* and *Trichoderma* were the main *Hypocreales* genera observed. Interestingly, many of the *Fusarium* strains identified as N_2O-producers were *Fusarium oxysporum*. Even within this species, the production rate was highly variable, ranging from 2.8 to 34.7 mg N_2O-N g^{-1} dry fungal biomass. This species was reported to be one of the dominant fungal taxa in several studies[32], and accounted for up to 43% of the ITS pyrosequencing dataset retrieved from Mediterranean soils[33]. *F. oxysporum* includes non-pathogenic and pathogenic strains, with the latter causing disease to a broad range of host plants, but no plant-based bioassay has been conducted on the tested strains to discriminate pathogenic and non-pathogenic *F. oxysporum*. In any case, the high number of N_2O-producing *F. oxysporum* individuals suggests this species is involved in greenhouse gas emissions, and therefore are potentially detrimental in terms of both primary production and climate regulation. In both *Trichderma* and *Fusarium* species capable of living in plant tissue, respiration of nitrogen oxides is likely due to the adaptation to hypoxic conditions. Indeed, the oxygen concentration near or within plant tissue is low ($<1\%$)[34], and several studies have reported that it is critical to the fungal infection of plants that the infecting fungus possess strategies to overcome hypoxia[35]. The other positive strains identified in this work belong to the *Eurotiales* (8), *Sordariales* (5), *Chaetosphaeriales* (3), *Mucorales* (1), *Pleosporales* (1), *Glomerellales* (1) and *Ophiostomatales* orders (1). *Sordariales* has also been reported as a dominant order in agricultural soil, while the order *Pleosporales* is frequently detected in that environment[32]. Collectively, our results indicate that N_2O production is a common trait in fungal taxa that

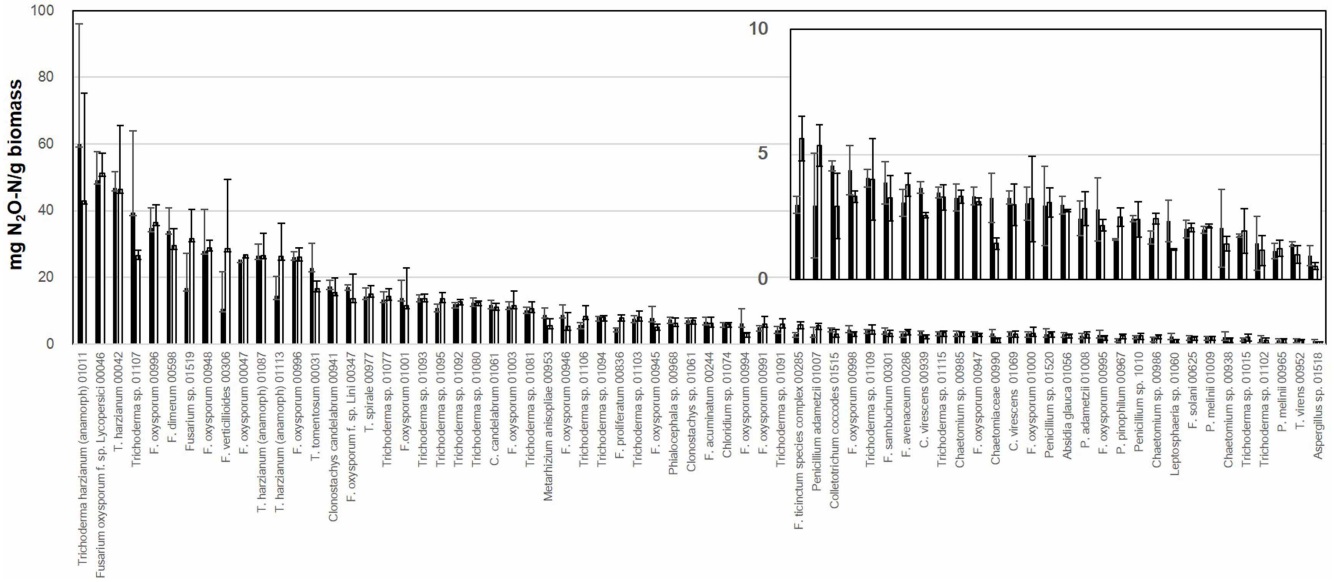

Figure 1 | N_2O production by the positive fungi strains with (white) and without (black) 10% C_2H_2 in the headspace. Error bars indicate the standard deviation (n = 3). Both strain names and MIAE numbers are indicated.

are frequently abundant in soils, although this feature seems to be more a strain-specific than a species-specific trait. It also underlines the importance of quantifying the fungal contribution to terrestrial N_2O emissions.

