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ORIGINAL PAPER

Biological nitrification inhibition by sorghum root exudates impacts ammonia-oxidizing bacteria but not ammonia-oxidizing archaea

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Abstract

Sorghum has a great capacity to release biological nitrification inhibitors (BNIs), but the inhibitory effect on nitrification and ammonia oxidizer populations under planted soil conditions is unclear. A pot experiment with three nitrogen (N) application rates (0, 50, and 200 mg N kg^{-1}) was set up to detect the influence of sorghum growth on soil nitrification and investigate the function of blocking the activity of ammonia oxidizers. A ^{15}N-labeled experiment was also conducted to detect the N form absorbed by sorghum. Sorghum root exudates were collected at 30 days after transplanting to hydroponic culture and added into cultured soil to determine the shifts in the populations of nitrifiers. The ^{15}N labeling experiment showed that the uptake rate by sorghum of ammonium N fertilizer was 24% and that of nitrate N fertilizer was 9%, indicating that sorghum was an ammonium using plant. Compared with unplanted soil, sorghum planting had a significant inhibitory effect on the nitrification process even at the high-N fertilizer rates. Autotrophic nitrification was the prevailing process, and sorghum root exudation inhibited this process as much as dicyandiamide (DCD, 10 mg kg^{-1}). Root exudates had a significant inhibitory effect on ammonia-oxidizing bacteria (AOB) but had no effect on ammonia-oxidizing archaea (AOA).

Keywords Biological nitrification inhibitors (BNIs) · Sorghum · Nitrification · Nitrifiers

Introduction

Relatively immobile ammonium (NH_4^+) conversion to highly mobile nitrate (NO_3^-) is the result of nitrification, which may lead to potential harmful impacts on the environment by NO_3^- leaching and nitrous oxide (N_2O) emissions (Schlesinger 2009). Thus, restraint of nitrification is very important to improve fertilizer N retention in soils and improve in N use efficiency. The most significant way to mitigate N loss through nitrification is by suppressing the activity of nitrifying microorganisms (Sahrawat and Keeney 1985; Subbarao et al. 2008).

The application of synthetic nitrification inhibitors (SNIs), for instance, dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP), and nitrapyrin, can inhibit the nitrification process effectively, thus reducing N loss by nitrate leaching and N_2O emissions and improving N use efficiency by plants (Sun et al. 2015). However, high cost and the potential for environmental contamination induced the restricted use of these SNIs. In particular, some hydrosoluble SNIs can leach into water and pollute aquatic ecosystems (Qiu et al. 2015).

Currently, some plants, such as brachiaria, sorghum, and rice, can release biological nitrification inhibitors (BNIs) (Subbarao et al. 2006a, 2013a; Sun et al. 2016). Plant-derived BNIs are low-cost and environmental-friendly and inhibit soil nitrification effectively (Gopalakrishnan et al. 2007; Subbarao et al. 2008). Sorghum (*Sorghum bicolor*) roots can release both sorgoleone and methyl 3-(4-hydroxyphenyl) propionate (MHPP), which are effective BNIs (Subbarao et al. 2013a; Zakir et al. 2008).

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Sorgoleone's effect and mode of inhibition on the ammonia-oxidizing bacterium *Nitrosomonas europaea* have been characterized (Tesfamariam et al. 2014). The ED_{80} (effective dose for 80% reduction of *Nitrosomonas*) values of sorgoleone and nitrapyrin in an in vitro assay were 12 and 17.3 μg ml^{-1}, respectively, which indicated that this BNI was the more effective nitrification inhibitor (Subbarao et al. 2008, 2013a). Zakir et al. (2008) found that MHPP only inhibited ammonia monooxygenase (AMO) but not hydroxylamine oxidoreductase (HAO) in *Nitrosomonas*, thus acting like most SNIs (e.g., DMPP and nitrapyrin), whereas sorgoleone blocked both the AMO and HAO enzymatic pathways (Subbarao et al. 2013a).

Some factors, such as pH and N form, influence the synthesis and secretion of BNIs (Subbarao et al. 2013b). The greatest BNI activity by sorghum was shown when the rhizosphere pH ranged from 5.0 to 6.0, whereas sorghum did not release inhibitors when the pH was higher than 7.0 (Subbarao et al. 2013a). The release of BNIs only occurred when sorghum grew with NH_4^+ as the N source, but not with NO_3^- as its N source (Subbarao et al. 2007a; Zakir et al. 2008). However, there can be considerable differences between varieties in the production of sorgoleone (Sarr et al. 2020), and it also depends on N availability, since *Brachiaria humidicola* root only released inhibitory compounds in N limited grassland ecosystems (Subbarao et al. 2006b).

