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Fungal denitrification: *Bipolaris sorokiniana* exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen

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ABSTRACT

Fungi may play an important role in the production of the greenhouse gas nitrous oxide (N_2O). *Bipolaris sorokiniana* is a ubiquitous saprobe found in soils worldwide, yet denitrification by this fungal strain has not previously been reported. We aimed to test if *B. sorokiniana* would produce N_2O and CO_2 in the presence of organic and inorganic forms of nitrogen (N) under microaerobic and anaerobic conditions. Nitrogen source (organic-N, inorganic-N, no-N control) significantly affected N_2O and CO_2 production both in the presence and absence of oxygen, which contrasts with bacterial denitrification. Inorganic N addition increased denitrification of N_2O (from 0 to 0.3 μg N_20-N h^{-1} g^{-1} biomass) and reduced respiration of CO_2 (from 0.1 to 0.02 mg CO_2 h^{-1} g^{-1} biomass). Isotope analyses indicated that nitrite, rather than ammonium or glutamine, was transformed to N_2O. Results suggest the source of N may play a larger role in fungal N_2O production than oxygen status.

Keywords: nitrite; glutamine; nitrous oxide; carbon dioxide; organic nitrogen; co-denitrification

INTRODUCTION

Fungal denitrification may contribute more to soil emissions of the greenhouse gas nitrous oxide (N_2O) than bacterial denitrification (Laughlin and Stevens 2002; Chen, Mothapo and Shi 2015), but physicochemical factors potentially altering fungal nitrogen (N) cycling require investigation. While many soil bacteria contain the N_2O reductase gene (*nosZ*) (required for N_2O to N_2 conversion), this gene is not found in fungi. Nonetheless, N_2 production by soil fungi has been reported (Shoun *et al.* 2012; Long *et al.* 2013). Some fungi, particularly for genera in the *Hypocreales* order (such as *Fusarium*), are capable of producing N_2O (Maeda *et al.* 2015) under anaerobic (Zumft 1997; Morozkina and Kurakov 2007; Shoun *et al.* 2012) or microaerobic conditions (Zhou *et al.* 2001; Morozkina and Kurakov 2007; Takaya 2009). Two pathways of fungal denitrification have been reported: (a) classical denitrification of nitrate or nitrite when oxygen is limited and insufficient to support aerobic respiration and (b) hybrid formation of N_2O, where two N sources are combined (Spott, Russow

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and Stange 2011). It is not clear if induction of anaerobiosis or changes in N source (Takaya 2002; Wei *et al.* 2014) cause a shift from one pathway to another. Hybrid N_2O and/or N_2 formation (also referred to as co-denitrification) has been observed in the presence of inorganic and organic N (Su, Takaya and Shoun 2004; Spott, Russow and Stange 2011; Long *et al.* 2013) but may also occur in the presence of two inorganic forms of N (Spott, Russow and Stange 2011). Data are lacking that indicate how inorganic and organic sources of N affect fungal denitrification and co-denitrification to N_2O pre- and postinduction of anaerobiosis. Addition of two N sources (one enriched with ^{15}N) would allow us to determine if N_2O formation could be attributed to denitrification or co-denitrification.

While a number of fungal strains have been tested for denitrification potential (Maeda *et al.* 2015), many strains, such as *Bipolaris sorokiniana*, have not been tested. *Bipolaris sorokiniana* is the asexual form (anamorph) of the fungus *Cochliobolus sativus*, a common plant pathogen in the order *Pleosporales* with a wide range of plant hosts. It is particularly common on cereals in the *Poaceae* family and is widely known as the causal agent for the common root rot. It can also survive as thick-walled conidia or as saprotrophic mycelium in soil or crop debris. Geographic distribution of *B. sorokiniana* is worldwide, particularly near mid-latitudes, in grasslands and agricultural fields (Kumar *et al.* 2002). It is not known if this soil fungus will denitrify inorganic or organic forms of N and if aerobic respiration and denitrification co-occur under controlled conditions. While a large body of research has reported bacterial N_2O and CO_2 production under microaerobic and anaerobic conditions (Butterbach-Bahl *et al.* 2013), few reports are available indicating if rates of fungal CO_2 and N_2O production vary with inorganic and/or organic N. It is unclear if fungi incubated with O_2 will respond to induction of anaerobiosis in a manner similar to denitrifying bacteria, with increased rates of N_2O production occurring within hours of induction (Firestone and Tiedje 1979; Smith and Tiedje 1979). The goal of this research was to test how N source (organic or inorganic) influences production of N_2O and CO_2 by *B. sorokiniana* pre and post anaerobiosis. Results add to the emerging body of knowledge regarding controls on fungal denitrification and co-denitrification processes.

