Revisiting plant biological nitrification inhibition efficiency using multiple archaeal and bacterial ammonia-oxidising cultures

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Abstract
Nitrification is a major process within the nitrogen (N) cycle leading to global losses of N, including fertiliser N, from natural and agricultural systems and producing significant nitrous oxide emissions. One strategy for the mitigation of these losses involves nitrification inhibition by plant-derived biological nitrification inhibitors (BNIs). Cultivation-based studies of BNIs, including screening for new compounds, have predominantly investigated inhibition of a single ammonia-oxidising bacterium (AOB), *Nitrosomonas europaea*, even though ammonia oxidation in soil is usually dominated by ammonia-oxidising archaea (AOA), especially in acidic soils, and AOB *Nitrosospira* sp., rather than *Nitrosomonas*, in fertilised soils. This study aimed to assess the sensitivity of ammonia oxidation by a range of AOA and AOB pure cultures to BNIs produced by plant roots (methyl 3-(4-hydroxyphenyl) propionate, sakuranetin and 1,9-decanediol) and shoots (linoleic acid, linolenic acid and methyl linoleate). AOA were generally more sensitive to BNIs than AOB, and sensitivity was greater to BNIs produced by shoots than those produced by roots. Sensitivity also varied within AOA and AOB cultures and between different BNIs. In general, *N. europaea* was not a good indicator of BNI inhibition, and findings therefore highlight the limitations of use of a single bioassay strain and suggest the use of a broader range of strains that are more representative of natural soil communities.

Keywords AOA · AOB · BNI · Thaumarchaeota · Plant · Culture · *Nitrosomonas europaea*

Introduction
The global N cycle is largely driven by soil microbial N transformations within which nitrification, the oxidation of ammonia (NH₃) to nitrate (NO₃⁻), is a key process. Nitrification involves initial oxidation of NH₃ to nitrite (NO₂⁻) by ammonia-oxidising archaea and bacteria (AOA and AOB), which is then oxidised to NO₃⁻ by nitrite-oxidising bacteria (NOB), while comammox can perform both steps. A key factor driving the soil N cycle, and global agricultural production, is the application of N fertilisers, which comprise more than 50% of N input. N fertiliser applications are projected to increase from 105 Mt (million tonnes) per year in 2010 to ~180 Mt per year by 2050 (Subbarao et al. 2015), and nitrification is responsible for N losses of up to 70% in natural and agricultural systems (Subbarao et al. 2015; Coskun et al. 2017). The mobility of anionic NO₃⁻ leads to significant leaching and pollution of water bodies or, under anaerobic conditions, to reduction of NO₃⁻ to dinitrogen (N₂) and nitrous oxide (N₂O), a highly potent greenhouse gas with an estimated global warming potential 265 times greater than CO₂ (IPCC 2014). Significant N₂O emissions are also associated directly with the activity of AOB and/or AOA, through nitrifier denitrification, incomplete oxidation of hydroxylamine and non-enzymatic conversion of nitrification products and intermediates (Prosser et al. 2019). Strategies for the control of nitrification in agroecosystems are therefore required to reduce the N footprint, reduce N₂O emissions and increase fertiliser nitrogen use efficiency (NUE).

One strategy employed to increase NUE is the application of synthetic nitrification inhibitors (SNIs), such as nitrapyrin, dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) (Ruser and Schulz 2015). However, efficient inhibition requires high amounts of SNIs, and their relatively high costs, low solubility in water, susceptibility to leaching and potential degradation by soil microbial communities restrict
their widespread use in farming systems (Ruser and Schulz 2015). Nonetheless, competition for N in soils has led to the evolutionary adaptation in a wide range of grasses, including important crops (sorghum rice, wheat and maize), to improve N uptake and assimilation by production of biological nitrification inhibitors (BNIs) from roots or following decomposition of shoots (Subbarao et al. 2009; Coskun et al. 2017; Subbarao et al. 2015).

