***Highlights (for review)**

Background: The greenhouse gas N_2O (nitrous oxide) is produced during denitrification, i.e. the sequential reduction of nitrate or nitrite to N-gases under anoxic conditions in soil. N_2O is the final denitrification product of fungal denitrification. However, the contribution of fungi to denitrification is not yet sufficiently investigated and precise methods to differentiate between N_2O fluxes from bacteria and fungi are lacking to date. Although recently developed marker gene methods are well suited to describe the genetic potential for fungal denitrification in soils, approaches to assess active fungal denitrifiers are lacking. Furthermore, the correlation between fungal *p450nor* marker gene expression and N_2O production rate was likewise unclear, although important for understanding implications of gene expression data for N_2O fluxes.

Highlights:

- New quantitative PCR assay targeting fungal *p450nor*
- *Fusarium oxysporum p450nor* expression correlates with nitrous oxide production
- *F. oxysporum* produced nitrous oxide with high ^{15}N site preference
- *F. oxysporum* tolerates high concentrations of nitrite (> 5 mM)

We developed and used a quantitative PCR based *p450nor* gene expression assay to determine active fungal denitrification and showed that fungal *p450nor* expression correlates with nitrite concentrations and N_2O emission rates in the model denitrifier *Fusarium oxysporum*. *P450nor* encodes for a nitric oxide reductase central to the denitrification pathway of fungi. Primers developed for the qPCR assay target a broad range of fungal *p450nor* and data suggest an amplification of *p450nor* from environmental samples, allowing for the application of our qPCR assay to soil.

Nitrite induced transcription of *p450nor* during denitrification by *Fusarium oxysporum* correlates with the production of N_2O with a high ^{15}N site preference

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Keywords: cytochrome *p450nor*, fungal denitrification, NO reduction, gene expression, ^{15}N site preference

Abstract

The greenhouse gas nitrous oxide (N_2O) is produced in soil as a consequence of complex co-occurring processes conducted by diverse microbial species, including fungi. The fungal *p450nor* gene encodes a nitric oxide reductase associated with fungal denitrification. We thus hypothesized that *p450nor* gene expression is a marker for ongoing fungal denitrification. Specific PCR primers and quantitative PCR (qPCR) assays were developed targeting *p450nor* genes and transcripts. The novel PCR primers successfully amplified *p450nor* from pure cultures, and were used in an mRNA targeted qPCR to quantify *p450nor* gene transcription (i.e., gene expression) during denitrification activity in cultures of the fungal model denitrifier *Fusarium oxysporum*. Gene expression was induced by high (5 mM) and low (0.25 mM) nitrite concentrations. Nitrite stimulated N_2O production rates by *F. oxysporum*, which correlated well with an up to 70-fold increase in *p450nor* gene expression during the first 12-24 hours of anoxic incubation. The relative *p450nor* gene peak expression and peak N_2O production rates declined 20- and 2-fold on average, respectively, towards the later phase of incubation (48-120 h). The ^{15}N site preference of N_2O ($SP(N_2O)$) was high for *F. oxysporum* and independent of reaction progress, confirming the fungal origin of N_2O produced. In conclusion, the developed fungal *p450nor* gene expression assay together with the analysis of $SP(N_2O)$ values provide a basis to improve current tools for the identification of fungal denitrification and/or N_2O production in natural systems like soils.

1. Introduction

Denitrification is an anaerobic respiration, usually coupled to energy conservation in ATP, and one of the main processes in soil producing the greenhouse gas nitrous oxide (N_2O). This pathway represents the reduction of nitrate (NO_3^-) and/or nitrite (NO_2^-) via nitric oxide (NO) to N_2O and dinitrogen (N_2) (Knowles, 1982; Zumft 1997). Many pure culture and soil studies gathered extensive knowledge on bacterial denitrification. Studies focusing on fungal denitrification attracted more attention only in recent years (Stein and Yung, 2003; Hayatsu et al., 2008; Ma et al., 2008; Chen et al., 2014; Maeda et al., 2015; Higgins et al., 2016), although it is well known since the 1990s that certain fungi and archaea are likewise capable of denitrification (Bollag and Tung, 1972; Shoun et al., 1992; Cabello et al., 2004; Mothapo et al., 2015). Recently, only *Fusarium* strains among 22 fungal strains tested were found to produce significant amounts of N_2O during anaerobic NO_2^- respiration, i.e. denitrification;

N_2O production from other tested fungal strains were marginal (Keuschnig et al., 2020). N_2O-production by non-denitrifying fungi lacking a NO_2^- reductase is considered to be associated with P450nor catalysed secondary metabolism/ NO detoxification rather than energy conservation by anaerobic respiration (Higgins et al., 2018). Fungi producing large amounts of N_2O like *Trichoderma hamatum* not assessed by Keuschnig et al. (2020) suggest that further fungi using the canonical denitrification pathway for anaerobic respiration remain to be identified (Rohe et al., 2014a; Rohe et al., 2014b; Mothapo et al., 2015; Rohe et al., 2017). The lack of N_2O reductase results in N_2O as the end product of fungal denitrification (Shoun et al., 1992), allowing for a high fungal contribution to N_2O emission from a soil community. This suggestion was supported by laboratory studies using growth inhibitors for either bacteria or fungi, showing that denitrification in specific environments, like acidic soil, appeared to be dominated by fungal compared to bacterial contributions (Laughlin and Stevens, 2002; Crenshaw et al., 2008; Chen et al., 2014; Huang et al., 2017). Understanding fungal N_2O producing pathways and their regulation in more detail is thus very important.

So far, identification of N_2O production via different microbial pathways is very challenging and impossible by measurement of N_2O fluxes only. Monitoring N_2O emissions alone implies many uncertainties, like the presence of co-occurring N_2O producing processes, N_2O reduction and diverse organisms associated with N_2O metabolism (Stein and Yung, 2003; Wallenstein et al., 2006; Baggs, 2011). Developing methods to identify sources for N_2O would help for a better quantification of different sources, but also to improve existing models to predict denitrification activities. Improved understanding of N_2O producing activities in soil may also lead to improved mitigation strategies for N_2O emission in the future.