Our understanding of the BNI's inhibition mechanisms is mainly based on ammonia-oxidizing bacteria (AOBs) (i.e., *Nitrosomonas* sp.) (Subbarao et al. 2013b). Ammonia-oxidizing archaea (AOA) and heterotrophic nitrifiers (fungi, e.g., *Penicillium* sp.) play a critical role in nitrification in many ecosystems, especially in acidic soils (Daims et al. 2015; Leininger et al. 2006; Li et al. 2018). Moreover, AOA have the AMO enzymatic pathway for nitrification, but not the HAO (Walker et al. 2010; Vajrala et al. 2013). Sarr et al. (2020) have recently reported that sorgoleone decreased the abundance of AOA but not that of AOB, which contrasts with the previous observations of the significant impacts on *Nitrosomonas* (Subbarao et al. 2013a; Tesfamariam et al. 2014). Hence, it is unclear whether BNIs, particularly those of sorghum, can inhibit AOB or AOA, or both, and under what conditions. Additionally, the impact on heterotrophic nitrifiers is unknown.

Most of the previous research involved the isolation and characterization of BNIs using hydroponics and the study of their effects on *Nitrosomonas*. Compared to hydroponics, soil is a much more complex ecosystem with a mixture of organic compounds, minerals, plants, animals, and microorganisms. Soil microorganisms play an important role in soil nutrient cycling and organic matter accumulation, and complex interactions occur between microorganisms and environmental factors (i.e., pH, organic matter, N content, root exudates) (Powlson et al. 2001). The effect of BNIs on nitrification and the abundances and community compositions of AOA and AOB, particularly under cropped soil conditions, are unknown. In the current work, we hypothesized that sorghum releases BNIs under soil conditions and inhibits nitrification, AOA, and/or AOB abundances and that this inhibition is affected by different N rates. Therefore, the objectives of this study were (1) to determine soil nitrification inhibitory activity in situ during the growth of sorghum with different rates of N supplied, including a high-N input treatment (200 mg N kg^{-1}) and (2) to determine the effect of BNIs on the abundance and composition of nitrifiers and so to understand the underlying microbial mechanisms.

Materials and methods

Evaluation of BNI capacity during sorghum growth with different rates of N fertilizer

A loess soil was collected from Beilun District in Ningbo city (121°51′6″ E, 29°54′43″ N), Zhejiang Province, China, in April 2018. The fresh soil was air-dried, sieved through a 2.0-mm mesh, and stored at room temperature. The main physicochemical properties of the soil are shown in Table 1. Soil pH in water was determined by a pH meter (1:5 w/v, soil/water). Total organic C was detected by dichromate oxidation (Nelson and Sommers 1982), and total N was determined using a CNS Element Analyzer (vario MAX C/N, Elemental, Germany). Soil exchangeable NH_4^+ and NO_3^- were extracted from fresh soil samples with 1 M KCl (soil: KCl = 1:10), shaken for 1 h, and detected colorimetrically with a continuous flow injection analyzer (FLA star 5000 Analyzer, Foss, Denmark).

The pot experiment included three treatments: 0, 50, and 200 mg N kg^{-1} with N applied to the soil as urea. Each treatment was replicated three times. In each treatment, 200 g air-dried soil was weighed into a PVC pot and the water holding capacity adjusted to 60%. Before planting, the soil was amended with monopotassium phosphate and potassium chloride, giving a rate of 100 mg K_2O kg^{-1} soil and 50 mg P_2O_5 kg^{-1} soil. Sorghum seeds (*Sorghum bicolor* (L.) Moench, cv. JB No.29) were surface sterilized with 10% H_2O_2 for 10 min and then rinsed and soaked with deionized water. After 4 days of incubation at 25 °C in the dark, the germinated seeds were placed into the pots (four seeds in each pot). Plants were grown in a growth chamber (day: night temperature regime of 28 °C: 24 °C; photoperiod, 14 h light; humidity, 65%). N fertilizer was added after 10 days. A second set of treatments involved unplanted soil. Soil samples were collected at 0, 3, 10, and 25 days after adding the urea. Each soil sample was divided into three portions. The first portion was immediately extracted with KCl for inorganic N measurement, the second portion was stored at − 80 °C for

Table 1 Main physicochemical properties of the used soil

pH	Organic C (g/kg)	Total N (g/kg)	NH_4^+-N (mg/kg))	NO_3^--N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	Particle size distribution (%)		
							Sand	Silt	Clay
6.10	29.1	2.30	3.56	3.85	43.4	90.8	64.28	21.75	13.97

DNA extraction, while the rest of the fresh soil was stored at 4 °C for other analyses.