All currently characterised BNIs inhibit ammonia monooxygenase (AMO), which catalyses the first and rate-limiting step of ammonia oxidation, oxidation of NH₃ to hydroxylamine (NH₂OH). However, subsequent energy-generating pathways for the conversion of NH₂OH to NO₂⁻ differ in AOA and AOB (Stein 2019). In addition, the two most active AO groups in soil, AOA and Nitrosospira differ in AOA and AOB (Stein 2019). In addition, the two groups of AO, and the very low abundance of Nitrososmonas in soil, BNI inhibition bioassays (e.g. AO inhibition assay through incubation of cultivated strains with root or shoot extracts or exudates) have been performed using a single AOB strain, Nitrosomonas europaea (Subbarao et al. 2015). These bioassays often utilise a genetically modified N. europaea that carries genes for bioluminescence activity from the marine bacteria Vibrio harveyi coupled to ammonia oxidation (Iizumi et al. 1998). This approach presents certain constraints, including the outsourcing of the genetically modified N. europaea strain and the laboratory requirement to use genetically modified organisms. In contrast, the Griess reagent test is used, which measures the nitrification activity of non-genetically modified ammonia oxidisers by determining the accumulated nitrite, an alternative high-throughput approach that is easily applicable to one or multiple ammonia-oxidiser strains.

Cultivation-based studies have shown variability in sensitivity to SNIs within AOB and AOA, with the AOB Nitrosospira multiformis being more sensitive to some SNIs than the AOA Nitrososphaera viennensis (Shen et al. 2013). In contrast, soil studies indicate greater sensitivity of AOA to BNI produced by some Brachiaria humidicola (Bh) hybrids, but not others (Subbarao et al. 2009). There is also evidence that the inhibition efficiency of BNIs depends on several factors, including BNI concentration (Nardi et al. 2012), soil pH (Lu et al. 2019), plant genotype (Subbarao et al. 2006, 2009; O’Sullivan et al. 2016; Sun et al. 2016) and AOA or AOB community composition (Lu et al. 2019). BNIs can be released as secondary metabolites from plant roots and stem tissues and as tannins, phenolic acids or flavonoids released from decaying leaves and stems (Coskun et al. 2017). The former BNIs are likely to be concentrated near the rhizosphere, while the latter will either be evenly distributed throughout the soil or be more abundant in upper soil horizons. There is evidence for the stimulation of AOA growth in the rhizosphere, possibly through increased nutrient supply or protection against abiotic stress (Taffner et al. 2019), and for production, by AOA, of secondary metabolites promoting plant growth and protection against pathogenic bacteria and fungi (Song et al. 2019). In addition, a greater abundance of AOA than AOB has been reported in the rhizosphere of several plants, including rice (Chen et al. 2008; Ke et al. 2013), wheat (Ai et al. 2013), maize (Wattenburger et al. 2020) and grasses (Thion et al. 2016).

These findings suggest that meaningful assessment of the efficiency of BNIs should focus on a larger and more representative set of soil ammonia oxidisers than N. europaea, and lead to the following hypotheses: (H1) AOA are more sensitive than AOB to BNIs; (H2) relative inhibition of AOA and AOB will depend on the source of BNIs, with greater sensitivity of AOB to root-derived BNIs and similar responses of AOA and AOB to those derived from shoots. (H3) N. europaea is not an appropriate model AO for bioassay of BNI inhibition of soil AO. This study aimed to test these hypotheses using ammonia-oxidiser cultures (rather than soil communities).

**Materials and methods**

**Cultivation of ammonia oxidisers**

Three AOA strains were investigated (see Table 1): Nitrososphaera viennensis, isolated from an Austrian pH 8 garden soil (Tourna et al. 2011), Candidatus Nitrosotalea sinensis, isolated from an acidic agricultural soil (Lehtovirta-Morley et al. 2011), and Ca. Nitrosocosmicus franklandus, isolated from a Scottish pH 7.5 agricultural soil (Lehtovirta-Morley et al. 2016). AOA were grown in a modified freshwater medium described by Tourna et al. (2011) (N. viennensis) and Lehtovirta-Morley et al. (2011, 2016) (Ca. N. sinensis and Ca. N. franklandus). Four AOB strains were also investigated: N. europaea ATCC 19718 and N. multiformis ATCC 25196, obtained from NCIMB (http://www.ncimb.com/); Nitrosospira tenuis NV12, isolated from a Hawaiian soil (Harms et al. 1976); and Nitrososphaera briensis 128, isolated from an acid agricultural soil (Rice et al. 2016). AOB were grown using the modified Skinner and Walker medium obtained from NCIMB (http://www.ncimb.com/); Nitrososphaera tenuis NV12, isolated from a Hawaiian soil (Harms et al. 1976); and Nitrososphaera briensis 128, isolated from an acid agricultural soil (Rice et al. 2016). AOB were grown using the modified Skinner and Walker medium
(Skinner and Walker 1961) containing phenol red pH indicator, which was periodically readjusted to pH 8 with 5% (wt/vol) Na₂CO₃.

