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ORIGINAL PAPER

Syringic acid from rice as a biological nitrification and urease inhibitor and its synergism with 1,9-decanediol

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Abstract

The type, functions, and mechanisms of biological nitrification inhibitors (BNIs) from rice were investigated using a combination of chemical and molecular techniques, bacterial bioassays, and soil microcosm experiments. We report the discovery of an effective nitrification inhibitor, syringic acid, in the root exudates of rice. Nitrification inhibition activity by syringic acid was verified in both weakly acidic and neutral pure cultures of *Nitrosomonas europaea*, and was superior to the widely used synthetic nitrification inhibitor, dicyandiamide (DCD). Moreover, syringic acid exhibited a dual inhibitory effect on ammonia monooxygenase (AMO), active in ammonium/ammonia oxidation, and on urease, active in urea hydrolysis. Nitrification inhibition by syringic acid was also demonstrated in a paddy soil system, and the abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) was significantly inhibited under all syringic acid treatments. A synergistic effect of syringic acid and another rice BNI, 1,9-decanediol, on nitrification was found in two pure *Nitrosomonas* cultures and a paddy soil. Together, our results enhance our understanding of BNI production by rice and enable the design of natural inhibitor formulations that regulate soil N transformation in a concerted manner.

Keywords Biological nitrification inhibitor · Urease activity · Syringic acid · Ammonia oxidizer · Synergism · Rice

Introduction

The economic and environmental costs of low nitrogen (N) use efficiency (NUE) and high N loss from agricultural ecosystems have become global concerns (Subbarao et al. 2006a; Galloway et al. 2008; Yan et al. 2014; Coskun et al. 2017a, b). The majority of crop fertilizers provide N in the chemically reduced form and liberate ammonium (NH_4^+) and ammonia (NH_3). As the subsequent microbiological conversion of ammonium (NH_4^+) to nitrate (NO_3^-), nitrification is one of the most important steps in the global N cycle. Nitrification inhibition leads to the improvement of N retention due to NH_4^+ binding to negatively charged soil particles via cation exchange, and thereby greatly enhances the availability of N to plants and reduces N loss via NO_3^- run-off and leaching and gaseous losses (Subbarao et al. 2015; Coskun et al. 2017a, b). Although several synthetic nitrification inhibitors (SNIs) have been developed and applied in the field (Zaman et al. 2009; Sun et al. 2015; Min et al. 2021), their high cost, limited availability, microbial degradation, adsorption to soil particles, adverse effects on beneficial soil microorganisms, and potential environmental and food safety risks are major constraints and have prevented more widespread adoption (Subbarao et al. 2006a; Fillery 2007). It is therefore highly desirable to develop nitrification inhibitors that might be produced by the crops themselves, which would circumvent most economic and environmental downsides and, as biological products, lead to easier acceptance by consumers, such as fertilizer producers and farmers, particularly organic agricultural producers.

It is well established that certain plants can secrete specific compounds that retard nitrification, termed biological nitrification inhibitors (BNIs) (Subbarao et al. 2006b). Field crops, tropical pasture plants, and trees have been evaluated for their BNI capacity (Subbarao et al. 2007; Tanaka et al. 2010; O'Sullivan et al. 2016; Laffite et al. 2020). Several

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BNIs exuded from roots and their release mechanisms have been identified in *Brachiaria humidicola* (brachialactone) and sorghum (sorgoleone and methyl 3-(4-hydroxyphenyl) propionate) (Zakir et al. 2008; Subbarao et al. 2009; 2013; Zhu et al. 2012; Egenolf et al. 2021). However, relatively few studies have focused on BNIs in the three major food crops (rice, wheat, and maize).

Rice (*Oryza sativa*) is grown worldwide and is a staple food crop for about half of the world's population. Given the economic importance and intensity of N use in rice systems, it is of great significance to develop management strategies that limit N losses from rice fields and improve NUE in rice, from flooded paddy to upland systems. It should be noted that while the bulk soil in paddy soils tends to present an anaerobic environment with little nitrification activity, the importance of the nitrification process in paddy field microsites and in the rhizosphere of rice plants can be significant, and rice shows excellent ability to utilize nitrate–N (Kronzucker et al. 1999, 2000; Kirk and Kronzucker 2005). Nitrification in paddy systems takes place in aerobic microsites, such as the soil–water interface, and in particular in the rice rhizosphere, as extensive aerenchyma tissue in mature rice plants allows oxygen to diffuse from shoot tissue into roots, from where it is then released into the soil (Kronzucker et al. 1998, 1999, 2000; Li et al. 2007). Once oxygen is introduced, nitrification starts quickly in previously anoxic niches, and, as a result, nitrogen mixtures of varying proportions can be expected in soil solution both in upland and paddy fields (Jensen et al. 1993; Kronzucker et al. 1999). Moreover, anaerobic paddy soils transition to an aerobic environment in the midseason aeration stage to suppress rice tillering. Thus, there is a need for nitrification inhibition in rice systems both in upland and paddy fields (Sun et al. 2016; Coskun et al. 2017a, b).

Evidence for rice's ability to produce BNIs comes from culture-based assays showing that root exudates of about 50% of rice genotypes possessed some nitrification inhibition ability, tested across 36 rice genotypes (Tanaka et al. 2010). Recently, our team identified a new BNI, 1,9-decanediol, from rice root exudates, a fatty alcohol compound with potential to inhibit nitrification by blocking ammonia monooxygenase (AMO) (Sun et al. 2016). The release of 1,9-decanediol from rice roots is induced by both NH_4^+ and nitrifying bacteria (Zhang et al. 2019). Moreover, the nitrification inhibition of 1,9-decanediol was demonstrated in a soil matrix and shown to target both ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Lu et al. 2019). Correlating 1,9-decanediol amounts in root exudates and BNI ability showed that 1,9-decanediol alone comprises about 36% of the BNI activity of tested rice root exudates (Sun et al. 2016), clearly implicating the existence of other BNIs in rice. However, the types and structures of which have not hitherto been explored.