MATERIALS AND METHODS

We used pure culture of *B. sorokiniana* (Sacc.) Shoemaker [telemorph: *C. sativus* (S. Ito & Kurib.) Drechsler ex Dastur] to test how organic N [glutamine ($C_5H_{10}N_2O_3$)] and inorganic N [ammonium sulfate ($(NH_4)_2SO_4$) and sodium nitrite ($NaNO_2$)] sources might influence fungal production of N_2O and CO_2 under sterile conditions. We controlled O_2 status with air-tight laboratory incubation vessels to evaluate rates of N_2O and CO_2 production pre and post anaerobiosis on the same samples (Firestone and Tiedje 1979; Smith and Tiedje 1979; Zhou *et al.* 2001; Butterbach-Bahl *et al.* 2013). We obtained isolate ICMP 6809 from the ICMP culture collection (http://www.landcareresearch.co.nz/resources/collections/icmp) in its anamorphic state. It was grown under aerobic conditions in the same media previously used by Rohe *et al.* (2014a,b) and Shoun *et al.* (1992), containing 1% glucose, 0.2% peptone, 0.02% $MgSO_4{\cdot}7\ H_2O$, 2 ppm $CaCl_2{\cdot}6\ H_2O$, 2 ppm $FeSO_4\ {\cdot}7\ H_2O$ and 0.01 mol potassium phosphate (pH 7.4). This media, which contained N as peptone, was used only for fungal growth. Another media, where the N source was omitted, was used for the incubations and referred to here as no-N media. After 7 days, cultures were washed, drained and stored in a reduced volume of the no-N media. Using the no-N media, four solutions were prepared for fungal inoculation: (a) no-N media (b) 0.5 mmols N as $C_5H_{10}N_2O_3$, (c) 0.25 mmols N as $Na^{15}NO_2$ (99.5 atom%; Cambridge Isotope Laboratory, Andover, MA) and 0.25 mmols N as $(NH_4)_2SO_4$, or (d) 0.25 mmols N as $Na^{15}NO_2$ and 0.25 mmols N as $C_5H_{10}N_2O_3$. The $Na^{15}NO_2$ was used to determine if these fungi would preferentially use NO_2 to form N_2O (thus forming $^{46}N_2O$) or if other sources of N would be utilized.

Approximately 10 ml of fungal biomass were transferred to 125 ml serum bottles, and four replicate bottles were inoculated with 1 ml of each sterile solution and 1 ml of no-N media. Additional bottles containing media solutions without fungi were also prepared. All bottles were sealed and the headspace evacuated and flushed with ultrapure helium, and then injected with O_2 to achieve 0.4% O_2 headspace before setting up on a robotic gas chromatograph (GC) fitted with electron capture and thermal conductivity detectors (Phillips *et al.* 2014; McMillan *et al.* 2015). Bottles were placed on the GC and measured every 6 hours for N_2O, CO_2 and O_2 at 19°C. Following this 48-h incubation experiment, bottles were then evacuated and flushed three times to create an anaerobic headspace for the second experiment, where the only difference between experiments was headspace O_2 concentration. Sterility was maintained and conditions remained constant during both incubations, including pH (ranged from 6.2 to 6.9). Data were normalized to the corresponding dry weight of fungal biomass in each bottle. The N isotopes for N_2O gas in the headspace of each sample at the end of the incubations were measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V; Thermo Scientific, Waltham, MA) in line with an automated gas bench interface (Thermo Gas Bench II) to determine if $^{45}N_2O$ or $^{46}N_2O$ were present. Precision of the isotopic analysis was <1 atom%. Data were analysed for effects of N source treatment on N_2O and CO_2 production rates for microaerobic and anaerobic experiments separately with a generalized linear model and means were compared with Tukey's test.