Potential nitrification rate (PNR) was determined by the shaken-slurry method as follows: 15 g fresh soils were mixed with 7.5 ml of KH_2PO_4 (0.2 M), 17.5 ml of K_2HPO_4 (0.2 M), and 75 ml of $(NH_4)_2SO_4$ (0.05 M) and shaken (180 rpm) for 24 h in the dark at 25 °C. Suspending liquid samples of 10 ml were centrifuged (1650×*g*), collected, and filtered through a filter paper (0.45 μm pore size) at 2-, 4-, 22-, and 24-h incubation. Nitrate concentration in the supernatant was immediately detected by a continuous flow injection analyzer (Persson and Wiren 1995; Yao et al. 2011).

Influence of N forms (NH_4^+ versus NO_3^-) on plant growth

Before planting, the soil was amended with nutrient solution containing (mg kg^{-1}) 100 N, 50 P, and 100 K. The 100 mg N kg^{-1} soil was applied in two treatments ($^{15}NH_4Cl + KNO_3$ and $NH_4Cl + K^{15}NO_3$, ^{15}N 10.3 atom % excess) with three replicates each. Conditions for sorghum germination and plant growth were as above. After 30 days growth, plant shoots and roots were collected separately, washed with water, denatured for 30 mins at 105 °C to stop enzyme activity, then oven dried at 65 °C to a constant weight, and ground to a powder. The total N concentration and ^{15}N values in the shoot and root were determined using isotope ratio mass spectrometry (Integra CN; Sercon Ltd., Cheshire, UK). The ^{15}N uptake by plants was calculated as follows:

$$^{15}N_{\text{intake}}(\text{mg}) = \frac{M \times C_N \times (^{15}N_{atom} - 0.3663)}{10 - 0.3663}$$

where M is the plant dry weight (g), C_N is the concentration of N in plants (mg/kg), and $^{15}N_{atom}$ is the ^{15}N atom%.

Estimation of autotrophic nitrification in the soil

Acetylene (C_2H_2) was used to inhibit soil chemoautotrophic nitrification (Berg et al. 1982). Soils were pre-incubated at 60% water holding capacity in the dark at 25 °C for 2 days, and then 20 g soil was placed in a glass bottle, treated with 100 mg urea-N kg^{-1} or with urea + C_2H_2 (0.1% in the headspace), and sealed and incubated at 25 °C in triplicate. Controls without urea and with and without C_2H_2, were also carried out. Each treatment was replicated three times. After 0, 1, 2, 4, and 5 days incubation, samples were collected for determining the NO_3^--N concentration.

Collection of root exudate

Conditions for sorghum germination and plant growth were the same as above except that seedlings were planted into 1.1-l plastic pots (one seedling per hole and four holes per plastic pot) containing aerated nutrient solution. The shoots of sorghum were held and supported with sterilized sponges. Nutrients concentration (mg l^{-1}) in the solution were KH_2PO_4, 38.31; $MgSO_4.7H_2O$, 36.93; K_2SO_4, 31.02; Fe-EDTA, 15.1; $CaCl_2.2H_2O$, 10.5; $MnSO_4.6H_2O$, 2.35; H_3BO_3, 0.57; $ZnSO_4.7H_2O$, 0.22; $Na_2MoO_4.2H_2O$, 0.126; and $CuSO_4.5H_2O$, 0.078 (Sun et al. 2016). The pH of the culture solution was adjusted to 6.10, and the culture solution was changed every week. After 30 days, the roots were washed gently in water. Since both the amount and composition of root exudate may be changed by mechanical injury, extreme attention was paid to the manipulation. Subsequently, the leaching solution (0.5 mM KCl, 1 mM $CaCl_2$, and 1 mM NH_4Cl) was used for the collection of root exudates. After 24-h incubation, shoots and roots were sampled, separated, washed, and freeze-dried for weighing. The collected solution was passed first through a cation-exchange column (16 mm × 14 cm) filled with 5 g Amerlite IR-120B resin (H^+ form, Amdas, Switzerland) and then through an anion-exchange column filled with 2.5 g Dowex 1 × 8 resin (200–400 mesh, Aladdin, USA). The root exudates retained in the anion-exchange resin were eluted using 5 ml 1 M HCl (Yang et al. 2011), and then the eluent was concentrated to dryness using a rotary evaporator at 40 °C (Sun et al. 2016). The residue was re-dissolved in 200 μl of dimethyl sulfoxide (DMSO), filtered (0.2 μm), and stored at − 20 °C until analysis.