Phenolic acid derivatives are an important class of allelochemicals in many soil ecosystems, both natural and agricultural, and can play a significant role in N cycling and ecosystem dynamics (Rice and Pancholy 1972, 1973; Lodhi 1978; Baldwin et al. 1983; Vitousek et al. 1989; Schimel et al. 1996; Kronzucker et al. 1997; Hattenschwiler and Vitousek 2000). However, the role of phenolic acids in suppressing nitrification is still a matter of debate, with some authors having reported clear nitrification inhibitory effects of phenolic acids in soils (Rice and Pancholy 1972; Castaldi et al. 2009), while others have reported little effect (McCarty et al. 1991; Gopalakrishnan et al. 2007). These differences may be attributed to the wide variety of chemical structures among phenolic acids, different concentrations and environmental persistence in varying soil ecosystems, co-presence of other secondary metabolites that might either enhance or suppress nitrification activity, and differences in the soil microbiome (Rice and Pancholy 1972; Badri et al. 2013). While several studies have shown a negative correlation between the total amounts of phenolics in plant extracts and the expression of *amoA* genes in soil-resident AOA and AOB (Chen et al. 2020), specific phenolic acids from root exudates and their effects on ammonia oxidizers remain largely unidentified.

Due to different solubilities and affinities, BNI compounds with different chemical structures are expected to differ in their mobility in soil. In the diverse sorghum root exudates, hydrophobic BNIs, such as sorgoleone, may remain close to the root systems, whereas hydrophilic BNIs, such as methyl 3-(4-hydroxyphenyl) propionate (MHPP), are more likely to move out of the rhizosphere (Subbarao et al. 2013), indicating that different BNIs in the rhizosphere may play complementary functional roles (Subbarao et al. 2015). As well, soil pH affect which kinds of BNIs are excreted—hydrophobic BNIs tend to be excreted more under alkaline conditions and hydrophilic ones preferentially under more acidic conditions (Subbarao et al. 2013). Additive or synergistic effects are believed to occur when biochemically distinct BNIs with similar mobilities coexist in the rhizosphere (Nardi et al. 2013; Coskun et al. 2017b), with the potential of enhancing the effect and persistence of single nitrification inhibitors. A recent study by Duncan et al. (2016) showed that the application of a composite inhibitor consisting of the SNI DCD and a guanyl thiourea inhibitor improved the efficacy of nitrification inhibition in soil. However, the nature of the interactions between different BNIs on nitrification has not been studied in detail.

Although the use of BNIs has the potential to increase crop NUE and reduce N loss (Subbarao et al. 2017), research into BNIs is still in its infancy (Coskun et al. 2017b). It should be noted that a limited arsenal of BNIs and lack of study on their interactions will restrict the potential application of BNIs. In the current study, we hypothesize that, in addition to 1,9-decanediol, rice roots also secret other types

of BNIs and synergistically inhibit nitrification, in concert with 1,9-decanediol. The main objectives of our study were (1) to identify novel types of BNIs exuded from rice roots; (2) to clarify the inhibitory function and mechanisms underlying the inhibitory role of rice BNIs; and (3) to explore the interactions of novel BNIs from rice with the known BNI 1,9-decanediol in the inhibition of nitrification.

Methods and materials

Plant materials and growth conditions

Two representative rice (*Oryza sativa* L.) cultivars, Wuyunjing7 (WYJ7, high BNI activity) and Wuyujing3 (WYJ3, no BNI activity), were used in this study (Sun et al. 2016). Seeds were surface-sterilized with 10% H_2O_2 for 30 min, washed with sterile water, and then germinated in 0.5-mM $CaCl_2$ solution at 30 °C in the dark. After 3 days, the germinated seeds were transferred into 1/2 modified Kimura's solution for a week (Sun et al. 2016). The full-strength solution composition was as follows: 0.5-mM NH_4NO_3, 0.18-mM KH_2PO_4, 0.54-mM $MgSO_4{\cdot}7H_2O$, 0.18-mM KCl, 0.36-mM $CaCl_2$, 0.2-μM $CuSO_4{\cdot}5H_2O$, 0.5-μM $MnCl_2{\cdot}4H_2O$, 0.4-μM $ZnSO_4{\cdot}7H_2O$, 3-μM H_3BO_3, 1-μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 20-μM Na_2EDTA-Fe, pH 5.8. To maintain the pH of 5.8 during cultivation, 0.2 g L^{-1} MES was added. The nutrient solution was changed every 3 day, and the solution volume was restored daily with deionized water. Plants were grown in a growth chamber, with a 14 h/10 h, 28 °C/25 °C light/night cycle, a light intensity of 400 μmol m^{-2} s^{-1}, and a relative humidity of 65%. Subsequently, three 10-day-old seedlings were bundled and transferred to normal modified Kimura's solution (not bubbled).

Collection and identification of root exudates

The static collection of root exudates was performed as described earlier (Sun et al. 2016). In short, thirty 6-week-old seedlings of WYJ7 and WYJ3 (equivalent to 5-g fresh weight of root) were rinsed with deionized water and then gently transferred into dark flasks containing 1-L 0.1-M $CaCl_2$ solution. Deionized water was replenished after 12 h to avoid excessive evapotranspiration. After 24 h, the collection solutions were filtered using 0.45-μm and 0.22-μm filter membranes to remove cellular debris and external microorganisms. The filtered samples were evaporated to dryness using a rotary evaporator (Eyela, N-1300D-WB, Tokyo, Japan) at 40 °C. The residues (not obvious solids) remaining in the round-bottom flask were redissolved in 10 mL of methanol and stored at − 20 °C. Samples (2 mL) of root exudates in methanol were evaporated to dryness under N_2, and then subjected to N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) derivatization at 60 °C for 1 h. The mixture was evaporated to dryness again, redissolved in 200 μL of hexane, and subjected to GC–MS.