RESULTS AND DISCUSSION

Table 1 and Fig. 1 show effects of treatment on CO_2 and N_2O production for the microaerobic incubations were similar to effects of treatment for the anaerobic incubations. Fungi inoculated with only organic-N ($C_5H_{10}N_2O_3$) or no-N media only linearly produced CO_2 (1–2 mg CO_2 h^{-1} g^{-1}) but not N_2O. Fungi inoculated with inorganic-N ($Na^{15}NO_2$ or ($(NH_4)_2SO_4$) or inorganic-N plus organic-N ($Na^{15}NO_2$ and $C_5H_{10}N_2O_3$) linearly produced N_2O (0.2–0.3 μg $N_2$0-N h^{-1} g^{-1}) but an order of magnitude less CO_2. The mass $^{46}N_2O$ comprised 94.7%–97.3% of all N_2O in the headspace, yielding an equivalent ^{15}N of 98 to 99 (±0.3) atom%. This enrichment was effectively equivalent to enrichment of the $^{15}NO_2$ used in the experiment. The lack of headspace $^{45}N_2O$ indicated hybrid formation of N_2O by *B. sorokiniana* did not occur under any of these conditions, and NO_2^- was the only N form used to form N_2O. Headspace O_2 (Fig. 2) over the aerobic time course indicated rapid declines for the no-N media and organic-N treatments but not the inorganic-N treatments. Minimal rates of CO_2 respiration (Table 1) in the presence of inorganic-N were consistent with lack of O_2 utilization by *B. sorokiniana* (Fig. 2). We also observed chemodenitrification (6%–8% of total biologically produced N_2O) for media amended with inorganic-N only (van Cleemput 1998; Kampschreur *et al.* 2011; Jones *et al.* 2015).

Table 1. Rates of N_2O and CO_2 produced during (1) aerobic and (2) anaerobic incubation experiments by treatment per gram of fungal biomass. Average rates followed by different letters for each experiment represent significant differences between treatments. The same letters indicate no differences between treatments.

Experiment	Treatment	Mean ng N_2O-N h^{-1} g^{-1} biomass	Std. Dev.	Mean μg CO_2 h^{-1} g^{-1} biomass	Std. Dev.
Anaerobic	No N	0.51^a	0.26	896.45^a	206.08
	Organic N	0.29^a	0.11	728.46^a	148.66
	Inorganic N	311.30^b	18.98	6.17^b	1.34
	Inorganic N + organic N	211.79^c	14.44	7.87^b	1.48
Aerobic	No N	0.57^a	0.23	1179.89^a	262.62
	Organic N	0.32^a	0.23	988.19^a	222.95
	Inorganic N	354.55^b	26.00	18.26^b	1.48
	Inorganic N + organic N	306.33^c	28.61	31.52^b	4.03