### BNI preparation and supplementation

Three root-derived BNIs [methyl 3-(4-hydroxyphenyl) propionate (MHPP), 1,9-decanediol (DD) and sakuranetin (SKNT)] and three shoot-derived BNIs [methyl linoleate (ML), linoleic acid (LA) and linolenic acid (LNA)] were investigated (see Supplementary Fig. 1 for chemical structures). MHPP, ML, LA, LNA and SKNT were obtained from Sigma-Aldrich© and DD from Molport, USA. Stock solutions of all chemicals were prepared in 100% dimethyl sulfoxide (DMSO). Ten microlitres of stock solution was added to 10 ml medium, resulting in 0.1% (vol/vol) DMSO with BNI supplementation. BNIs were prepared to solubilise BNIs, determining the concentration vs. time, using data from at least four time points during exponential nitrite production. Initial growth experiments were performed to test potential effects of DMSO, which was used to solubilise BNIs, determining the μₘₐₓ of each AO as described above after supplementation of medium with 0.1% DMSO. Inhibition was quantified as the percentage reduction in μₘₐₓ in the presence of DMSO (μₘₐₓavgw/DMSO) (Eq. 1).

\[
\text{Percentage inhibition} = 100 - \left( \frac{\mu_{\text{DMSO}}}{\mu_{\text{NoDMSO}}} \right) \times 100
\]  

The inhibition of a particular strain by BNIs was assessed as the reduction in μₘₐₓ in BNI-supplemented medium as a percentage of the μₘₐₓ of that strain in the presence of DMSO (μₘₐₓavgw) (determined using Eq. 2).

\[
\text{Percentage inhibition} = 100 - \left( \frac{\mu_{\text{BNI}}}{\mu_{\text{avgw}}} \right) \times 100
\]

The concentration of each BNI leading to 80% inhibition (IC₈₀) was determined from a plot of percentage inhibition vs. BNI concentration, using data from at least four concentrations and assuming a linear relationship between percentage inhibition and inhibitor concentration (see Supplementary Tables S1, S2, S3, S4, S5, S6, S7 and S8). The μₘₐₓ and IC₈₀ were determined separately for each individual replicate.

### Statistical analysis

The significance of the effect of DMSO on each AO was tested using analysis of variance followed by Tukey post hoc analysis. Hypotheses were tested by comparing the IC₈₀ values obtained for each strain growing in the presence of each BNI using a multiple pairwise Student’s t test with the p value adjusted for Bonferroni correction. Prior to analysis of variance, the homoscedasticity and normality of data distribution were assessed. Hypothesis H1, that AO are more sensitive to BNIs than AOB, was tested by comparing the IC₈₀ values for BNIs from the two sources. Hypothesis H2, that AOA and AOB respond differently to BNIs from roots and shoots, was tested by comparing the IC₈₀ values for BNIs from the two sources. Hypothesis H3, assessing the validity of N. europaea as a model for bioassay of AO inhibition by BNIs, was tested by comparing IC₈₀ values of N. europaea with those of other strains for each BNI. All pairwise Student’s t tests were performed in R (R Core Team 2017) using the packages “tidyr” (Wickham and Henry 2019) and “dplyr” (Wickham et al. 2019). R package “ggplot2” (Wickham 2016) was used to plot the results.
Results