The GC–MS analysis was carried out using an Agilent 6890 gas chromatograph equipped with a DB-5 (30 m × 0.25 μm × 0.25 mm) capillary column and coupled to an Agilent 5975 mass spectrometer. The derivatized sample was injected (2 μL) in splitless mode at 280 °C, using helium as the carrier gas (1.0 mL min^{-1}). A GC oven temperature started from 60 for 2 min up to 300 °C at 10 °C min^{-1}, holding for 30 min. The mass-selective detector was operated at an ionization energy of 70 eV and in a range of 20–650 amu. Compounds were identified based on the comparison of their retention time and mass spectra to commercial standards in the NIST libraries. Specific compounds identified were then tested using authentic compounds to confirm their characteristics in GC–MS and to examine their BNI effects.

Evaluation of syringic acid for its inhibitory effect

Authentic syringic acid, shikimic acid, ferulic acid, methyl 3-(4-hydroxyphenyl) propionate, and DCD were obtained from Sigma-Aldrich. Authentic 1,9-decanediol was synthesized by WuXi AppTec (Shanghai, China). DCD was dissolved in sterilized Milli-Q water, and all the other authentic compounds were dissolved in dimethyl sulfoxide (DMSO).

Nitrosomonas europaea (NBRC 14,298) and *Nitrosomonas stercoris* (NBRC 110,753) were obtained from NITE Biological Resource Center (NBRC), Tokyo, Japan. The strain was grown aerobically in HEPES medium, as recommended by NBRC, containing the following nutrients: 2.5 g L^{-1} $(NH_4)_2SO_4$; 0.5 g L^{-1} KH_2PO_4; 11.92 g L^{-1} HEPES; 0.5 g L^{-1} $NaHCO_3$; 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$; 5 mg L^{-1} $CaCl_2{\cdot}2H_2O$; 75 mg L^{-1} Fe-EDTA; pH 8.0. Bacteria were cultured in 500-mL flasks containing 200 mL of HEPES medium using an incubation shaker at 200 rpm and 30 °C. A 7-day-old culture mix was centrifuged at 5000 g for 10 min and resuspended in sterile HEPES medium with OD_{600} 1.0.

In the BNI bioassay, a mixture of 5 μL of identified syringic acid in DMSO (500 μM and 2500 μM), 100 μL of HEPES medium, 200 μL of resuspended bacterial cells, and 195 μL of sterilized Milli-Q water was added to a 1.5-mL tube and statically incubated at 25 °C for 2 h in the dark. The reaction was stopped by the addition of 20 μL of 0.1-mM allylthiourea (AT), a reference nitrification inhibitor. Control experiments were performed by adding 5-μL DMSO. NO_2^- production was then determined using a modified Griess nitrite test method (Sastry et al. 2002). The nitrification inhibition of the specific compounds was calculated using the following equation:

$$\text{Nitrification inhibition (\%)} = [1 - (C_{\text{inhibitor}}/C_{\text{DMSO}})] \times 100$$

where $C_{inhibitor}$ is the amount of NO_2^- produced in the inhibitor treatments and C_{DMSO} is the amount of NO_2^- produced in the DMSO control.

The BNI activity of syringic acid was investigated under weakly acidic (pH 6.0), neutral (pH 7.0), and alkaline conditions (pH 8.0). The pH of the HEPES medium used in the BNI assay was adjusted with 1-M HCl or 1-M NaOH. The inhibitory effects of two other phenolic acids, shikimic acid and ferulic acid, were also compared with syringic acid under pH 6.0 and pH 8.0, at concentrations of 500 µM and 2500 µM.

A dose–response curve of syringic acid (with concentrations of 0, 100, 200, 400, 500, 1000, 2000, and 5000 µM) was established using the method discussed under the above BNI assay, and the effects of various nitrification inhibitors (1,9-decanediol, syringic acid, MHPP, and DCD) were compared using the same method under pH 6.0. Using a modification of a method reported by Kaur-Bhambra et al. (2021), the concentration of each inhibitor leading to 50% inhibition (ED_{50}) was determined from a plot of nitrification inhibition (%) vs. inhibitor concentration, using data from at least four concentrations and assuming a linear relationship between nitrification inhibition (%) and inhibitor concentration. The combination effect of syringic acid (500 µM) and 1,9-decanediol (280 µM) on the nitrification of *N. europaea* and *N. stercoris* was also determined.

Inhibition mode of syringic acid on Nitrosomonas

To further explore which specific process of ammonia oxidation is influenced by syringic acid, an experiment was performed by incubating *N. europaea* in the presence and absence of hydroxylamine, using the modified method described by Subbarao et al. (2006b). If the inhibitor only affects the AMO pathway, the inhibitory effect can be alleviated in the presence of hydroxylamine; if the inhibitor affects both AMO and HAO enzymes, the inhibition of *Nitrosomonas* will be maintained in the presence of hydroxylamine (Subbarao et al. 2013). Specifically, 100 µL of 1-mM hydroxylamine was added to the assay mixture before incubation, as well as 1250 µM of syringic acid or 0.22 µM of the standard inhibitor AT was used, which both possess an 80% inhibition.