Analysis of the N_2O isotopomer ratios (relative abundance of $^{14}N^{15}N^{16}O$ or $^{15}N^{14}N^{16}O$ to that of $^{14}N^{14}N^{16}O$) has been proposed as a powerful method to obtain more information on the sources of this greenhouse gas[36]. However, the site preference (SP) values due to fungal N_2O production have only been determined in 2 strains (*F. oxysporum* and *C. tonkinense*)[37], and a more comprehensive analysis of the variability of the fungal ^{15}N site preference is required for a robust distinction between the bacterial and fungal contributions to N_2O emissions. The SP values of the 67 fungal strains tested herein varied from 15.8–36.7‰ with an average value of 30.0 ± 4.8‰. Our results are partly consistent with the previous study reporting a positive SP of 36.9–37.1‰[37]. However, we found a larger range of variation, with SP values as low as 15.8 ± 2.6‰ for *Penicillium melinii* and as high as 36.7 ± 2.2‰ for *F. sambucinum*. These strains with low SP values often had more acidic conditions at the end of the incubation (pH 3–5; Fig. S1). Therefore, we cannot rule out that these lower SP values resulted from the higher contribution of abiotic N_2O production under more acidic conditions[38]. Interestingly, a significant taxa effect ($P < 0.01$ by Student's *t*-test) with lower SP values was observed for the *Penicillium* strains compared to the other fungal genera. We thus confirmed that the N_2O isotopic signature can be used to determine which organisms, *i.e.*, fungi or bacteria, are producing N_2O by denitrification because of their distinct SP (about 0–10‰ for bacterial denitrification[39]). However, it will be difficult to distinguish N_2O emissions from nitrification and fungal denitrification in the environment, since SP values for the N_2O produced by both bacteria and archaea during ammonia oxidization ranged between 13.1 and 30.2‰[40,41]. Since fungi are microaerophilic denitrifiers, our results suggest that a stable isotopic approach alone is not enough to decipher whether nitrification or fungal denitrification is contributing to N_2O emissions in environments where fungi are abundant.

To further confirm that fungi could actually produce N_2O in soil and not only in liquid culture, 15 of the positive strains were selected based on their high activity in pure culture and/or their taxonomic affiliation for inoculation into an arable, a grassland and a forest pre-sterilized soils. After allowing soil colonization by the inoculated fungal strains for a month, nitrite was added to induce denitrification and the concentration of N_2O in the headspace was measured after 2, 4 and 7 days. Seven strains belonging to the *Trichoderma, Fusarium, Penicillium* and *Phialocephala* genera produced N_2O in at least one of the soils, up to a maximum amount of 82.1 ng N_2O-N g^{-1} h^{-1} (Fig. 2), which was significantly higher ($F = 15590$, $P < 0.0001$) than the amount produced by chemical denitrification in the non-inoculated sterile soils. This is comparable to previous studies focusing on fungal contribution[15–17,42] or net N_2O production from soil[43–45]. In our study, the highest amount of N_2O produced was observed in the forest soil for *Fusarium verticillioides* and *F. dimerium*, while the grassland and the arable soils were the soils with the highest emissions for *Trichoderma harzianum* and *Phialocephala* spp. on one hand, and *F. oxysporum* f. sp. *lini*, *Metarhizium anisopliae* on the other. These significant strain-by-soil effects ($F = 4.69$, $P < 0.0001$) likely reflect differences in fungal nutrient requirements and/or preferences for different soil physico-chemical characteristics. Indeed, previous studies demonstrated that several soil physico-chemical parameters, such as extractable P concentrations, C/N ratio[46], pH, sand content or litter cover[47], can affect the soil fungal communities or fungal biomass[48]. The fungal N_2O-producing capacity observed in our study could also be influenced by biotic interactions occurring in the natural ecosystems between fungi or with bacteria and other organisms.