Effect of root exudates on nitrification and ammonia oxidizers

Five treatments were set up as follows: urea (100 mg urea-N kg^{-1}), root exudate (0.21 μg C), urea + root exudate, urea + DCD (10 mg kg^{-1}), and control. Urea, root exudate, and DCD were added to the glass bottles which contained 20 g soil (with soil water content amended to 60%). Samples were collected

after 0, 3, 5, 7, and 10 days for NO_3^--N and quantitative PCR (qPCR) analysis.

DNA extraction and PCR amplification

Soil DNA was extracted from approximately 0.5 g soil sample using the Fast DNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions. DNA concentration was determined by NanoDrop (Thermo Scientific, Waltham, MA, USA). The *amoA* gene abundances of AOB and AOA were determined by qPCR with a Light Cycler 480 (LC480) (Applied Biosystems, Foster City, CA, USA). The absolute 16S rRNA abundances of bacterial and crenarchaeal genes were determined by using primers 515F/907R, Arch349f/Arch806R (Takai and Horikoshi 2000; Zhou et al. 2011), respectively. The *amoA* gene copy numbers of AOB and AOA were quantified using primers amoA-1F/amoA-2R and CrenamoA23f/CrenamoA616r, respectively (Xi et al. 2017). Each qPCR reaction was performed in a 20 μl volume containing 1 μl of template DNA, 0.5 μl of bovine serum albumin (BSA, 0.2 mg ml^{-1}), 0.5 μM of each primer, 10 μl of SYBR Premix EX Taq (Takara, Japan), and 7.5 μl of deionized water. The qPCR conditions were denaturation at 95 °C for 5 min, then 40 cycles of 5 s at 95 °C, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Melting curve analysis (67 to 95 °C) was used to confirm the specificity of the amplification product before visualization by agarose gel electrophoresis. Standard curves were generated with known copy numbers of 16S rRNA and *amoA* genes. For all assays, amplification efficiencies were > 90%, and r^2 values were in the range from 0.97 to 0.99.

Statistical analysis

All data are the means of three replicates. Quantitative PCR data were log-transformed before further analysis. Statistical tests were performed using Microsoft Excel 2010 (Microsoft, USA) and SPSS 19.0 (IBM, USA). ANOVA tests (least significant difference test, $P < 0.05$) were performed using SPSS to compare different treatments. Pearson correlation analysis ($P < 0.05$) was also performed to test the correlation between soil nitrification rate, fertilizer level, and microbial abundances.

Results

Soil inorganic N concentration and potential nitrification rate

In the first 3 days, the exchangeable NH_4^+-N concentrations of planted soils increased in the urea treatments, especially in the 200 mg N kg^{-1} treatment, and then decreased until the end of the experiment (Fig. S1a). The NO_3^--N concentrations in planted soils were rather low during the growth of sorghum in the three treatments (Fig. S1b). In unplanted soils, the exchangeable NH_4^+-N concentration increased to 102.1 mg N kg^{-1} during the 10 days incubation and then decreased in the 200 mg N kg^{-1} treatment. In the 50 mg N kg^{-1} treatment, the exchangeable NH_4^+-N concentration increased to 39.5 mg N kg^{-1} during the 5 days incubation and then decreased (Fig. S1c). The NO_3^--N concentrations gradually increased from day 3 to day 25 in the three treatments, and the highest concentration was 140 mg N kg^{-1} (Fig. S1d). These results indicated that the soil used in this study had a high rate of nitrification when the substrate concentration was sufficient.

We compared the soil potential nitrification rates with or without sorghum at the three different N application rates. The soil potential nitrification rates increased as the N application rates increased, and unplanted soils had higher soil potential nitrification rates than planted soils (Table 2). These results suggested that sorghum growth significantly inhibited soil nitrification.

^{15}N labeling was used to determine the N uptake by sorghum. There was no difference in plant biomass between $^{15}NO_3^-$ and $^{15}NH_4^+$. However, compared with the $^{15}NO_3^-$ treatment, ^{15}N uptake of shoot and root was two times higher with the $^{15}NH_4^+$ than with the $^{15}NO_3^-$ treatment (Fig. 1), indicating that sorghum prefers NH_4^+-N to NO_3^--N.