Inhibition of syringic acid on soil nitrification and ammonia oxidizers

To characterize the inhibitory function of syringic acid in soil, soil incubation experiments were conducted. The soil used for the incubation studies was a paddy soil classified as silt loam (pH 6.25, clay 17.4%, silt 41.9%, sand 40.7%; total C, 13.4 g kg^{-1} soil; total N, 1.7 g kg^{-1} soil; organic matter, 22.7 g kg^{-1} soil; and CEC, 11.2 cmol kg^{-1}), collected from a research experimental farm (31° 17′ N, 119° 54′ E) in Yixing, Jiangsu Province, China. The soil (0–20 cm) was air-dried and passed through a 2-mm sieve before use.

Soil microcosms consisted of 100-mL glass bottles containing 6 g of soil (oven dry equivalent). Treatments were (1) nitrogen 200 mg kg^{-1} soil, added as ammonium sulfate ($(NH_4)_2SO_4$ as the control); (2) nitrogen plus syringic acid at a concentration of 100 mg kg^{-1} soil (SA-low dose); (3) nitrogen plus syringic acid at a concentration of 250 mg kg^{-1} soil (SA-medium dose); and (4) nitrogen plus syringic acid at a concentration of 500 mg kg^{-1} soil (SA-high dose). We designed the doses of syringic acid based on the BNIs concentrations in other soil incubation experiments that range from 0 to 2000 mg BNI kg^{-1} soil (Subbarao et al. 2008, 2013; Nardi et al. 2013; Tesfamariam et al. 2014). The syringic acid was added to soil in the same manner as described earlier (Lu et al. 2019). The microcosms were incubated at 25 °C in the dark for 14 days and maintained with a 60% water-filled pore space (WFPS). Soil samples were collected on day 14 for determination of soil-exchangeable NH_4^+-N and NO_3^--N, potential nitrification activity (PNA), and molecular analysis. Soil NH_4^+-N and NO_3^--N were extracted with 2-M KCl and determined on a continuous flow analyzer (Skalar, Breda, Netherlands). The combination effect of syringic acid (100 mg kg^{-1} soil) and 1,9-decanediol (100 mg kg^{-1} soil) on soil nitrification at day 7 and day 14 was also determined. The inhibition of soil nitrification based on the amount of NO_3^--N produced was calculated.

Potential nitrification activity (PNA) was determined using the shaken slurry method as described in our previous study (Lu et al. 2019). Briefly, 15 g of soil per sample was mixed with 100 mL of phosphate buffer solution (50-mM KH_2PO_4; 50-mM K_2HPO_4; pH 7.2) with 37.5-mM $(NH_4)_2SO_4$. The suspensions were shaken at 180 rpm on a shaker at 25 °C. After 2, 4, 22, and 24 h, aliquots of 10-mL liquid suspension were taken and centrifuged at 8000 g for 10 min at 4 °C. The NO_3^--N concentration was determined by continuous flow analysis (Skalar, Breda, Netherlands). PNA was calculated as mg NO_3^--N h^{-1}.

Total genomic DNA was extracted from 0.25 g of soil using MoBio PowerSoil DNA isolation kits (Qiagen). To obtain the ammonia oxidizer community size, the abundance of AOA and AOB was assessed by qPCR of the *amoA* gene, using the same primers and protocol described in Lu et al. (2019), on a LightCycler 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). Real-time PCR was performed in triplicate, and amplification efficiencies of 93.5–103.6% were obtained, with R^2 values > 0.99.

Urease inhibition assay

The urease activity was determined by measuring urea consumption. One milliliter of the test compounds (syringic acid, MHPP, linolenic acid, DCD), 0.5 mL of the enzyme (jack bean urease from Sigma, 1 U/mg), and 0.5 mL of urea (100 mM) were added into 3 mL of phosphate buffer (50-mM K_2HPO_4, 50-mM KH_2PO_4, pH 7.0).

The final assay mixture was 5 mL, containing 0.1 U/mg enzyme and 10-mM urea, and inhibitors with concentrations of 10–5000 μM were incubated for 30 min at 30 °C. After incubation, the remaining urea in the reaction mixture was colorimetrically determined using the *p*-dimethylaminobenzaldehyde method (Roijers and Tas 1964). The absorbance of the solution was measured at 420 nm after 20 min, using a microplate reader (Tecan Spark, Austria). All reactions were performed in triplicate. Percentage inhibition was calculated by using the formula:

$$\text{Percentage inhibition} = [(OD_{testwell}/OD_{control}) - 1] \times 100$$

The inhibition of urease was expressed as an ED_{50} (i.e., the concentration of each inhibitor leading to 50% inhibition), which was determined from a plot of percentage inhibition vs. inhibitor concentration, using data from at least four concentrations and assuming a linear relationship between percentage inhibition and inhibitor concentration. N-(n-Butyl)thiophosphoric triamide (NBPT) was used as the standard inhibitor of urease.

Evaluation of synergism

The synergistic effect of syringic acid and 1,9-decanediol was analyzed by applying the modified Bürgi formula (Jin equation) (Jin 2004; Wu et al. 2016). The formula is $q = E_{A+B}/(E_A + E_B - E_A \times E_B)$, where E_{A+B}, E_A, and E_B are the average effects of the combination treatment, syringic acid alone, and 1,9-decanediol alone, respectively. The q values < 0.85, 0.85–1.15, and ≥ 1.15 indicate antagonism, additive effects, and synergism, respectively.

Statistical analysis

Statistical analyses were carried out using the SPSS 18.0 software package for Windows. Normality of data distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test) assumptions were satisfied. Statistically significant differences among treatments were determined by one-way ANOVA and least significant difference (LSD) calculations at a 5% confidence level.