Both copper-containing nitrite reductase (encoded by the *nirK* gene) and P450nor (nitric oxide reductase) are key enzymes involved in fungal denitrification. However, P450nor belongs to a superfamily of proteins that are widely distributed among fungi and known to be involved in a wide variety of physiological reactions[10], which prevents the use of the corresponding genes as molecular markers to target denitrifying fungi. To date, several primers targeting the *nirK* denitrification gene have been described in the literature, but none of them was designed to amplify denitrifying eukaryotes[25,49]. Despite the low number of fungal *nirK* sequences available in the databases (less than 30) and the high diversity of the tested fungal strains, the amplification of the fungal *nirK* denitrification gene was successful in 45 out of 70 strains using our newly designed primer set EunirK-F1 and EunirK-R2. This supports our findings that these fungal strains are capable of denitrification and that N_2O was not produced by other processes. Notably, when used to amplify DNA extracted from soil, our primer set also amplified the bacterial *nirK* in part due to the lower proportion of fungi in soil compared to bacteria (data not shown). A phylogenetic tree was constructed using these fungi *nirK* sequences and bacterial *nirK* sequences from available databases (Fig. 3). Our fungal *nirK* sequences clustered with the other fungal *nirK* homologues retrieved from the database, and were distinct from the bacterial *nirK* sequences. The phylogeny also shows that fungal *nirK* sequences are closer to other eukaryotic sequences (amoeba, protozoa or green alga) than to bacterial ones, indicating that fungal *nirK* sequences branched from bacterial *nirK* sequences at a very early stage of their evolutional history, as suggested by Kim et al.[50]. In addition, we also found a strong congruence between the *ITS* and the *nirK* phylogenies (Fig. S2), indicating a vertical inheritance of *nirK* genes. Interestingly, we found no correlation between the genetic distance of the *nirK* genes and the N_2O production rates. Similarly, weak or no relationships were observed between the bacterial genotypes and denitrification phenotypes in previous studies[31,51]. Phenotypic convergence within similar ecological niches of distantly related organisms can lead to such a discrepancy between genetic and phenotypic distances. In depth investigation of the ecology of denitrifying fungi would undoubtedly help clarify which environmental factors lead to a convergent denitrification phenotype.

In conclusion, the analysis of a vast collection of fungi showed that N_2O production is a common and widespread trait in fungi. Nitrite instead of nitrate was the preferred substrate, while N_2O was always the end-product of denitrification. We showed that the range of variation of the N_2O isotopic signature was taxa-dependent and larger than previously reported, with values as low as 15‰. Inoculation of 15 strains into previously sterilized arable, forest and grassland soils demonstrated the ability of fungi to contribute to soil N_2O emissions with fluxes potentially as high as those reported in natural soils. Further studies are clearly warranted to elucidate the significance of denitrification in fungi and its consequences for N_2O emissions.

Methods

Fungal strains and in vitro incubation experiments. The fungal strains tested in this study were previously isolated from agricultural soils or plant roots. The details of the isolating procedures have been described previously[52]. The strains were purified by single-spore isolation and preserved in the MIAE collection (INRA, Dijon, France). Fungal strains were first cultured under an aerobic condition in a 147 ml plasma flask containing 50 ml of liquid malt medium (pH 7.5). The flasks were incubated with a rotator shaker at 25°C, 120 rpm. After 3 days to 2 weeks depending on their growth, 50 ml of liquid malt medium with 10 mM $NaNO_2$ (pH 7.5) was added to the plasma flasks, which were capped with a butyl rubber stopper. The headspace gas was then replaced by pure N_2 gas and 1 ml of pure O_2 to obtain microaerobic conditions. The flasks were incubated again at 25°C, 120 rpm for one additional week. The incubations were performed in triplicate flasks for each fungal strain. After the second incubation round, 0.5 ml of headspace gas was sampled and N_2O concentrations were determined by gas chromatography. Three previously characterized