Many laboratory studies and also several field studies used analysis of N_2O isotopocule values ($\delta^{15}N$, $\delta^{18}O$ and ^{15}N site preference of N_2O ($SP(N_2O)$, i.e. difference between isotopic ratio of $^{15}N/^{14}N$ of central and outer N atom) to differentiate between pathways (Baggs, 2008; Decock and Six, 2013; Lewicka-Szczebak et al., 2020). Pure culture studies revealed much lower $SP(N_2O)$ values from bacterial denitrification and nitrifier denitrification compared to nitrification and fungal denitrification (Sutka et al., 2006; Ostrom et al., 2007; Sutka et al., 2008; Decock and Six, 2013; Toyoda et al., 2015; Rohe et al., 2017). When applying these methods to N_2O fluxes from soils, knowledge on the isotopic signatures of the N_2O precursors and proportion of N_2O reduced to N_2 is necessary. N_2O precursors and isotopic fractionation of different pathways affect the isotopic composition of N_2O, which is again altered by N_2O reduction (Ostrom and Ostrom, 2011; Denk et al., 2017). Furthermore, even if all these parameters are known, isotopic signatures of N_2O produced by pure cultures showed an

overlapping range for fungal denitrification and nitrification in SP(N_2O) values (16 to 37 ‰), which also applies for bacterial denitrification and nitrifier denitrification (-11 to 0 ‰; Toyoda et al., 2005; Sutka et al., 2006; Sutka et al., 2008; Frame and Casciotti, 2010; Rohe et al., 2017; Yu et al., 2020). Nonetheless, the unique fungal NO reductase (P450nor) was found to be responsible for higher SP(N_2O) values compared to that from bacterial denitrification (Stein and Yung, 2003; Shoun et al., 2012; Yang et al., 2014).

Another very promising tool is the molecular-based analysis of denitrifying microorganisms by analysing the N_2O producing community or identifying and analysing the enzymes being involved in N_2O production, their encoding genes and transcripts (Butterbach-Bahl et al., 2013). The genetic potential of bacterial or fungal denitrification is routinely analysed by amplifying and sequencing genes encoding the NO_3^-, NO_2^- and N_2O reductases (*narG*/*napA*, *nirK*/*S*, and *nosZ*), respectively (e.g. Enwall et al., 2005; Wei et al., 2015; Chen et al., 2016; Ma et al., 2019). So far most studies focused on bacterial Cu- and cytochrome cd_1-NO_2^- reductase genes (*nirK* and *nirS*, respectively), NO reductase genes (*norBC*) or N_2O reductase genes (clade I and II *nosZ*; Braker et al., 2012; Palmer et al., 2015; Hallin et al., 2018). As mentioned before, the *p450nor* gene differs from bacterial NO reductases and was thus identified as a marker for fungal denitrification potentials. PCR assays with *p450nor* specific primers with environmental DNA have recently been established (Higgins et al., 2016; Novinscak et al., 2016; Chen and Shi, 2017). However, to study regulation of N_2O production and improve predictions of N_2O emissions, not only the genetic potential of essentially all organisms present is important, but also its expression. Although urgently needed, such methods to study *p450nor* gene expression are lacking to date.

The aim of the study was thus to develop a molecular marker based approach targeting the specific cytochrome *p450nor* of fungi, in particular *Fusarium spp*., to be used in gene expression analysis as a proxy of N_2O production by fungal denitrification/ NO reduction. Specific objectives were to (a) develop primers and quantitative PCR (qPCR) assays specific for *p450nor* in fungal DNA and RNA, (b) to quantify *p450nor* expression as a proxy for fungal N_2O production activity, and (c) determine potential correlation of *p450nor* expression and fungal N_2O production. As mentioned before, *Fusarium* strains were recently found by Keuschnig et al. (2020) to represent the only tested fungi that produced significant N_2O via anaerobic respiration. Among those *Fusarium* strains, *Fusarium oxysporum* is one of the best studied fungal denitrifiers (Shoun and Tanimoto, 1991; Kobayashi and Shoun, 1995; Zhou et al., 2001; Takaya et al., 2003; Matsuoka et al., 2017), thus representing an ideal model

organism to study *p450nor* expression when incubated anaerobically with NO_2^- as electron acceptor.

2. Material & Methods

2.1 Primer design, DNA extraction, and amplification of p450nor

Gene sequences of fungal and two algal (*Chlamydomonas* and *Chlorella*) *p450nor* were identified by BlastX with a reference sequence of *F. oxysporum* (Figure S1) and used to create a custom data base in ARB (Ludwig et al., 2004; Table S1). The CODEHOP strategy (Rose, 2003) inspired design of *p450nor* specific primers. Five conserved sequence motifs were identified (Figure S1) and many primers delineated (Tables S2-3). The theoretical PCR products of the different developed *p450nor* specific primer pairs ranged from 155 and 875 bases (Table 1, primer sequences Table S3).

Best primer sequences were selected by using the NCBI tool Primer BLAST (Ye et al., 2012) and the Oligonucleotide Properties Calculator 'Oligo Calc' (Kibbe, 2007) by calculating the primer melting temperature (T_m). Primers showing hairpin or dimer formation with more than 5 base pairs (bp) and primer combinations producing amplicons larger than 200 bp were not considered for quantification. *P3.04fw* ($^{5'}$GCCACCATGGTVAAYATGAT$^{3'}$, degeneracy 6, T_m 47.7 - 51.8°C, no hairpin, primer dimer 3 complementary bp) and *p5.02rev* ($^{5'}$GTGTAGATGATRTADGANGG$^{3'}$, degeneracy 24, T_m 45.6 - 51.8°C, no hairpin, primer dimer 2 complementary bp) with an amplicon of approximately 155 bp were used for qPCR (Tables 1, S2 and S3).