Results

Identification of BNI substance from rice root exudates

The root exudate of WYJ7 showed a significant nitrification inhibitory activity, whereas WYJ3 exhibited a stimulation (Fig. S1), consistent with Sun et al. (2016). We therefore compared the root exudate profile of these two rice varieties by GC–MS analysis. Of particular interest was the peak at 27.44 min, which was detected in WYJ7 root exudates but was not found in WYJ3 (Fig. S2). Comparing to the NIST mass-spectral library revealed that the compound belonged to the phenolic acids, and that, based on 99% mass-spectral similarity (Fig. S3), it was identified as syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid). This chemical identity was then ascertained by comparing the retention time with authentic standards.

To determine the inhibitory function of syringic acid, authentic compounds were supplied to pure *N. europaea* cultures under three pH conditions. As can be seen from Fig. 1, syringic acid had a significant inhibitory effect of 56.0% when provided at 500 μM and of 87.6% when provided at 2500 μM, at pH 6.0. The inhibition rates decreased to 2.5% and 31.6% at pH 7.0, respectively. Low-dose and high-dose syringic acids lost their nitrification inhibitory activity as pH rose to 8.0. These results indicate syringic acid displays a strong nitrification inhibitory effect under neutral and weakly acidic conditions. Similarly, the other two phenolic acids, ferulic acid and shikimic acid, had 44.5–87.9% inhibitory activity under weakly acidic conditions, whereas no effect was detected under alkaline conditions (Table 1).

Comparative evaluation of syringic acid and other NIs

In order to evaluate the BNI potential of syringic acid, its ED_{50} value was compared with that of other inhibitors

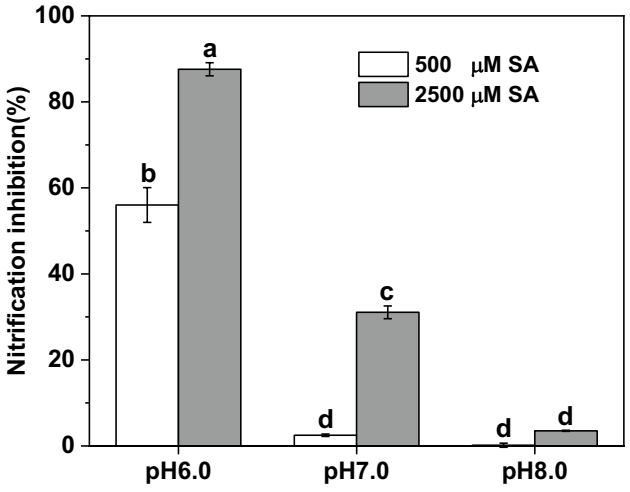

Fig. 1 Nitrification inhibitory effects of authentic syringic acid (SA) under different pH conditions. Data are presented as means ± SE ($n = 3$). Lowercase letters represent significant differences (LSD test, at $P < 0.05$) between treatments

Table 1 Nitrification inhibitory effects of syringic acid, shikimic acid, and ferulic acid

Compounds	Nitrification inhibition (%)			
	500 μM	2500 μM	500 μM	2500 μM
	pH 6.0		pH 8.0	
Syringic acid	51.96 ± 5.47	86.30 ± 2.33	1.54 ± 0.28	7.43 ± 1.12
Shikimic acid	56.34 ± 2.54	87.93 ± 2.54	5.32 ± 1.85	2.52 ± 0.09
Ferulic acid	49.81 ± 2.06	80.36 ± 3.03	0.65 ± 0.08	0.32 ± 0.09

Data are presented as means ± SE ($n = 3$).

Table 2 Comparison of the nitrification inhibition of syringic acid and that of other biological and synthetic inhibitors on *Nitrosomonas europaea*

Compound	ED_{50} (μM)
Syringic acid (SA)	444
1,9-Decanediol	280
Methyl 3-(4-hydroxyphenyl) propionate (MHPP)	295
Dicyandiamide (DCD)	1223

(Table 2). The results revealed a superior inhibitory effect of syringic acid to that of the well-known synthetic nitrification inhibitor DCD. However, syringic acid was less effective than the previously identified BNI 1,9-decanediol from rice. Although weaker than biological inhibitors MHPP by ED_{50}, the maximum inhibitory effect of syringic acid was higher than that of MHPP (data not shown).

Mode of inhibition of syringic acid

Ammonium oxidation, which is catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), is the first and rate-limiting step of nitrification. The standard inhibitor allylthiourea (AT) can block the AMO pathway specifically (Subbarao et al. 2006b). When adding the intermediate hydroxylamine to the reaction mixture, the inhibition by AT was alleviated from 79.8 to 47.2%. Similarly, the inhibition by syringic acid was alleviated by the addition of hydroxylamine, from 81.1 to 50.3%, indicating that syringic acid specifically blocks the AMO process.

Effect of syringic acid on soil nitrification and ammonia oxidizers

To verify whether syringic acid is effective to suppress soil nitrification, a 14-day microcosm incubation of paddy soil supplemented with syringic acid was performed. Compared to the control, lower soil nitrate (NO_3^--N) concentrations were observed under the low-dose, medium-dose, and high-dose syringic acid treatments, with an inhibition rate of 5.3–8.0% (Fig. 2a, $P < 0.05$). This was accompanied by a significant trend of increasing soil-exchangeable NH_4^+-N at day 14 (Fig. 2b, $P < 0.05$). The nitrification inhibition by syringic acid was also confirmed in potential nitrification activity (PNA) data. As shown in Fig. 2c, PNA was 1.5-mg NO_3^--N kg^{-1} h^{-1} in the control treatment of paddy soil, and it decreased to 1.3-, 1.0-, and 0.9-mg NO_3^--N kg^{-1} h^{-1} in the low-dose, medium-dose, and high-dose syringic acid treatments, respectively.