Relationship between N application rates, nitrification rates, and nitrifiers

Acetylene (C_2H_2) can be used to distinguish autotrophic and heterotrophic nitrification (Jia and Conrad 2009). The inhibitory effect of C_2H_2 on nitrification was significant in this study. The control treatment (without C_2H_2) showed a significantly increasing trend in NO_3^--N concentrations, especially after 2 days, whereas C_2H_2 decreased NO_3^--N concentrations (Fig. 2). The effect was even more marked after adding urea-N. This result indicated that autotrophic nitrification was the main process in this soil.

Bacterial abundance ranged between 5.23×10^9 and 1.81×10^{10} copies g^{-1} dry soil during the growth of sorghum, and the abundance was higher with than without N (Fig. S2a). The abundance of AOB *amoA* genes increased during the 10 days incubation with N application and then decreased until the end of the experiment (Fig. S2c). The abundances of total archaea and AOA *amoA* gene showed no significant difference among all treatments (Fig. S2b and Fig. S2d). The soil potential nitrification rate was positively correlated with N application rate ($r = 0.926$, $P < 0.01$), bacteria abundance ($r = 0.245$, $P < 0.05$), and AOB abundance ($r = 0.538$, $P < 0.01$) (Table 3), but not with AOA and archaea abundances.

Table 2 Soil potential nitrification rate (mg kg^{-1} d^{-1}) (mean ± standard error)

Days	0 mg N kg^{-1}		50 mg N kg^{-1}		200 mg N kg^{-1}	
	Planted	Unplanted	Planted	Unplanted	Planted	Unplanted
3	0.90 (±0.03)a	1.45 (±0.01)b	1.05 (±0.15)a	1.16 (±0.2)a	1.47 (±0.10)a	1.82 (±0.3)a
10	1.05 (±0.07)a	1.48 (±0.02)b	1.07 (±0.05)a	2.18 (±0.08)b	2.27 (±0.06)a	5.89 (±0.1)b
25	1.12 (±0.3)a	1.87 (±0.07)b	1.12 (±0.11)a	2.14 (±0.3)b	2.37 (±0.12)a	7.16 (±0.19)b

Different letters within each row at each N rate indicate a significant difference at $P < 0.05$. The pot experiment included three treatments (0, 50, and 200 mg N kg^{-1}) which were applied to the soil as urea. Each N rate had two sub-treatments: "planted" means soil growth with sorghum and "unplanted" means soil without sorghum

Effects of root exudates on nitrification and microbial functioning

The NO_3^--N concentrations increased with time in the five treatments (root exudate, control, urea + DCD, urea + root exudate, urea only), and values were significantly the highest with urea alone. Addition of either root exudate or DCD reduced the formation of nitrate significantly, with the greatest effect due to the treatment with root exudate (Fig. 3).

Both the AOA and AOB abundances in the five treatments were measured on days 0, 3, 5, 7, and 10 (Fig. 4). The AOB *amoA* gene copy numbers were the highest (6.30×10^8 copies g^{-1} dry soil) in the urea alone treatment and the lowest (3.51×10^7 copies g^{-1} dry soil) in the soil treated with root exudate added. Overall, root exudate decreased the AOB *amoA* gene copy numbers by 60.3% compared to the urea treatment. DCD also decreased the AOB *amoA* gene copy numbers to the same extent (56.3%) as the root exudate treatment. The AOA *amoA* gene copy numbers ranged from 4.42×10^6 to 5.48×10^6 copies g^{-1} dry soil, indicating that the gene abundance of AOA was much lower than that of AOB. There was no significant difference among the AOA abundances in the soils of all treatments.

Discussion

The influence of BNI on soil nitrification

Biological nitrification inhibition can enhance soil fertility and primary production by improving N uptake and reducing N loss in a sustainable way (Coskun et al. 2017). The bioluminescence assay using a recombinant strain of *Nitrosomonas europaea*, carrying *Vibrio harveyi lux*AB genes, was used for detecting and quantifying BNI activity released from roots (Subbarao et al. 2006a, 2013b). Subbarao et al. (2007b, 2009) estimated that the inhibition of potential nitrification by *Brachiaria humidicola* was equivalent to the application of about 6.2–18 kg of nitrapyrin ha^{-1} $year^{-1}$, thus suggesting that BNI by this plant is adequate to inhibit soil nitrification and promote utilization of the NH_4^+ form. However, this method used *Nitrosomonas europaea*, and not soil, which is