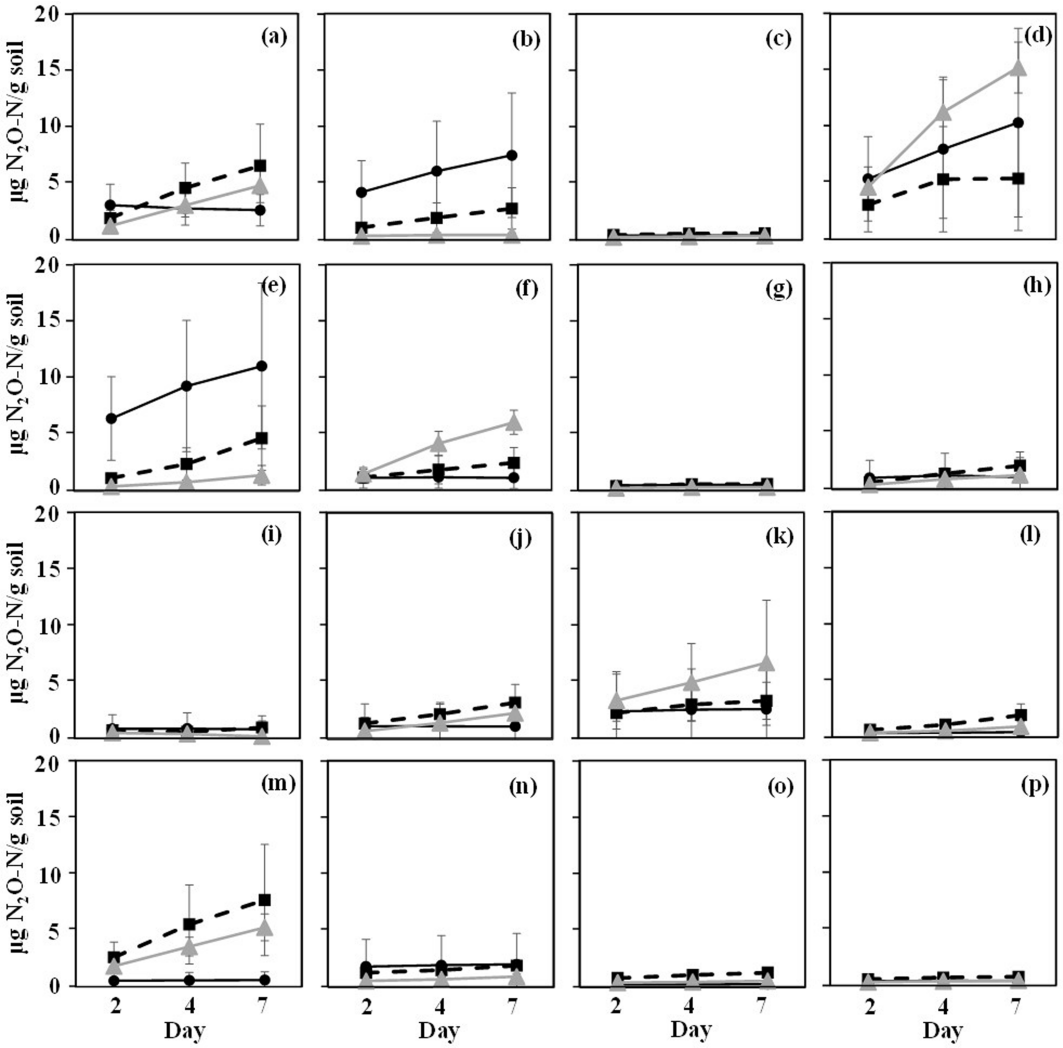

Figure 2 | N_2O production by 15 selected fungi strains inoculated in 3 different sterile soils. NO_2^- was used as the electron acceptor. The strains were incubated for 7 days, and headspace N_2O concentration were measured 3 times (2, 4 and 7 days after NO_2^- addition). Code for the soils: Black circle: Sweden (Slogaryd), forest soil; grey triangle: France, arable soil; black rectangle: The Netherlands, grassland soil. Code for the fungi: a: *Trichoderma harzianum* (MIAE00042); b: *Fusarium verticillioides* (MIAE00306); c: *Penicillium adametzii* (MIAE01008); d: *F. oxysporum* f. sp. *lini* (MIAE00347); e: *F. dimerum* (MIAE00598); f: *Metarhizium anisopliae* (MIAE00953); g: *Chaetomium* sp. (MIAE00985); h: *T. harzianum* (anamorph) (MIAE01011); i: *Leptosphaeria* sp. (MIAE01060); j: *T. tomentosum* (MIAE00031); k: *Fusarium* sp. (MIAE01519); l: *Clonostachys candelabrum* (MIAE00941); m: *Phialocephala* sp. (MIAE00968); n: *Colletotrichum coccodes* (MIAE01515); o: *Aspergillus* sp. (MIAE01518);); p: No fungi control. The error bar represents the standard deviation (n = 3).