A quantitative PCR assay was carried out to estimate the influence of syringic acid on the abundance of ammonia oxidizers in paddy soil. As shown in Fig. 2d, a dose–response relationship was also found between syringic acid levels and AOB and AOA populations. Compared to the control, the low, medium, and high dose of syringic acid significantly inhibited the abundance of AOB by 60–87% and AOA by 50–66% ($P < 0.05$).

Effect of syringic acid on urea hydrolysis

In order to evaluate the inhibition of urea hydrolysis by syringic acid, its ED_{50} value was compared with that of other inhibitors (Table 3). Among all the test BNIs compounds, syringic acid exhibited the strongest urease inhibitory activity, although not as effective as the standard urease inhibitor NBPT. The BNIs MHPP and LN showed a moderate inhibition. By contrast, the addition of the SNI DCD had no significant effect on urease.

Synergism between syringic acid and 1,9-decanediol

The interaction between the two rice-derived BNIs syringic acid and 1,9-decanediol on nitrification was further examined under both pure *Nitrosomonas* cultures and soil incubation conditions using Jin's formula. Compared to syringic acid (500 μM) and 1,9-decanediol (280 μM) alone, their combination yielded synergistic nitrification inhibition of *N. europaea* ($q = 1.52$) and *N. stercoris* ($q = 1.36$) (Fig. 3a). This synergism was also observed in the paddy soil at the 7- and 14-day incubation times (Fig. 3b). The combined administration of syringic acid and 1,9-decanediol was more effective than either agent alone ($P < 0.05$).

Discussion

Plant BNI exudation holds the promise of increasing agronomic NUE in crop plants and minimizing N pollution (Coskun et al. 2017a, b). As compared with the

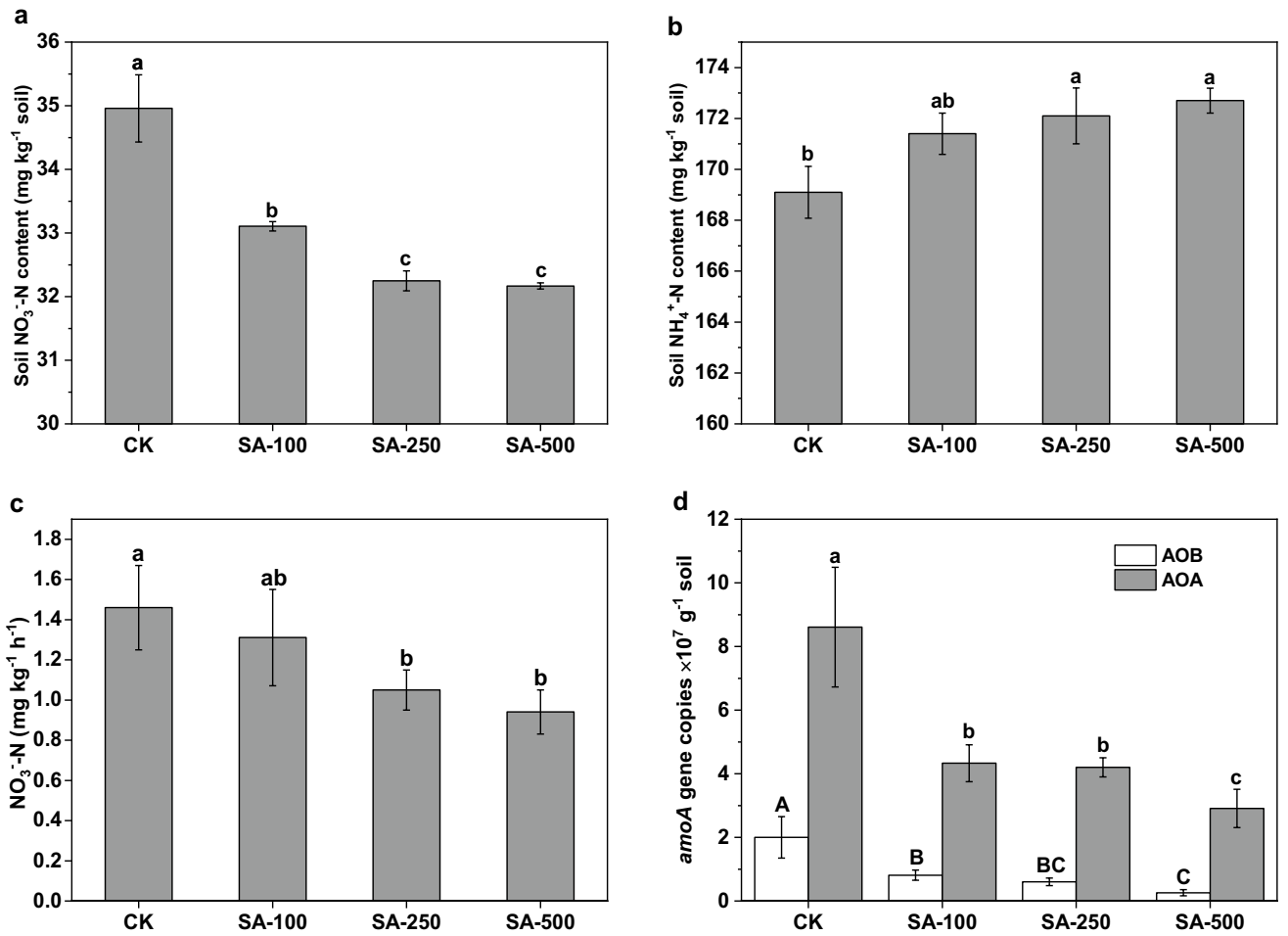

Fig. 2 NO_3^--N content (**a**), exchangeable NH_4^+-N content (**b**), potential nitrification activity (**c**), and *amoA* gene copies of ammonia-oxidizing bacteria (AOB) and archaea (AOA) (**d**) in paddy soil sampled after 14-day incubation. Data are presented as means ± SE ($n = 3$). a.b.c. lowercase letters represent significant differences (LSD test, at $P < 0.05$) between treatments; d. Lowercase and capital letters represent statistical differences (LSD test, at $P < 0.05$) of *amoA* gene copies of AOB and AOA, respectively