This primer set was tested with DNA extracted from five fungi hosting *p450nor* or related genes: *Fusarium oxysporum* (JCM 11502), *Fusarium solani* fsp. *pisi* (NBRC 9425), *Fusarium decemcellulare* (NBRC 31657), *Trichoderma hamatum (*JCM 1875) and *Chaetomium funicola* (JCM 22733). Prior to DNA extraction, fungal hyphae were grown on agar in complex medium (Shoun et al., 1992; Rohe et al., 2014a) and 15 g L^{-1} agar Kobe I (CAS 9002-18-0). The hyphae were carefully scrapped off with a sterile scalpel and transferred to a 2 mL screw cap microcentrifuge tube containing 3 glass beads (Ø 3 mm) and 0.5 g zirconia beads (Ø 0.5 mm). DNA extraction was performed according to a phenol/chloroform protocol (Griffiths et al., 2000). Gel electrophoresis served to verify the successful extraction of DNA as well as the performance of a PCR with known fungal-specific ITS primers (ITS1 and ITS4) (White et al., 1990). Negative control DNA of *Escherichia coli, Ranunculus glacialis, Bacillus subtilis, Pseudomonas fluorescens, Clostridium intestinale,* and *Chlamydomonas reinhardtii* was included in test PCRs (see Supplementary Material Figure S2). The test PCRs

were performed with a Primus 96 Plus Thermal Cycler (MWG AG Biotech, Ebersberg, Germany). The reaction volume was 25 µL containing 12.5 µL AccuStart^{TM} II PCR Tough Mix (Quanta Biosciences, Beverly, MA (USA)), 0.3 µL of 25 mM magnesium chloride solution ($MgCl_2$, Thermo Scientific), 3 µL of 20 mg mL^{-1} bovine serum albumin solution (BSA, New England Biolabs GmbH, Frankfurt, Germany), 1.25 µL of 10 µM of each primer solution, 5.7 µL H_2O and 1 µL template. The PCR started with an initial denaturation step at 95 °C for 6 minutes followed by 40 cycles with denaturation at 95 °C for 30 seconds. Optimal annealing temperature for amplification with primers *P3.04fw* and *P5.02rev* was 53 °C after analysing a temperature range between 44.7 and 72 °C (Supplementary Material Figure S2a). Thus, annealing at 53 °C for 30 seconds and elongation at 72 °C for 1 minute and a final elongation step at 72 °C for 5 minutes were applied. The amplification of the correct *p450nor* gene fragment size was confirmed by gel electrophoresis (1 % agarose in TAE buffer, stained with ethidium bromide, 90 V for 60 min; Supplementary Material Figure S2) and by cloning and sequencing according to published protocols (Zaprasis et al., 2010) and section 2.3.5 below.

2.2 Experimental setup to study gene expression of p450nor by Fusarium oxysporum

F. oxysporum (JCM 11502) was preincubated in liquid medium after Shoun et al. (1992) in 150 mL in 300 mL Erlenmeyer flasks with cotton stopper at 22 °C on a rotary shaker (100 rpm) in the dark. After 3 days, 5 mL culture were used to inoculate 150 mL fresh medium to prepare an overnight culture. This overnight culture of *F. oxysporum* was used for the incubation under anaerobic conditions.

In experiment 1, 66 serum bottles (120 mL with crimp-top) were prepared, containing 47 mL fresh medium and 2 mL medium of the overnight culture. Two treatments with a total volume of 50 mL in 120 mL were prepared by adding (1) 1 mL $NaNO_2$ solution to 33 of these bottles to achieve 5 mM NO_2^- as the electron acceptor in the liquid medium and (2) 1 mL deionized H_2O to the remaining 33 bottles serving as controls without electron acceptor. All flasks were closed with butyl rubber stoppers before flushing with filter-sterilised (0.2 µm) N_2 for 10 minutes to expel O_2. Incubation took place in the dark on a rotary shaker (100rpm) as described above. Additionally, 36 flasks serving as controls of abiotic N_2O production were prepared; 18 flasks containing medium only without NO_2^- addition and 18 containing medium with 5 mM NO_2^- were prepared. This experimental setup did not include additional controls with autoclaved fungal biomass, since previous studies showed that the impact of dead fungal biomass on abiotic N_2O production was negligible (Rohe et al., 2017). The gas phase of three replicates of each treatment (with or without NO_2^-) was sampled at selected time points, i.e.,

after 2, 4, 6, 8, 10, 12, 24, 48, 72, 96 and 120 h for N_2O analysis. Gas sample collection for controls of chemical N_2O production was after 12, 24, 48, 72, 96 and 120 h.

In a second experiment (experiment 2), *F. oxysporum* was incubated with 4 different NO_2^- concentrations, i.e., 0.05, 0.2, 0.5 and 5 mM in 50 mL total liquid volume according to the protocol provided above. Three replicates of each treatment (0.05, 0.2, 0.5 and 5 mM NO_2^-) were sampled for N_2O analysis after 6, 12, 24 and 30 h.

2.3 Analytical methods

2.3.1 Gas chromatography

Gas phase samples from the headspace of one flask were collected with a double syringe (2x 14 mL) and transferred to 12 mL exetainers (Labco®, Lampeter, UK) for analysis of N_2O concentration and isotopic composition of N_2O. Gas samples were analysed for N_2O concentration using gas chromatography (Agilent 7890A Technologies, Santa Clara, USA) with a helium ionization detector (HID). The detection limit was 0.55 ppm and the precision was 1 %. N_2O production rates per gram (g) dry weight of *F. oxysporum* were calculated during experiment 1 considering Henry's law to account for gaseous and dissolved N_2O (Davidson and Firestone, 1988). 1 ppm N_2O in the gas phase (0.07 L) was equivalent to 0.128 µg total N_2O per flask including physically dissolved N_2O (0.05 L liquid phase) in our experiments. N_2O production rates were calculated as change of N_2O in µmol of the current to the preceding sampling per unit time between the two measurements, and normalized to the mean biomass of the same samplings. During experiment 2, fungal biomass was analysed at the end of incubation only, resulting in one N_2O production rate per g dry weight biomass determined at the end of incubation.

2.3.2 Isotopic composition of N_2O produced

Analysis of stable isotopes of N_2O was performed with a pre-concentrator (PreCon, ThermoFinnigan, Bremen, Germany), which was interfaced to a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany; (Brand, 1995; Toyoda and Yoshida, 1999; Köster et al., 2013)). Information about isotopic measurement and normalization was already described elsewhere (Lewicka-Szczebak et al., 2016; Rohe et al., 2017). Ratios of $^{15}N/^{14}N$ or $^{18}O/^{16}O$ in N_2O in relation to the international standards (air-N_2 or VSMOW, respectively) are expressed as $\delta^{15}N^{bulk}(N_2O)$ and $\delta^{18}O(N_2O)$ values, respectively. Analysis of 31/30 ratio of the NO fragment was used to determine the $\delta^{15}N$ value of the central N within N_2O molecule ($\delta^{15}N^{\alpha}(N_2O)$), while the of $\delta^{15}N$ value of the peripheral N within N_2O molecule ($\delta^{15}N^{\beta}(N_2O)$) derives from $\delta^{15}N^{bulk}(N_2O)=((\delta^{15}N^{\alpha}(N_2O)+$

200 $\delta^{15}N^{\beta}(N_2O))/2$) (Toyoda and Yoshida, 1999). The $SP(N_2O)$ value is defined as $\delta^{15}N^{\alpha}(N_2O)$–
201 $\delta^{15}N^{\beta}(N_2O)$.