strains[24,53,54], *F. oxysporum* MT811 (JCM11502), *Cylindrocarpon lichenicola* (NBRC30561) and *Aspergillus oryzae* RIB40 (NBRC100959), were used as positive controls. At the end of the incubation, mycelia were collected and dried for 24 h at 105°C for fungal biomass determination, and the pH of the medium was determined by using a commercial electrode.

Strains capable of producing N_2O were also incubated as described above, but with NO_3^- as electron acceptor ($NaNO_3$ at a final concentration of 5 mM), and biomass and pH determination were performed as described above. Gas measurements were done with and without 10% C_2H_2 gas in the headspace[55] to verify whether or not they were capable of reducing N_2O into N_2.

Fungal N_2O production in sterile soil. The physical and chemical parameters of the three soils used in this study are described in Table 1. Triplicate samples were collected from the top 10 cm of three different soils, sieved to <2 mm and sterilized by γ -radiation (35 kGy; Conservatome, Dagneux, France).

Denitrifying strains were incubated with liquid malt medium (400 mL) in 1 L flasks at 25°C, 120 rpm for 7 to 14 days so that enough biomass was obtained. Liquid cultures were centrifuged (12,000 rpm for 10 min) in 50 mL tubes and washed twice with 30 mL of sterile physiological water (0.9% NaCl). For each soil, fungal pellets were resuspended with sterile water and inoculated into two series of triplicate microcosms containing the γ-ray sterilized soil (5 g in 147 mL sterile bottles). Soil moisture was adjusted in order to obtain a water holding capacity (WHC) of 60% after the inoculation, and all the inoculated soil microcosms were incubated for 3–4 weeks at room temperature to allow soil colonization by the fungi. After this pre-incubation, 2 mL of $NaNO_2$ solution (10 mM) was added and the soil moisture was adjusted at a WHC of 90%. In half of the replicated microcosms (three for each soil), 10 mL of the ambient air in the headspace was replaced with 10 mL C_2H_2, and all bottles were incubated at 25°C for one additional week. Production of N_2O was also monitored in all three sterile soils without inoculation of fungi (negative controls).

Nitrous oxide emission measurements. Nitrous oxide production by fungal pure culture was determined by analysing the gas samples collected in the headspace gas using a gas chromatograph (TRACE GC Ultra; Thermo) equipped with an electron capture detector (GC-ECD).

In the inoculated soil microcosms, gas samples were collected 3 times (2, 4, and 7 days after NO_2 addition) from the headspace and were analysed with GC-ECD to determine the N_2O concentrations. The N_2O concentrations were analysed by ANOVA using the GLM procedure in SAS[56]. For the liquid medium culture experiment, strain and substrate (NO_2 or NO_3) were used for the fixed effect. For the sterilized soil incubation experiment, strain and soil were used for the fixed effect and strain*soil was included in the model. Tukey's multiple comparisons test was used to separate the means. The significance level was 0.05.

Nitrous oxide isotopic signature measurements. Ten ml of each headspace gas sample was taken at the end of the incubation experiments and stored in a pre-evacuated vial. The samples were then introduced into a gas chromatograph-isotope