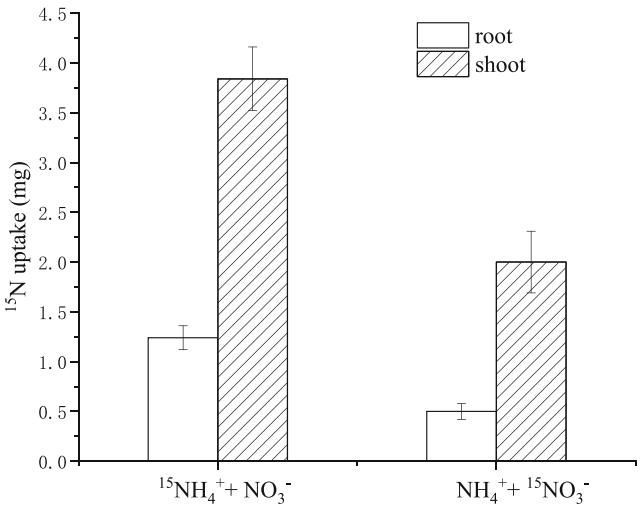

Fig. 1 The N uptakes in sorghum shoots and roots with differently labeled fertilizer N in the ^{15}N-labeled soil pot experiment. Error bars indicate standard deviations ($n = 3$). ^{15}N-labeled NH_4Cl or KNO_3 (10.30% atom ^{15}N excess) at a rate of 100 mg N kg^{-1} soil was applied

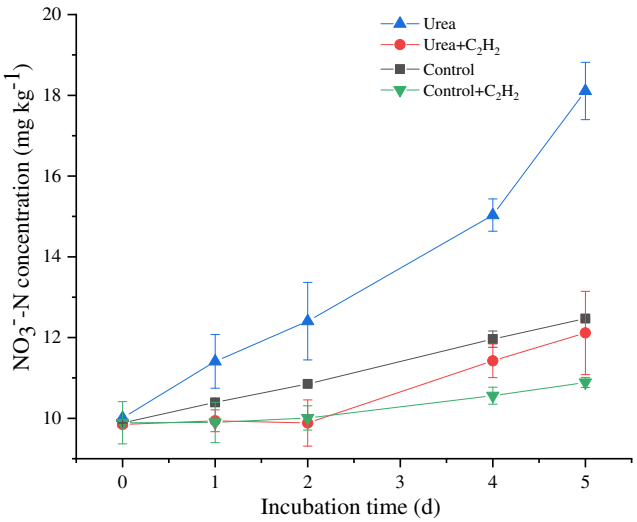

Fig. 2 The production of NO_3^--N with and without C_2H_2 added. Error bars indicate standard deviations ($n = 3$). Urea: 100 mg urea-N kg^{-1}; C_2H_2: 0.1% in the headspace

Table 3 Pearson correlation between target gene copies, potential nitrification rates, and different fertilizer levels in soils planted with sorghum

Item	N rate	AOB	Bacteria	AOA	Archaea	Potential nitrification rate
N rate	1	0.691**	0.105	−0.063	−0.067	0.926**
AOB	0.691**	1	0.614*	0.358*	0.353*	0.538*
Bacteria	0.105	0.614**	1	0.708*	0.537*	0.245*
AOA	−0.063	0.358*	0.708*	1	0.415*	−0.105
Archaea	−0.067	0.353*	0.537*	0.415*	1	−0.231
Potential nitrification rate	0.926**	0.538*	0.245*	−0.105	−0.231	1

*Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level (two-tailed). N rate: 0, 50, and 200 mg N kg^{-1} soil; AOB: the *amoA* gene of ammonia-oxidizing bacteria; Bacteria: the 16S rRNA gene of bacteria; AOA: the *amoA* gene of ammonia-oxidizing archaea; Archaea: the 16S rRNA gene of crenarchaea

inhabited by a microbial nitrifier community (i.e., AOB, AOA, or heterotrophic nitrifiers).

Few studies have determined the inhibitory effect of BNIs in agricultural soils. A 3-year field experiment revealed that nitrification rate in soil under *B. humidicola* was much lower than soil beneath soybean or *Panicum maximum*. In addition, compared with soybean soil, that under *B. humidicola* decreased nitrous oxide emissions by 90% (Subbarao et al. 2009). Wild sorghum (sp. *S. arundinaceum*) markedly inhibited soil nitrification potential as 67% of the added ammonium N persisted at the end of 60 days incubation (Subbarao et al. 2013b). In a greenhouse experiment, Sarr et al. (2020) showed that high sorgoleone-producing strains of sorghum reduced soil nitrate concentration and that sorgoleone concentration was significantly correlated with the reduced potential nitrification rate. In our study, we found that the growth of sorghum decreased the potential nitrification rate by 19.2%, 61.5%, and 66.9% after 3, 10, and 25 days, respectively (Table 2).