AOA are more sensitive to BNIs than AOB

The potential inhibition of AO growth by DMSO was investigated by comparison of $\mu_{\text{max}}$ of three AOA and four AOB in the presence and absence of 0.1% DMSO (Table 1). DMSO significantly reduced the $\mu_{\text{max}}$ of N. viennensis by 9% ($p = 10^{-14}$). The $\mu_{\text{max}}$ of all other strains was not significantly affected by the addition of DMSO (Table 1, Supplementary Fig. 2). The percentage inhibition of each strain increased linearly with increasing BNI concentration up to ~90% inhibition; this linear relationship was used to estimate the BNI concentration resulting in 80% reduction in $\mu_{\text{max}}$ (IC$_{80}$) (Supplementary Tables S1, S2, S3, S4, S5, S6, S7 and S8). H1 proposed that AOA were more sensitive than AOB to BNIs, with the null hypothesis that AOA and AOB sensitivities are not significantly different. Indeed, AOA sensitivity to BNIs was significantly higher than for AOB ($p = 0.2 \times 10^{-5}$), with mean IC$_{80}$ values of 201 and 506 $\mu$M, respectively (Fig. 1).

BNI inhibition varies between strains and with BNI source

There was no significant difference in overall AO sensitivity to BNIs produced by roots and shoots across all treatments (Fig. 2a). However, while there was no significant difference in inhibition between AOA and AOB for the root-derived BNIs, AOA were significantly more sensitive than AOB to shoot-derived BNIs ($p = 1.59 \times 10^{-12}$) (Fig. 2b, Supplementary Table S9), providing only partial support for hypothesis H2, that AOA and AOB respond differently to BNIs. Within each AO group, IC$_{80}$ values for AOA were significantly lower for the three shoot-derived BNIs, LA, LNA and ML, and two root-derived BNIs, DD and SKNT, with values of 15 s.e. 19, 11 s.e. 9, 18 s.e. 6, 377 s.e. 269 and 119 s.e. 77 $\mu$M, respectively, compared to AOB at 510 s.e. 201, 259 s.e. 79, 832 s.e. 259, 793 s.e. 500 and 354 s.e. 255 $\mu$M, respectively, where s.e. represents standard error (Fig. 2c, Supplementary Table S10). However, AOB were significantly more sensitive than AOA to the root-derived BNI MHPP, with IC$_{80}$ values of 287 s.e. 214 and 667 s.e. 405 $\mu$M respectively. The relative sensitivities of AOA and AOB were significantly different for each BNI with AOA being more sensitive than AOB to 5 of the 6 tested BNIs (all except MHPP), irrespective of the source (Fig. 2c, Supplementary Table S11).

The inhibition by root-derived BNIs was strain-dependent for both AOA and AOB (Fig. 3). The inhibition by shoot-derived BNIs was also strain-dependent for AOB, but not for AOA (Fig. 3). Inhibition of N. europaea varied with BNIs and was significantly different from that of AOA for all of the BNIs tested, confirming the hypothesis H3 that N. europaea is not a good model for bioassay of AO inhibition by BNIs. In addition, the inhibition of N. europaea was similar to the inhibition of one AOB strain (N. multiformis), but only for 2 out of 6 BNIs tested. Overall, the inhibition of N. europaea was significantly different from that of other AOB strains (Fig. 3, Supplementary Table S12).

Discussion

AOA are more sensitive than AOB to BNIs

Hypothesis H1 predicted that AOA would be more sensitive than AOB to BNIs, based on reported greater sensitivity of AOA in soil planted with some B. humidicola genotypes (Subbarao et al. 2009). Within both AOA and AOB, there was a considerable strain variation in inhibitory concentrations, and relative sensitivities differed between BNIs. However, as a group, AOA were significantly more sensitive than AOB to BNIs and were more sensitive to five of the six BNIs investigated. The exception was MHPP, for which N. viennensis and Ca. N. sinensis were less sensitive than the other strains, although Ca. franklandus was more sensitive than the four AOB strains. Strain variation in sensitivity has been observed within AOA and AOB for different SNIs (e.g. Taylor et al. 2013; Wright et al. 2020; Zhao et al. 2020) and may be related to the chemical structure of the compound. For example, Shen et al. (2013) reported a greater sensitivity of AOA for the aromatic SNI, nitrapyrin, but the reverse situation for linear SNIs allylthiourea (ATU), amidinothiourea (ASU) and dicyandiamide (DCD). In addition, differences in sensitivities of AOA and AOB to alkynes of different chain length provide the basis for use of octyne as a differential inhibitor of...
AOB (Taylor et al. 2013). The chemical structure may influence the mechanism of inhibition of ammonia monooxygenase which may stem from the differences in the enzyme’s active site (Wright et al. 2020), but there is currently no evidence for the specific effects of chemical structure on inhibition.