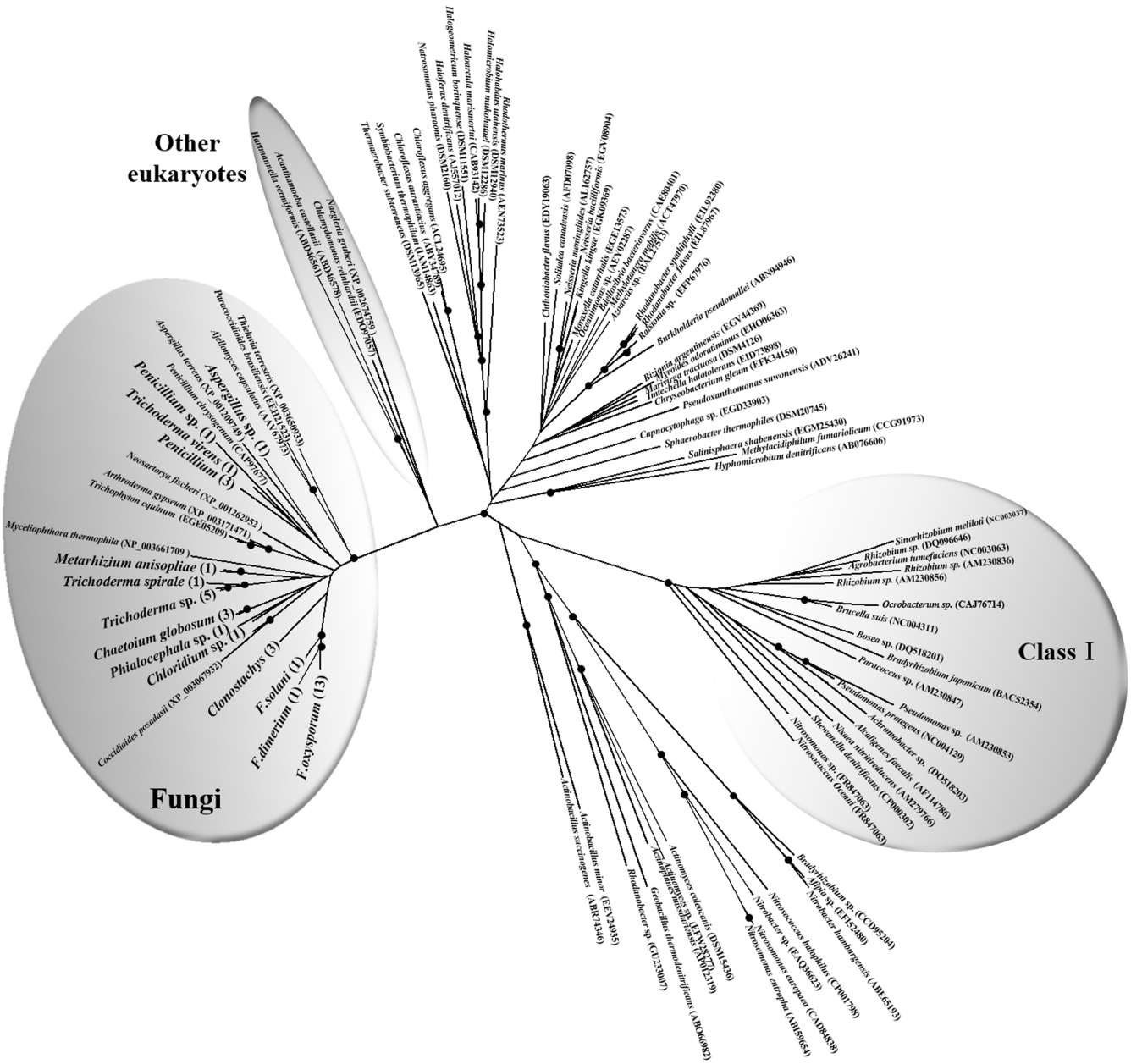

Figure 3 | Neighbour-joining phylogenetic tree of *nirK* amino acid sequences constructed by Clustal W with 1000 bootstrap samplings. Strain names in bold indicate the sequences obtained in this study. The numbers in parentheses indicate the number of the strains. Bootstrap values greater than 75% are indicated as black circles.

Table 1 | Soil characteristics and properties across a range of ecosystem types

		Clay	Loam	Sand	Water content	WHC		Total-C	Total-N		OM
Country	Description	%	%	%	g g^{-1} soil DW	g g^{-1} soil DW	pH	g kg^{-1} soil DW	g kg^{-1} soil DW	C:N ratio	g kg^{-1} soil DW
France	Non-irrigated arable land	18.4	66.4	15.1	0.11	0.50	6.6	10.2	0.97	10.6	17.7
The Netherlands	Permanent Grassland	3.4	2.7	93.8	0.17	0.44	5.1	20.3	1.25	16.3	35.1
Sweden	Coniferous forest	21.4	15.9	62.7	1.51	2.54	4.3	253.0	14.2	17.8	438.0

WHC: water holding capacity; OM: organic matter; DW: dry weight.