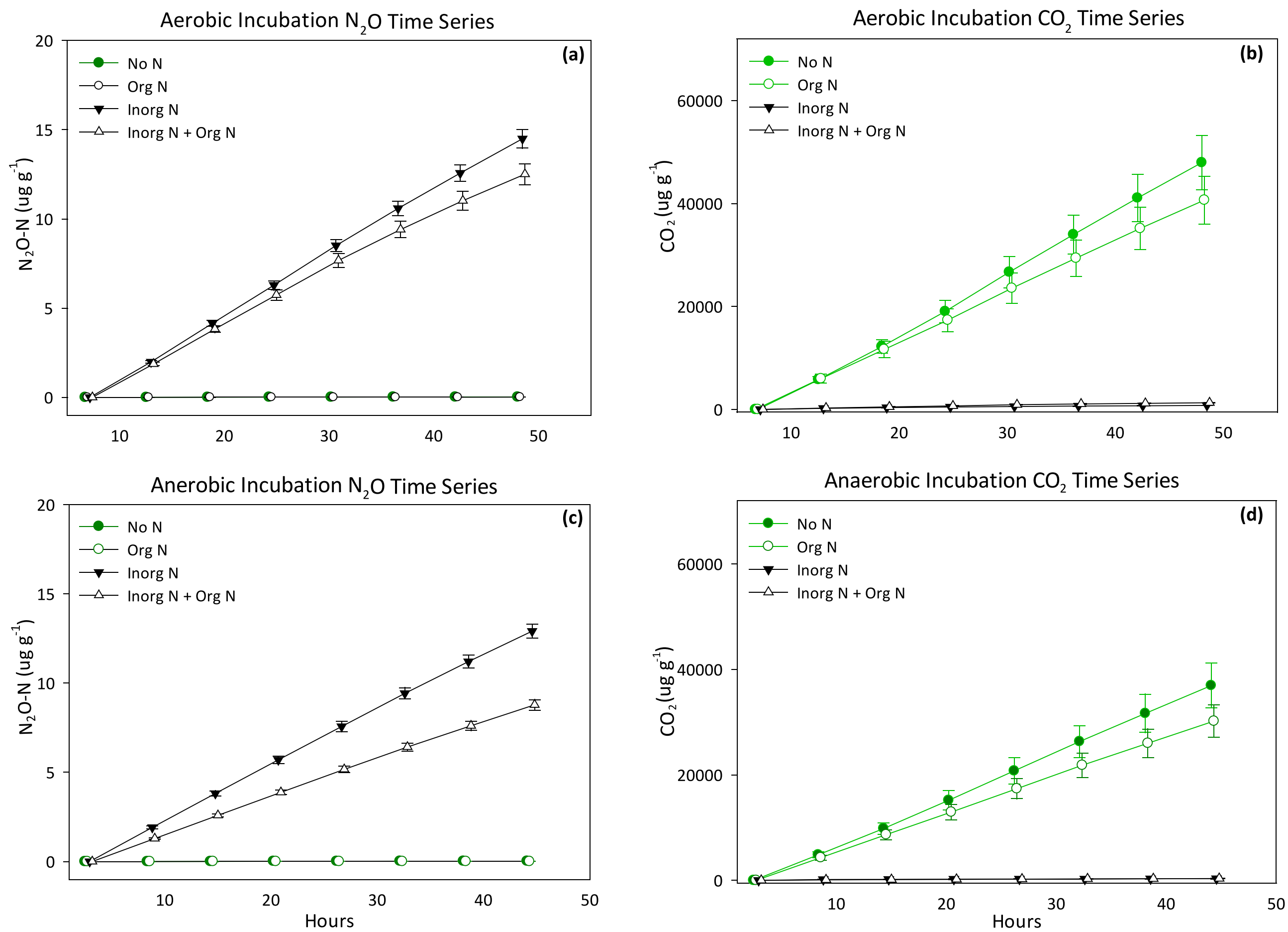

Figure 1. Average (std. dev; $n = 4$) N_2O and CO_2 measured in the headspace per g of fungal biomass by treatment during aerobic (**a**, **b**) and anaerobic (**c**, **d**) incubations.