**BNI inhibition is dependent on BNI source**

Hypothesis H2 predicted greater sensitivity of AOB to root-derived BNIs and similar responses of AOA and AOB to those derived from shoots, based on evidence for possible selection of AOA, rather than AOB, in the rhizosphere. There was, however, little evidence for these predictions. There was no significant difference in the sensitivity of AOA and AOB to the three root-derived BNIs, when treated as a group, but effects varied within BNIs, with greater sensitivity of AOA for DD (from rice) and SKNT (from sorghum), but greater sensitivity of AOB to MHPP (also from sorghum). This suggests that the chemical nature of the BNI may be more important for resistance than adaptation of AOA to growth in the rhizosphere and that any selection for AOA in the rhizosphere is not due to increased resistance to BNIs. In contrast, AOA were significantly more sensitive than AOB to all three shoot-derived BNIs investigated. The inhibitory concentrations of shoot-derived fatty acids LNA and LA were similar within each group, potentially through...
similarities in chemical structure (Supplementary Fig. 1) implying a similar mode of action for inhibition, as previously observed (Subbarao et al. 2008).

Choice of ammonia oxidisers for BNI bioassays

Although *N. europaea* is the most extensively studied bacterial ammonia oxidiser, it is common in sediments and wastewater treatment plants but rare in soil, raising concerns regarding its ecological relevance in bioassays for BNIs in soil. Our findings demonstrate significant variation in sensitivity to BNI both within and between AOA and AOB and significant differences between sensitivity of *N. europaea* and strains that are more representative of natural soil AO communities (Fig. 3). The significant differences in inhibitory concentrations for different strains certainly suggest that a wider range of representative organisms is required for bioassays, rather than relying on a single strain.

A similar variation in relative sensitivities of individual strains to different BNIs also suggests that, without greater understanding of mechanisms of inhibition, generalisations cannot be made from studies of individual BNIs. This, combined with the niche partitioning amongst AOA and AOB, complicates BNI application in soil
systems when inhibition studies are based on a single microorganism. Certainly, the greater resistance of AOA to MHPP observed in this study is consistent with the dominance of soil ammonia oxidation by AOA and reports of much greater concentrations of MHPP required to inhibit soil nitrification than in *N. europaea* bioassays (Subbarao et al. 2012). We therefore suggest that future BNI testing should involve several ammonia-oxidiser strains of both AOA and AOB to obtain an accurate picture of the potential effectiveness of BNIs in suppressing soil nitrifier activity, soil nitrification and N₂O emissions.

A number of studies reported differences between BNI potential determined in bioassays in pot experiments and field studies. For example, the inhibition of nitrification by DD and MHPP in soil differed significantly from the inhibition in culture bioassays (Sun et al. 2016; Lu et al. 2019). Sakuranetin, a BNI produced by sorghum, also strongly inhibited activity in bioassays, but not in soil incubations (Subbarao et al. 2012).

In contrast, *N. europaea* is much more resistant to nitrapyrin in soil (Powell and Prosser 1986), in part through cell attachment to clay minerals (Powell and Prosser 1992). Lu et al. (2019) also suggested that AOA and AOB inhibition varied with soil pH and with AO community composition and complexity. A number of factors may influence inhibitory concentrations in soil. These discrepancies may be due to protection from inhibition by cells attached to soil particulate matter and/or in biofilms and/or to differences in abiotic characteristics, e.g., temperature, pH, to degradation of inhibitors and other factors. However, increasing knowledge of AO soil community ecology, niche partitioning and the influence of plants, soil conditions and overall microbial community activity (e.g., via competition, mineralisation) on AO communities suggest that differences in strain sensitivity to BNIs will be important in designing relevant bioassays and assessing the potential efficiencies of BNIs in soil. Future testing of both additional ammonia-oxidiser cultures and BNIs yet to be discovered is encouraged to better determine the effectiveness of the different sources of BNIs. This study demonstrates the significant limitations of reliance on a single bioassay strain that is not representative of natural soil AO communities. It also highlights the need for greater understanding of mechanisms of inhibition of BNIs as well as of the factors influencing differences in inhibition in laboratory culture and in the soil environment.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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248

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