202 *2.3.3 Biomass of* Fusarium oxysporum *and pH*

203 In experiment 1, fungal biomass was sampled from flasks immediately after gas sample
204 collection. To obtain sufficient biomass, the liquid from the 120 mL flasks was transferred to
205 50 mL tubes, put on ice, and centrifuged with 5236 x g for 30 min at 10°C. The supernatant of
206 three tubes was used to measure pH values of residual medium with a pH electrode (InLab®
207 Routine Pro, Mettler Toledo, Greifensee, Switzerland). pH values of the pure medium before
208 the experiment and from controls for chemical N_2O production was measured likewise. The
209 pellet containing the fungal biomass was washed twice with water before drying the pellet at
210 100 °C for 2 days to measure the dry weight of fungal biomass per flask. In experiment 2
211 (with varying NO_2^- concentration), biomass sampling, pH analysis of the supernatant and
212 extraction of mRNA was performed as described after 30 h of incubation.

213 *2.3.4 RNA extraction*

214 At the same time points as gas sample collections, three additional flasks of each treatment
215 were used to collect fungal biomass followed by total RNA extraction. Medium of these
216 flasks was transferred to RNAse and DNAse-free 50 mL tubes, put on ice, and centrifuged
217 with 5236 x g for 30 min at 10 °C. The supernatant was decanted, while the pellet was
218 transferred to RNAse and DNAse-free 2 mL screw cap tubes and flash-frozen in liquid
219 nitrogen. To disrupt fungal hyphae, bead beating with one glass bead (2.7 mm) and 0.5 g
220 zirconia beads (0.5 mm) was performed (5.5 $m\ s^{-1}$ for 30 sec; FastPrep-24TM, MP
221 Biomedicals, Eschwege, Germany). Total RNA was extracted using peqGOLD Total RNA
222 Kit (S-Line; Peqlab, VWR, Darmstadt, Germany). As a control for contamination, RNAse-
223 free water (Carl Roth, Karlsruhe, Germany) was treated like fungal biomass. RNA
224 concentration was analysed using 1 µL with microliter spectrometry (NanoDrop™,
225 ThermoFisher Scientific). Reverse transcription of 1 µL total RNA to cDNA was done using
226 the qScript™ cDNA Synthesis Kit (Quanta Bio, Berverly, MA, USA) with oligo-dT-priming
227 of polyadenylated mRNA, while 0.01 $\mu g\ \mu L^{-1}$ T4 gene 32 protein (Gp 32 protein, Roboklon,
228 Berlin, Germany) was added to the recommended mixture of the manufacturer. As controls,
229 RNAse-free water (Carl Roth, Karlsruhe, Germany) was used and one sample was prepared
230 without reverse transcriptase but containing fungal RNA.

231 *2.3.5 Quantitative polymerase chain reaction (qPCR) and sequencing*

The total reaction volume was 25 µL, containing 12.5 µL of the PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Beverly, MA (USA)), 0.25 µL of 25 mM $MgCl_2$ solution (Thermo Scientific), 3 µL of 20 mg mL^{-1} BSA solution (New England Biolabs GmbH, Frankfurt, Germany), 0.75 µL of each primer (10 µM), 2.75 µL RNAse-free H_2O (Carl Roth, Karlsruhe, Germany), and 5 µL cDNA template (1:5 diluted). qPCR was performed in technical triplicates with a CFX96 Touch™ Real-Time PCR cycler (Bio-Rad Laboratories Inc. Hercules, CA) with 6 minutes of an initial denaturation step at 95 °C followed by 40 cycles at 95 °C for 15 seconds and annealing as well as elongation at 53 °C for 30 sec. The Bio-Rad CFX Manager3.1 Software was used for data analysis. Cells of *E. coli* (Promega, JM 109 competent cells) containing the cloned DNA sequence of *F. oxysporum* between primers *P3.04fw* and *P5.02rev* were used to generate a standard curve. The qPCR product was purified (HiYield® Gel/PCXR DNA Extraction Kit, SLG; Gauting, Germany), cloned (CloneJET PCR Cloning Kit, Thermo Scientific) with *E. coli* (Promega, JM 109 competent cells), and sequenced (Sanger Sequencing, GATC Biotech AG, Köln, Germany). Nucleotide sequences were analysed with BLASTx (https://blast.ncbi.nlm.nih.gov/), confirming the identity of amplicons as *p450nor*. Amplicon sequences obtained by *P3.04fw* and *P5.02rev* have been deposed at Genbank (https://www.ncbi.nlm.nih.gov/Genbank/) under the accession numbers MW032687- MW032694. The relative gene expression was determined by the delta-delta-Cq method (Pfaffl (2001); Eq. 1), taking cycle quantification value (Cq) of controls, i.e. incubations of *F. oxysporum* without NO_2^- (Cq(control)), as well as that of treatments with NO_2^- (Cq(nitrite)) into account. In consequence, the abundance of *p450nor* mRNA in the presence of NO_2^- as electron acceptor was compared to that in the absence NO_2^- to estimate the induction of *p450nor* gene expression by NO_2^- (*ddCt(NO_2^-)*).

$$ddCt(NO_2^-) = 2^{(Cq(nitrite)-(Cq(control))} \quad (1)$$

The *ddCt(NO_2^-)* values were calculated for three biological replicates (i.e., fungal hyphae of *F. oxysporum* incubated in triplicates), and 3 technical replicates (i.e., cDNA of each biological replicates was used in triplicates for qPCR). Calculated *ddCt(NO_2^-)* values ≥2 were considered as significant *p450nor* gene expression induced by NO_2^-.