Table 3 Urease inhibition activities of syringic acid and other inhibitors

Compound	ED_{50} (μM)
Syringic acid (SA)	290
Methyl 3-(4-hydroxyphenyl) propionate (MHPP)	1484
Linolenic acid (LN)	1300
Dicyandiamide (DCD)	> 10,000
N-(n-Butyl)thiophosphoric triamide (NBPT)	157

commercial SNIs, BNIs can regulate soil nitrification process in a more environmental friendly and sustainable way (Subbarao et al. 2008). 1,9-Decanediol has been identified as the first BNIs from the "big three" crops (Sun et al. 2016), is derived from rice root exudates, and has the potential to increase N utilization and reduce N_2O emissions in rice fields (Lu et al. 2019). While it was obvious from earlier studies (Sun et al. 2016) that additional BNIs are produced by rice, these have to date remained uncharacterized.

Nitrification inhibitory activity of syringic acid

The secondary metabolite syringic acid, a product of the shikimic acid pathway, is widely distributed in the plant kingdom. It has been shown to exhibit many biological functions, such as allelopathic, antimicrobial, antioxidant, and plant defense ones (Cheemanapalli et al. 2018; Sharma et al. 2018). It has been found in root exudates, plant extracts, and in soils (Wu et al. 2001; Kong et al. 2006; Shi et al. 2016). The metabolite also has a myriad of plant-internal functions, such as in redox regulation, including in rice (Dey and Bhattacharjee 2020), and many of its biological functions appear to be related to the methoxy groups in positions 3 and 5 of the benzene ring structure (Cheemanapalli et al. 2018). Its

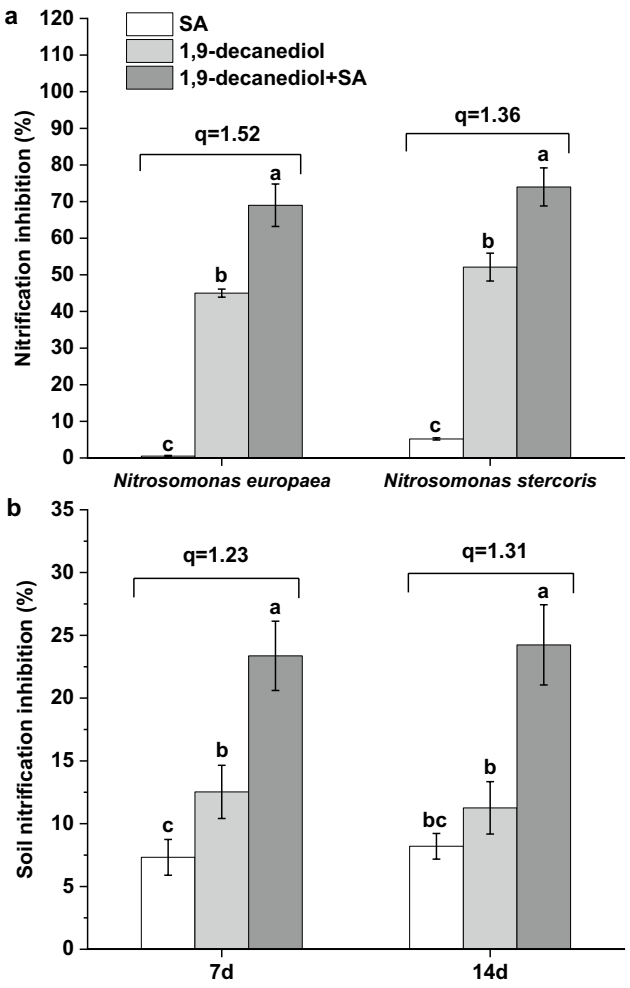

Fig. 3 Inhibition by syringic acid (SA), 1,9-decanediol, and their combination on nitrification under both *Nitrosomonas* pure culture system (**a**) and soil incubation conditions (**b**). Data are presented as means ± SE ($n = 3$). Lowercase letters represent significant differences (LSD test, at $P < 0.05$) between treatments. $q \geq 1.15$ indicates synergism (Jin's formula) between syringic acid and 1,9-decanediol

role in affecting the activity of soil microbes central to N cycling, however, has not been examined, while phenolic acids more generally are well-known to influence soil microbial activity, soil N cycling, and the dynamics of a variety of ecosystems, from natural grassland and forests to agricultural systems (Rice and Pancholy 1972, 1973; Lodhi 1978; Baldwin et al. 1983; Vitousek et al. 1989; Hattenschwiler and Vitousek 2000). Data specific to the potential effect of syringic acid in this regard, however, are scarce and indeed absent for leading agricultural species and systems. Only one study to date has reported that an addition of 990 mg kg^{-1} of syringic acid led to a 4–14.4% nitrification inhibitory effect on neutral Fayette soil and on Canisteo soil (Karmarkar and Tabatabai 1991).

The rice genotype (WYJ3) with no BNI activity lacked syringic acid (Fig. S2), and one may consider whether syringic acid is being actively produced specifically for its BNI activity or for different functions. Evidence that BNI secretion is induced under favorable nitrifying environments, such as by NH_4^+, oxygen, and inoculation with nitrifying bacteria (Subbarao et al. 2009; Zhu et al. 2012; Zhang et al. 2019) indicates that BNIs are released specifically for its BNI activity (Coskun et al. 2017a, b). Thus, syringic acid appears to be released in WYJ7 specifically in the BNI context, but whether such BNI specificity occurs in other rice genotypes remains unclear and warrants further study.