Evidence of chemodenitrification, where N_2O was produced under sterile conditions, may be due to reactions between NO_2^- and reduced metals in the media (van Cleemput 1998; Kampschreur *et al.* 2011). We chose the media for this experiment because it is commonly used for fungal denitrification investigations (Shoun *et al.* 1992; Rohe *et al.* 2014a,b). Kampschreur *et al.* (2011) used a chemostat to demonstrate that NO_2^- to NO is an equilibrium-based reaction, where emissions of NO and N_2O were coupled to iron oxidation under anoxic conditions. A recent review by Medinets *et al.* (2015) argued that NO_2^-, a precursor of NO, is central to processes associated with chemodenitrification. Additional research is needed to determine the reactants involved in this specific media and the prevalence of chemodenitrification in an oxygenated headspace.

Evidence of biological denitrification coupled with reduced CO_2 respiration in the presence of inorganic-N may be due to *B. sorokiniana* preferentially denitrifying as a defence against accumulation of NO_2^- (Geets, Boon and Verstraete 2006; Clark *et al.* 2012). Fungi are capable of converting NO_2^- to NO, the precursor to soil N_2O emissions (Russow, Stange and Nueue 2009), through expression of the NO_2^- reductase gene (*nirK*), and NO is a regulator of O_2 consumption in eukaryotes (Thomas *et al.*

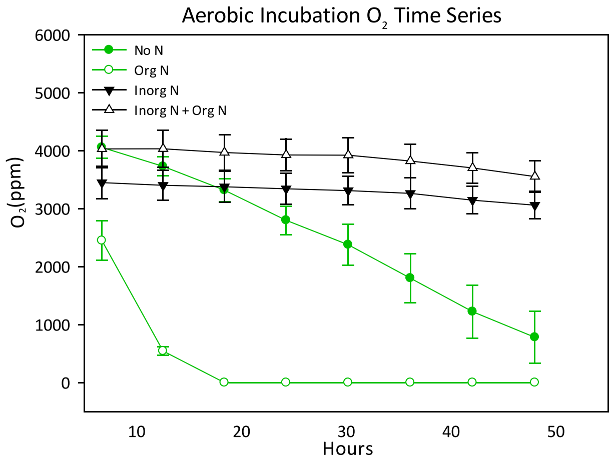

Figure 2. Average (std. dev; $n = 4$) O_2 measured in the headspace by treatment during aerobic incubation.

2001). We suspect that some NO_2^- was converted to NO (both abiotic and biotic), which inhibited mitochondrial respiration (Thomas *et al.* 2001; Medinets *et al.* 2015) and therefore CO_2 production, as shown in Fig. 1 and Table 1. One way of discerning the importance of NO_2^- reduction to NO and N_2O by *B. sorokiniana* would be to identify *nirK* gene expression in conjunction with NO measurements. If *B. sorokiniana* does not reduce NO_2^- to NO, then chemical transformation of NO_2^- may be the source of NO (van Cleemput 1998; Kampschreur *et al.* 2011). In either event, there is a need to identify chemical versus biological sources of NO, the precursor to N_2O (Russow, Stange and Nueue 2009).

Denitrification in soils and sediments is referred to as a keystone ecosystem service with positive water quality implications. However, when N_2O is the final end product of denitrification, there are negative impact on stratospheric ozone and radiative forcing in the troposphere (Erisman *et al.* 2013). Here, *B. sorokiniana*, a ubiquitous soil fungus, demonstrated the capacity to transform dissolved NO_2^- to gaseous N_2O at the expense of aerobic respiration, a more energetically favourable pathway. Denitrification and aerobic respiration for *B. sorokiniana* were closely linked via NO_2^-. Additional investigations into alternative respiration pathways and effects of inorganic N on C assimilation are warranted, particularly with respect to O_2 status. Here, we focused on how inorganic and organic N sources influence pathways to N_2O production before and after onset of anaerobiosis (Firestone and Tiedje 1979; Smith and Tiedje 1979; Zhou *et al.* 2001). Future work should be designed with sample in parallel to test how O_2 and N interact to affect fungal denitrification phenotype and genotype. Our results suggest that fungal denitrification may not be driven by anaerobiosis but instead by form of N; however, additional strains need to be tested to determine if this is widespread among soil fungi or unique to *B. sorokiniana*.

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