2.3.6 Analysis of data

Statistical analysis was conducted using R (R Core Team, 2018). Correlations were determined by Spearman's rank correlation for isotopic values, N_2O concentration as well as production rate, RNA, biomass, *ddCt(NO_2^-)* and incubation time. Experiment 2 was used to

evaluate a possible correlation of *ddCt(NO_2^-)* and NO_2^- concentration using data with *F. oxysporum* and NO_2^- only. The significance level was $p \leq 0.05$ unless otherwise stated.

3. Results

3.1 Primer specificity

Amplicons of the expected size potentially indicative for fungal *p450nor* were obtained with primers *P3.04fw* and *P5.02rev* from N_2O producing fungi, i.e., *F. oxysporum*, *F. solani* fsp. *pisi*, *F. decemcellulare*, and *T. hamatum* (Mothapo et al., 2015), but not from *C. funicola* (Figure S2b). Amplification was specific as suggested by *in silico* analyses (Table S2), detection of a correct sized PCR product of approximately 155 bp, and/or sequencing of amplicons. Amplicons of the expected size were likewise obtained from agricultural soils and green rot (composted lawn cuttings) (Supplementary Material, Figure S3). Controls with DNA from 6 organisms lacking *p450nor* showed no amplification.

3.2 Incubations with F. oxysporum *under denitrifying conditions*

3.2.1 Biomass and pH

The pH of the non-inoculated medium was 7.05 and did not change during incubation in sterile medium, i.e. control flasks without *F. oxysporum*. In treatments with *F. oxysporum*, the pH was always greater than or equal to 6.3 or 6.4 during incubations without or with NO_2^-, respectively. Biomass of *F. oxysporum* marginally increased during the first 48 h of incubation (from 0.001 to 0.007 g dry weight per flask), and remained essentially constant (0.008 – 0.01 g dry weight per flask) between 48 and 120 h of incubation (Tables S4 and S5).

3.2.1 N_2O production

In the treatment with NO_2^- and *F. oxysporum*, N_2O was detectable after 2 h (0.05 µmol g^{-1} h^{-1}; Figure 1, Table S5). *F. oxysporum* produced N_2O throughout the incubation with the maximal production rates approximating 3 µmol g^{-1} h^{-1} after 8 h. More than 300 ppm of N_2O accumulated after 120 h of incubation. Abiotic N_2O production in sterile controls without *F. oxysporum* but with NO_2^- was marginal after 48 h, and increased to 5 ppm until 120 h. Thus, the proportion of chemical N_2O production was negligible, i.e. smaller than 1.6 % of incubations with *F. oxysporum* at the end of incubation. N_2O production was not detectable in flasks without NO_2^- in the presence and absence of *F. oxysporum* (Table S5). In line with experiment 1, N_2O concentration increased with incubation time in treatments with 0.5 or 5

mM NO_2^-, while N_2O was not detectable with 0.05 or 0.25 mM NO_2^- after 30 h of incubation (Table 2).

3.2.2 Isotopic signature of N_2O

Isotopic analysis was possible after 8 h of incubating *F. oxysporum* with 5 mM NO_2^- (Figure S4, Table S5). As expected, $\delta^{15}N^{bulk}(N_2O)$ values slightly increased with incubation time (from -49.6 ‰ to -43.9 ‰; *Spearman's rho* = 0.93, $p < 0.05$) (Table S4). In contrast, $\delta^{18}O(N_2O)$ values peaked at 12 h of incubation (43.7 ‰), and then slightly decreased to 39.2 ‰ (*Spearman's rho* = -0.85, $p < 0.05$). Although $SP(N_2O)$ values varied between 8 and 12 h (31.1 to 36.9 ‰), it was essentially constant (34.2 ± 0.8 ‰) between 24 and 120 h of incubation (*Spearman's rho* = 0.38, $p = 0.07$).

3.3 Total RNA and p450nor *expression (ddCt(NO_2^-)*)

RNA yields per g dry weight of fungal biomass varied within the first 24 h of the incubation with highest values in treatments with and without NO_2^- after 4 h of incubation, and then sharply decreased with incubation time (Figure S5, Table S5). There was a significant positive association between RNA extracted (ng μL^{-1}) and fungal biomass (*Spearman's rho* = 0.49, $p < 0.05$) during the first phase of incubation, but this association was not found after 24 h of incubation (*Spearman's rho* = -0.09, $p = 0.74$). The semi-logarithmic calibration curve for the qPCR assay was linear between 10^2 and 10^8 *p450nor* genes per μL^{-1} with a PCR efficiency of 75-80 %. Transcripts of *p450nor* were found with and without NO_2^-, suggesting basal transcriptional activities even in the absence of NO_2^-. *ddCt(NO_2^-)* values were very low after 2 h of incubation, coinciding with undetectable N_2O production at the first measurement (Figure 1). However, *ddCt(NO_2^-)* values sharply increased after 4 h, i.e. without appreciable delay after NO_2^- supply (Figure 1). *ddCt(NO_2^-)* values peaked between 4 and 24 h of incubation, and decreased during the remaining incubation time. Similar patterns were reproduced in two further replicate incubations (Figures S6), demonstrating an induction of *p450nor* expression by NO_2^-. *ddCt(NO_2^-)* and non-normalized N_2O production rates (in µmol h^{-1}) showed a strong positive association during the first 30 h of incubation (*Spearman's rho* = 0.85; $p < 0.05$). *ddCt(NO_2^-)* and N_2O production rates normalized to biomass (in µmol h^{-1} g_{dw}^{-1}) showed only a weak positive association during the first 8 h of incubation, possibly due to variations in biomass data (*Spearman's rho* = 0.06; $p < 0.05$; Table S4). In line with such observations, linear regression analysis supported a correlation of *p450nor* gene expression and biomass-normalized N_2O production rate within the first 24h of incubation (Figure 2).