ratio mass spectrometer (GC-IRMS) (MAT 252; Thermo Fisher Scientific K.K., Yokohama, Japan) system as described elsewhere to measure the N_2O isotopomer ratios[57]. Site-specific N isotope analysis in N_2O was conducted using ion detectors that had been modified for the mass analysis of fragment ions of N_2O (NO^+) containing N atoms in the centre positions of N_2O molecules, whereas the bulk (average) N and oxygen isotope ratios were determined from molecular ions[58]. Pure N_2O (purity > 99.999%; Showa Denko K.K., Japan) was calibrated with international standards and used as a working standard for the isotopomer ratios. The notation of the isotopomer ratios is shown below. The measurement precision was typically better than 0.1‰ for $\delta^{15}N^{bulk}$ and $\delta^{18}O$, and better than 0.5‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$.

$$\delta^{15}N^{i} = \left({}^{15}R^{i}_{sample} / {}^{15}R_{std} - 1 \right) \quad (i = \alpha, \beta, \text{or bulk}) \tag{1}$$

$$\delta^{18}O = \left({}^{18}R_{sample} / {}^{18}R_{std} - 1 \right) \tag{2}$$

Here, $^{15}R^{\alpha}$ and $^{15}R^{\beta}$ respectively represent the $^{15}N/^{14}N$ ratios at the centre and end sites of the nitrogen atoms; $^{15}R^{bulk}$ and ^{18}R respectively indicate the average isotope ratios for $^{15}N/^{14}N$ and $^{18}O/^{16}O$. The subscripts "sample" and "std" respectively indicate the isotope ratios for the sample and the standard, atmospheric N_2 for N and Vienna Standard Mean Ocean Water (V-SMOW) for O. We also define the ^{15}N site preference (hereinafter SP) as an illustrative parameter of the intramolecular distribution of ^{15}N:

$$^{15}N - \text{site preference } (SP) = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}. \tag{3}$$

The N_2O concentration was measured simultaneously with the isotopomer ratios by comparing the peak area of the major ion (mass 44 and 30 in molecular ion analysis and fragment ion analysis, respectively) obtained with the sample gas and with a reference gas (349 nL/L N_2O in Air; Japan Fine Products Co., Ltd.)[57].

Primer design. Full-length *nirK* nucleotide sequences of fungal genomes were obtained from the Functional Gene Pipeline public database (http://fungene.cme.msu.edu//index.spr). These sequences were aligned using MEGA in order to design fungal specific −*nirK* primers. The primer sequences were as follows: EunirK-F1 (5′-GGB AAY CCI CAY AAY ATC GA-3′) and EunirK-R2 (5′-GGI CCI GCR TTS CCR AAG AA-3′).

DNA extraction, and PCR amplification of the nirK gene and ITS from positive strains. DNA extraction from the denitrifying fungal cultures was performed using a commercially available DNA extraction kit, DNeasy® Plant Maxi (QIAGEN). The extraction was performed according to the manufacturer's instructions. The purified DNA samples were stored at −20°C until further analysis.

The PCR protocol for the *nirK* gene was as follows: 10 min at 94°C and 40 cycles consisting of 1 min at 94°C, 30 s at 53°C, and 1 m at 72°C. For amplification of the fungi Internal Transcribed Spacer (ITS) region, the primer sets ITS5 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS4 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) were used[59]. The PCR protocol was as follows: 10 min at 95°C and 35 cycles consisting of 95°C for 15 s, 30 s at 52°C, and 72°C for 1.5 s. These amplicons were purified and sequenced with the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and the ABI Prism 3100 genetic analyser. The nucleotide sequence of the ITS regions of other fungi and bacterial *nirK* (both class 1 and 2) amino acid sequences were also obtained from the database described above, and the phylogenetic tree was constructed based on the maximum likelihood method using CLUSTALW. Congruence between the *ITS* and *nirK* phylogenies was graphically illustrated using the *cophyloplot* function from the "ape" R package[60].

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Author contributions

L.P. and C.S. designed the experiments. K.M., A.S. and L.P. wrote the paper. K.M., C.H., M.C.B. and F.B. screened the fungal isolates. V.E.H. verified the fungal isolates. K.M., S.T. and N.Y. worked on the isotopic experiments.

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