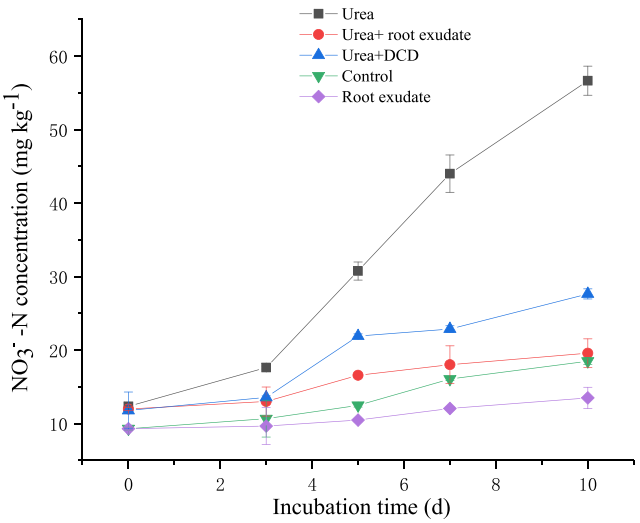

Fig. 3 The variations in NO_3^--N concentrations with different inhibitor treatments (control, root exudate, urea, urea + root exudate, and urea + DCD). Error bars indicate standard deviations ($n = 3$). Urea: 100 mg urea-N kg^{-1}; root exudate: 0.21 μg C; DCD: 10 mg kg^{-1}

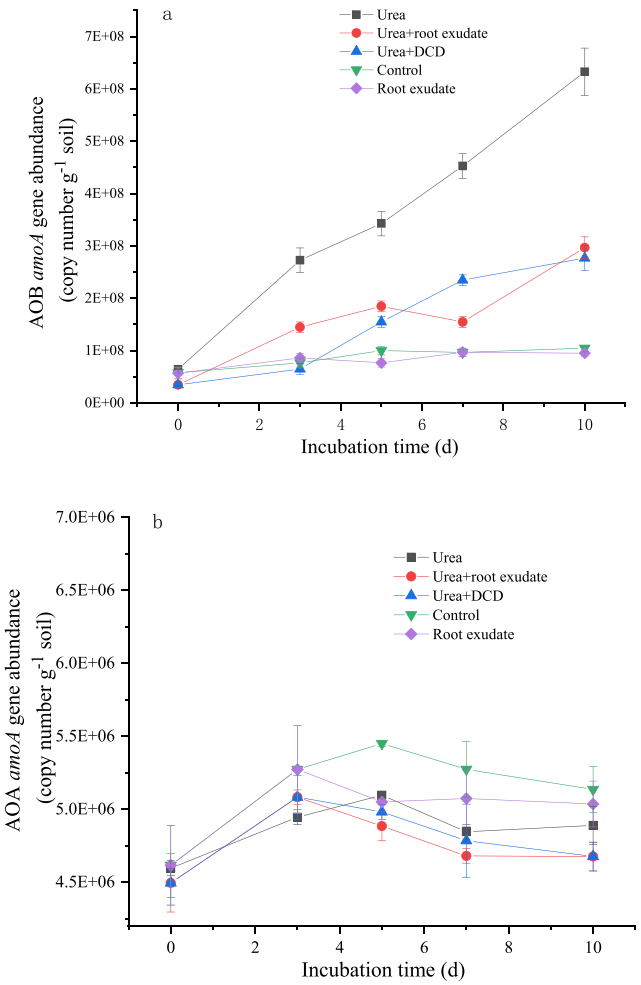

Fig. 4 Changes in copy number of AOA and AOB *amoA* genes with different inhibitor treatments (control, root exudate, urea, urea + root exudate, and urea + DCD). Error bars indicate standard deviations ($n = 3$). Urea: 100 mg urea-N kg^{-1}; root exudate: 0.21 μg C; DCD: 10 mg kg^{-1}

The root exudates from sorghum reduced the soil NO_3^--N concentrations, and the effect was stronger than that by the DCD treatment (Fig. 3). Gopalakrishnan et al. (2009) added BNI released from *B. humidicola* to soil and similarly found a significant inhibitory effect on soil nitrification during 60 days incubation.