The earliest time point when N_2O production rates were at or beyond peak production was around 30 h of incubation. Therefore, when testing different NO_2^- concentrations (experiment 2), *ddCt(NO_2^-)* was determined after 30 h of incubation (Table 2). 0.05 and 0.25 mM NO_2^- marginally increased *ddCt(NO_2^-)* values (1.1 and 3.2, respectively). Such low *ddCt(NO_2^-)* values coincided with undetectable N_2O levels at 30 h. C_q values of treatments with 0.25 and 0.5 mM NO_2^- were significantly higher than those of treatments without NO_2^- ($p < 0.01$; Welch two sample t-test). N_2O production was detectable with 0.5 mM and 5 mM NO_2^- after 30 h. High *ddCt(NO_2^-)* values were found again with 5 mM NO_2^- (Table 2). This confirmed the high gene expression of *p450nor* by *F. oxysporum* induced by high NO_2^- concentrations of experiment 1 (Figures 1 and S6) and showed detectable *p450nor* gene expression as well as an induction at ≤ 0.25 mM NO_2^- (Table 2). Spearman's rank correlation confirmed a positive association of *p450nor* gene expression and NO_2^- concentrations (*Spearman's rho* =0.69, $p<0.05$; Table S4).

4. Discussion

The present study successfully demonstrates the induction of *p450nor* expression in the model denitrifier *Fusarium oxysporum* by NO_2^- using a new qPCR assay and thus extends previous studies on diversity analyses of *p450nor* from pure cultures and environmental DNA (Maeda et al., 2015; Higgins et al., 2016; Novinscak et al., 2016; Chen and Shi, 2017). The genetic potential of fungal species to be capable of N_2O production was detected by primer systems targeting fungal *p450nor* or *nirK* (Maeda et al., 2015; Higgins et al., 2016; Novinscak et al., 2016; Chen and Shi, 2017). Available primer sets were designed to generate rather large amplicons (between 650 and 850 bp) allowing for a decent phylogenetic affiliation of amplified genes. However, such large amplicons are non-ideal for gene expression studies via qPCR demanding short amplicons. The primer sets *P3.04fw* and *P5.02rev* resulting in rather short amplicons of approximately 155 bp fill such a gap.

Fragments of the expected size were amplified from certain environmental samples (Figure S3), suggesting an applicability of the developed qPCR assay to study *p450nor* gene expression in soil, although this has not been attempted in this study. Primer specificity as well as target range remain to be studied in more detail. Specificity of *P3.04fw* and *P5.02rev* was successfully tested with a rather limited number of fungal pure cultures dominated by *Fusarium* sp. and failed to amplify *p450nor* from DNA of *C. funicola*. However, *C. funicola* belongs to the *Sordariales* and showed extremely small N_2O production as well as distinct

isotopic signature of N_2O (especially $SP(N_2O)$) compared to the other clearly N_2O producing fungi belonging to *Hypocreales* (e.g., *Trichoderma* and *Fusarium spp.*) that were also examined in the present study (Rohe et al., 2014a; Rohe et al., 2014b; Rohe et al., 2017). Thus, we previously assumed abiotic N_2O production rather than denitrification by *C. funicola* (Rohe et al., 2017). Data of the present study and recent genomic evidence (from *C. funicola* MPI-SDFR-AT-0129; https://mycocosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=Chafu1) confirm the suggestion that *C. funicola* is not capable to produce N_2O during denitrification due to the absence of canonical *p450nor* genes. Nevertheless, due to the huge genetic diversity represented in soils, specific analysis of *p450nor* gene expression in soils might be challenging. *p450nor* mRNA abundance below the detection limit of the presented qPCR assay (false negatives), unspecific amplifications (false positives), and selective amplification are possible biases (Smith et al., 2006; Asuming-Brempong, 2014). Pre-enrichment of polyadenylated eukaryotic mRNA from total RNA extracts using poly-dT coated magnetic beats (Chung et al., 2018) is one option to increase the likelihood of detecting *p450nor* genes expressed at low levels. Experimental testing of *P3.04fw* and *P5.02rev* primer specificity as well as universality was certainly only touched upon in this study. Such testing becomes particularly important when complex environmental samples such as soils are analysed. Soils are highly complex, spatially and temporally heterogeneous, and strongly differ in their genetic diversity. qPCR assays working accurately with one soil might thus fail to do so with another soil, requiring an evaluation of assay specificity on a per sample basis. Coupling of qPCR to amplicon sequencing and sequence analysis easily facilitates such an evaluation of primer and assay specificity (or target range) per qPCR reaction/sample and provides information on the diversity of expressed *p450nor* at one go.

A broad target range of our primers *P3.04fw* and *P5.02rev* is thus advantageous. It is a plus that a broad range of fungal *p450nor* genes are covered *in slilico* by *P3.04fw* and *P5.02rev* allowing for the detection of hitherto unknown canonical fungal denitrifiers and at the same time fungi using the P450nor for NO detoxification/secondary metabolism. The latter organisms likewise contribute to N_2O production, although to a lesser extent than canonical denitrifiers, and should thus not be fully excluded from analysis. Such *p450nor* genes are not expected to be inducible by NO_2^-, are consequently not expected to dominate *p450nor* gene expression under denitrifying conditions and might thus be discriminable. A broad detection range of *p450nor* gene expression studies will enable such analyses and shed light on the role of *Fusarium* spp. relative to other fungal taxa. Interestingly, of 22 tested fungi only those of the genus *Fusarium* showed significant N_2O production and anaerobic respiration activities

(Keuschnig et al., 2020), suggesting that the denitrification trait (producing N_2O as part of anaerobic respiration from NO_2^- rather than by NO-detoxification) is less common among fungi than previously thought, and that *Fusarium* sp. are important players for fungal denitrification associated N_2O production. This is in line with Higgins et al. (2018), who recently suggested fungal N_2O production by *p450nor* being part of a secondary metabolism rather than denitrification for most fungi, since growth in fungal biomass is not observed under anoxic conditions (Shoun and Tanimoto, 1991; Zhou et al., 2001). When exposed to anoxic conditions with NO_2^- serving as electron acceptor, *F. oxysporum* switched very fast to denitrification (Figure 1, S6). Gene expression of *p450nor* as well as N_2O production was detectable without appreciable delay and very high after 4 h of incubation. During the first 20 h of incubation, the *p450nor* gene expression continuously increased, coinciding with increasing N_2O production rates and suggesting buildup of the *p450nor* enzyme pool. However, after 30 h, *ddCt(NO_2^-)* values dropped to a constant lower level (minimum *ddCt(NO_2^-)* value of about 10), suggesting that the *p450nor* expression was still induced. The N_2O production rate was constant since then during the remaining incubation time. Thus, *F. oxysporum* reacts to anoxia by a strong induction of the *p450nor* gene during the first 20 h to quickly build up a P450Nor enzyme pool, and by using the existing enzymes whilst gene expression serves only hold the steady state for the rest of the incubation. Peak expression of catabolic genes during adaption to new conditions is also observed for model denitrifying prokaryotes (Baumann et al., 1996; Bergaust et al., 2008). Thus, denitrifying fungi show similar reaction patterns under changing conditions (i.e., during the transition from aerobic to anaerobic respiration).

The present study showed a clear link between NO_2^- concentrations, *ddCt(NO_2^-)* values, and N_2O production rates by *F. oxysporum* in the absence of N_2O reduction. This emphasizes good possibilities to use *ddCt(NO_2^-)* values for studies examining soil denitrification. However, under natural soil conditions in the presence of N_2O reduction, a positive correlation between N_2O production and the expression of genetic markers (e.g., *nirS*, *nosZ*) is often hindered as summarized by Chen et al. (2015). N_2O reduction by diverse N_2O-respirers in soil needs to be considered in future studies (Jones et al., 2014; Hallin et al., 2018). The contribution of fungi to N_2O emissions depends on their abundance, specific ability to denitrify, and their actual denitrifying activity. Existing methods based on growth inhibitor approaches (Laughlin and Stevens, 2002; Crenshaw et al., 2008; Chen et al., 2014) have a high uncertainty in estimating fungal or bacterial contribution on N_2O production, because, as summarized by Mothapo et al. (2015), bacterial growth inhibition may be insufficient under

certain conditions, apart from the very difficult task to find effective inhibitor concentrations without inhibiting other than target species above a certain concentration. Even a successful inhibition of growth will not necessarily coincide with an inhibition of respiration activities like denitrification (Ladan and Jacinthe, 2016). Therefore, more reliable methods are needed to study fungal denitrification in soil. Isotopic values of N_2O, especially SP(N_2O) values, have been widely used to identify several sources of N_2O (Decock and Six, 2013). The question arises whether combining studies using the expression of gene markers together with isotopic analysis is promising to obtain reliable estimates of fungal N_2O production. However, the quantification of fungal *p450nor* expression will be a good proxy for the occurrence of N_2O production by fungi, even if multiple N_2O production and consumption pathways are co-active.

SP(N_2O) values of N_2O produced by purified fungal NO reductase was found to increase from approximately 14 to 29 ‰ with ongoing reaction progress (Yang et al., 2014), suggesting that SP(N_2O) values might be more variable than assumed based on previous pure culture studies. We observed initial SP(N_2O) values after 8 h (32.6 ± 1.1 ‰) slightly lower compared to later values (32.4 to 34.0 ‰,) (Figure S4, Table S5) which might reflect the dynamics reported by Yang et al 2014. However, the SP(N_2O) values measured so far from N_2O produced by fungi were still higher than that produced by other denitrifiers.

Abiotic processes had only a minor impact on our data. Only a small N_2O production occurred in incubations without *F. oxysporum*, showing that the contribution of abiotic N_2O production was negligible, although the complex medium contained reduced metals such as Fe(II) that enhances abiotic N_2O production (Phillips et al., 2016). However, in the present study the pH of the medium was always >6.3 minimizing abiotic decomposition of N_2O producing intermediates (Su et al., 2019). Additionally, isotopic analysis of N_2O in this study confirmed not only the existing knowledge of high SP(N_2O) values by fungi (summarised in e.g. Decock and Six, 2013; Denk et al., 2017), but also proved that denitrification was the N_2O producing process in the presented system. Abiotic N_2O production was shown to be characterized by lower SP(N_2O) values compared to N_2O from fungal denitrification under similar experimental conditions (Rohe et al., 2017).

Besides SP(N_2O) values, $\delta^{15}N^{bulk}(N_2O)$ and $\delta^{18}O(N_2O)$ values of N_2O have also been used to better constrain N_2O processes (Decock and Six., 2013). We found increasing values of $\delta^{15}N^{bulk}(N_2O)$ with time which result from isotopic fractionation of the residual NO_2^- during denitrification (Denk et al., 2017). The $\delta^{15}N^{bulk}(N_2O)$ value of produced N_2O is thus potentially an independent tool, but only if the ongoing process is known. Methods exist

(isotopic mapping approach) using SP(N_2O) and $\delta^{18}O(N_2O)$ values to estimate the source partitioning even under natural conditions with simultaneously occurring processes in soil (Lewicka-Szczebak et al., 2017; Wu et al., 2019). However, several data on precursor signatures or the proportion of N_2O reduction have to be collected for such methods, and the uncertainty of the method is still quite high. Wu et al. (2019) suggested, especially when fungal denitrification is enhanced, the precision of prediction is reduced due to the overlapping ranges of N_2O isotopes from various sources (fungal denitrification, bacterial nitrification, abiotic N_2O production).

Despite the limitations outlined above, combining isotopic signature of N_2O together with *p450nor* gene marker expression will offer new possibilities for improved identification of fungal denitrification and possibly pave the way towards a more reliable quantification of this process in future studies. The current study provides the basis for identifying the transcription of *p450nor* in soil and study the regulation of fungal denitrification under various environmental conditions.

In conclusion, we developed new primers for gene expression analyses of fungal *p450nor*, demonstrated that *p450nor* expression is induced by nitrite and correlates with N_2O production in the model organism *Fusarium oxysporum*. *Fusarium* species are considered to represent denitrifying fungi producing significant amounts of N_2O during anaerobic respiration, a rare trait among fungi (Keuschnig et al., 2020). Coupling of qPCR to amplicon sequencing is recommended to evaluate the diversity of expressed *p450nor* and to assay primer specificity on a per sample basis. Thus, the new assay provides a basis to study regulation of denitrification in fungi and environmental samples, and to complement current methods to elucidate fungal contributions to denitrification in soils.

5. Acknowledgements

We thank Natalie Röder (Leibniz University Hannover) for technical support and Christoph Tebbe and his team Karin Trescher, Jana Usarek, and Britta Müller (Thünen-Institute for Biodiversity) for support with laboratory work. Anette Giesemann and Martina Heuer are acknowledged for IRMS analyses at the Thünen-Institute for Climate-Smart Agriculture. This project was funded by the Deutsche Forschungsgemeinschaft (DFG HO4020/4-1 and WE 1904/8-1; ”Role of fungal denitrification in N_2O fluxes from soils“; grant number 251282570) and from the Leibniz University Hannover.