Our study showed that sorghum showed a preference for NH_4^+ over NO_3^-, and this may explain the release of BNIs by the plants. The N availability in the soil may also regulate BNI release, since BNIs are released under low-N conditions (Subbarao et al. 2009). Subbarao et al. (2007b) found that *B. humidicola* and *B. decumbens* had the highest BNI capacity among the forage grasses under the low-N availability conditions of the South American savanna soils. In contrast, *P. maximum* exhibited the lowest BNI capacity and was adapted to high-N production environments (Subbarao et al. 2007a). However, our results found that BNIs could decrease nitrification rates even when 200 mg N kg^{-1} N was applied.

The influence of BNI on ammonia-oxidizing microorganisms

Soil nitrification is considered to be predominantly carried out by chemoautotrophic microorganisms, but in natural or semi-natural ecosystems, particularly acidic coniferous forest soils, the involvement of heterotrophic microorganisms is well documented (Killham 1990). Acetylene (C_2H_2) has been frequently used to inhibit autotrophic nitrification (Garrido et al. 2000; Walter et al. 1979). In our study, the addition of C_2H_2 to the soil significantly decreased NO_3^--N concentration, thus demonstrating that autotrophic nitrification was the dominant process in our soil.

AOB and AOA are the two most important autotrophic nitrifiers, affected by many environmental factors, such as temperature, soil pH, land utilization, organic matter, substrate concentration, and oxygen concentration (Li et al. 2018). AOA and AOB contribute to the nitrification process in a different way because they differ markedly in their oxidation of ammonia (Di et al. 2009; Prosser and Nicol 2008). Zhang et al. (2012) found that nitrification was driven primarily by AOA in highly acidic soils, while Di et al. (2009) found the opposite to be true in N rich, temperate grassland soils. In our experimental soil, the copy numbers of AOB *amoA* genes were much higher (about 100 times) than those of AOA, which combined with the much lower activity per copy number for AOA (Jia and Conrad 2009), suggested that AOB was likely the dominant population of soil nitrifiers.

Nitrapyrin, DCD, and DMPP reduced the net nitrification rates and decreased the abundances of AOB but exhibited no effect on AOA (Di et al. 2010; Kleineidam et al. 2011; Minet et al. 2016). In contrast, NBPT showed no significant effect on the AOA and AOB abundance and community composition (Xi et al. 2017). Up to now, the inhibitory effects and mechanism of BNIs on nitrifiers in soil are poorly known. Gopalakrishnan et al. (2009) used a plate-dilution frequency technique and found that the roots of *B. humidicola* could release BNIs and inhibited nitrifying bacteria (AOB and NOB) but could not inhibit other soil microorganisms. A 3-year field experiment with *B. humidicola* demonstrated that soil nitrification rates were suppressed > 90%, due to reduced populations of both AOA and AOB in soils (Subbarao et al. 2009). Sarr et al. (2020) found that sorghum, particularly high sorgoleone-producing strains, reduced AOA gene copy number but had no impact on AOB gene copy number. On the contrary, root exudates from sorghum suppressed the AOB *amoA* gene number but had no significant effect on AOA *amoA* gene number, likely because AOB was the main nitrifier in our soil, while the AOA was dominant in soils of the experiments by Subbarao et al. (2009) and Sarr et al. (2020).

Except for the direct inhibition of ammonia oxidizers, root exudates have indirect effects on soil nitrification (Nardi et al. 2020). Root exudates provide available C in the rhizosphere. The high C/N ratio can stimulate microbial activity, increase N immobilization, and inhibit nitrification (Li et al. 2016; Verhagen and Laanbroek 1991). In addition, some root exudates, i.e., phenolics, tannins, and monoterpenes, can inhibit nitrification rates indirectly by reducing gross N mineralization or increasing microbial N immobilization (Castells et al. 2004; Kraus et al. 2004; Uusitalo et al. 2008).

Conclusions

BNI secretion is supposed to be a survival mechanism in low-N environments, but our results show that sorghum has the BNI capability to inhibit soil nitrification even in high-N input soils. Sorghum root exudates inhibited AOB in an agricultural soil but did not influence AOA abundance, in contrast to a recent study that showed the reverse. We attribute this difference to be related to which nitrifiers dominate in any particular soil, either AOA or AOB, and suggest that sorghum can inhibit both groups. These results provide additional evidence for understanding BNI function under soil conditions. However, $^{13}CO_2$ and ^{15}N isotopes in combination with molecular-based tools should be used to clarify the interaction mechanisms between BNIs and nitrifiers in the future.

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