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Figure captions

Figure 1: Relative *p450nor* gene expression (*ddCt(NO_2^-)*; filled circles), N_2O concentration (ppm; empty circles), and N_2O production rate (µmol h^{-1} g^{-1}; grey squares) in anoxic *Fusarium oxysporum* incubations as a function of incubation time (h) from experiment 1 with 5 mM initial NO_2^-. Data from one representative biological replicate is shown; the other two biological replicates are shown in Figure S6.

Figure 2: N_2O production as a function of relative *p450nor* gene expression (*ddCt(NO_2^-)*) by *Fusarium oxysporum* during the first 24 h of anoxic incubation (experiment 1) with 5 mM initial nitrite. The linear regression model and the associated R^2 value are presented.

Tables

Table 1: Approximate length of PCR products (in base pairs, including introns) from selected combinations of primers complementary to conserved positions given in Figure S1, including introns.

	p1	**p2**	**p3**
p2	320		
p3	720	420	
p5	875	600	155

Table 2: N_2O concentration, N_2O production, and *p450nor* gene expression by *Fusarium oxysporum* at four nitrite (NO_2^-) concentrations after 30 h of incubation (experiment 2). Means of triplicate incubations and standard deviation in parentheses are shown.

Initial NO_2^- [mM]	**N_2O [µmol g^{-1} h^{-1}]**	**N_2O [ppm]**	***p450nor* gene expression [ddCt(NO_2^-)]**
0.05	n.d. [a]	n.d.	1.1 (<0.1)
0.25	n.d.	n.d.	3.2 (0.4)
0.5	1.87 (1.37)	20.4 (8.9)	1.7 (0.7)
5	15.04 (3.94)	62.6 (6.0)	83.9 (29.7)

[a] n.d., not detected

Figure 1

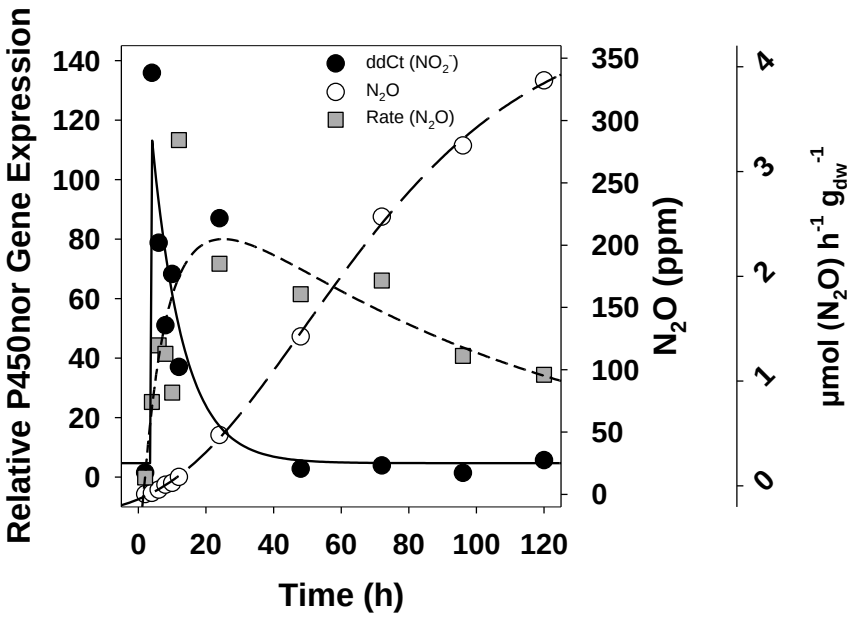

Figure 1: Relative *p450nor* gene expression (*ddCt(NO_2^-)*; filled circles), N_2O concentration (ppm; empty circles), and N_2O production rate (µmol h^{-1} g^{-1}; grey squares) in anoxic *Fusarium oxysporum* incubations as a function of incubation time (h) from experiment 1 with 5 mM initial NO_2^-. Data from one representative biological replicate is shown; the other two biological replicates are shown in Figure S6.

Figure 2

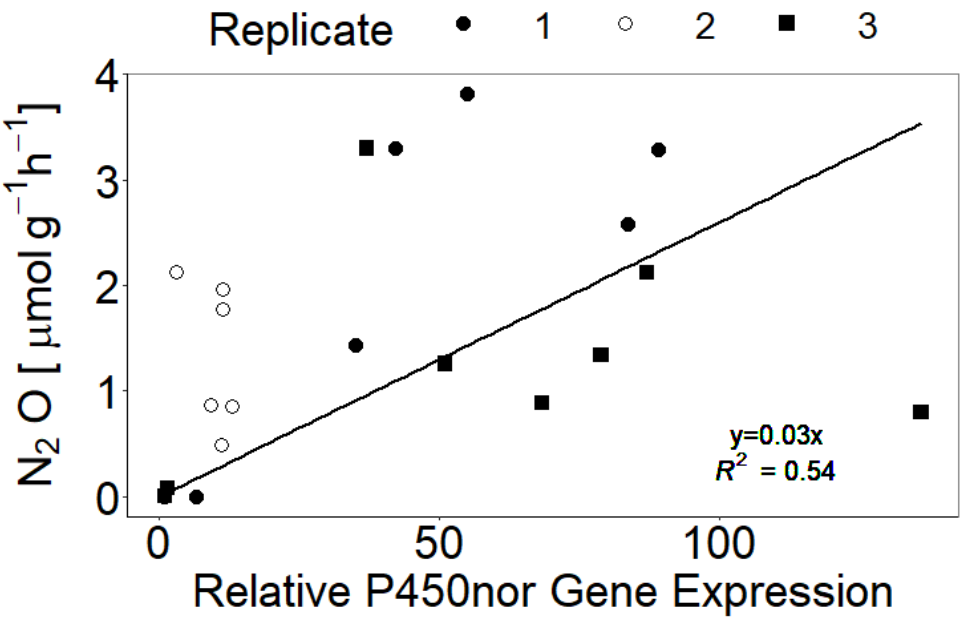

Figure 2: N_2O production as a function of relative *p450nor* gene expression (ddCt(NO_2^-) by *Fusarium oxysporum* during the first 24 h of anoxic incubation (experiment 1) with 5 mM initial nitrite. The linear regression model and the associated R^2 value are presented.