Given the importance of soil pH in influencing nitrification activity and that reports on pH dependence have delivered varying results (Mochizuki et al. 2002; Wu et al. 2019), we, here, also evaluated the performance of syringic acid under different pH conditions. We found that syringic acid inhibits nitrification by *N. europaea* under both neutral and weakly acidic conditions (Fig. 2), while no detectable activity was observed under alkaline conditions. This behavior is in accordance with the biological activity of another phenolic acid, *p*-coumaric acid, which showed a 29.9–35.6% inhibitory effect on nitrification on neutral pH soil (Karmarkar and Tabatabai 1991), whereas no significant inhibition was detected in *N. europaea* cultures at pH 8.0 (Gopalakrishnan et al. 2007). Thus, we propose that the alkaline culture systems commonly used to test BNI activity might have limitations in evaluating nitrification inhibitors.

To gain more insight, we tested the inhibitory activity of shikimic acid (Sun et al. 2016) and ferulic acid (Gopalakrishnan et al. 2007), previously demonstrated to show no nitrification inhibition under alkaline culture conditions. Similar to syringic acid, these two phenolic acids exhibited a dramatic inhibitory effect at pH 6.0 (Table 1). This underscores that pH is a critical factor in determining the nitrification inhibition potential of phenolic acids, including that of syringic acid. Since the phenolic hydroxyl group is the key determinant for nitrification inhibitory activity of the phenolic acid structure (Wu et al. 1999), it is likely that hydroxide ions (OH^-) in alkaline media facilitate dissociation and neutralization of the H^+ proton of the phenolic hydroxyl group, thereby altering the reactivity of the phenolic hydroxyl group. That may be the reason why the nitrification inhibitory potency of acidic BNIs remained masked in several previous studies. Kaur-Bhambra et al. (2021) also demonstrated significant limitations of using a single *N. europaea* strain in the inhibition bioassay, as the efficacy of BNIs varies among AOA and AOB cultures. A recommendation for future research on acidic nitrification inhibitors is, therefore, that these be carried out under weakly acidic conditions and with bacterial strains that are representative of natural soil ammonia oxidizer communities, as well as in soil environments.

The nitrification inhibitory activity of syringic acid was further confirmed in paddy soil under aerobic conditions

that favors nitrification, indicating that SA may also act as an effective inhibitor in aerated upland rice cultivation systems and dried paddy fields, or flooded rice fields (at the midseason aeration stage) where, due to large nitrous oxide emissions, strong BNI activity is of special importance. Although derived from rice, SA might also inhibit nitrification in other dryland fields, such as maize- or wheat-growing systems, as discussed for 1,9-decanediol (Lu et al. 2019). Compared with other inhibitors, SA was superior in efficacy to the widely used inhibitor DCD and the biological inhibitor methyl 3-(4-hydroxyphenyl) propionate from root exudates of Sorghum (Zakir et al. 2008). Although not as strong as the 1,9-decanediol previously identified from rice root exudates (Sun et al. 2016), the efficacy of syringic acid appeared to be enhanced owing to its excellent suppressive effect on urea hydrolysis via urease (Table 3). This is in agreements with a previous study that tropical medicinal plant extracts containing a high content of phenols exhibited a dual inhibition of urease and of enzymes of nitrification (Zhao et al. 2015). Other studies have shown that linoleic acid, another BNI from the shoot tissue of *B. humidicola*, inhibited both soil urea hydrolysis and nitrification (Subbarao et al. 2008). By contrast, the SNI DCD possessed no inhibitory activity on urease. In this regard, syringic acid exuded from rice roots may have potential as a dual natural nitrification and urease inhibitor, giving it a strong advantage over currently used SNIs.

Nitrification inhibitory mechanism of syringic acid

Although plant-derived BNIs compounds are assumed to inhibit nitrification by acting directly on ammonia oxidizers (Rice and Pancholy 1972, 1973), other indirect mechanisms, such as a reduction in NH_4^+ availability by virtue of increased NH_4^+ immobilization, have also been identified (Schimel et al. 1996; Nardi et al. 2020). It is known that phenolic acids can be used as a C source by soil microorganisms, which may stimulate microbial activity and increase their demand for N (Fierer et al. 2001). Thus, the possibility of NH_4^+ immobilization by syringic acid was investigated, and we provide several lines of experimental evidence to show that it is of minor importance and that, instead, the effect on ammonia oxidizers is of a more direct nature.

The first argument is that NO_3^- concentration increased concomitantly with a decline in NH_4^+ at day 14 (Fig. 2a, b), indicating that NH_4^+ immobilization did not occur in the syringic acid treatments in paddy soil. The second experimental test involved the analysis of potential nitrification activity (PNA), using the soil-slurry method. Compared to the N control, the addition of syringic acid led to a 13–40% inhibition in PNA (Fig. 2c), which is consistent with the NO_3^- decline in the soil incubation experiments. Given PNA was measured in soil samples supplemented with an excess of NH_4^+ and the assay involved continuous shaking to ensure an aerobic environment, the possibility that lack of substrate availability played an important role in the lower nitrification rates in the syringic acid treatments can be largely ruled out. A recent study by Vazquez et al. (2020) showed that the association between the BNI effect and PNR activity was inferior to that with gross nitrification rate. Thus, it will be worthwhile to analyze the influence of BNIs such as SA on gross nitrification rate in the future, in order to evaluate the BNI effect more comprehensively. The final line of evidence was provided by the observation that AMO enzyme activity was inhibited in the bioassay, suggesting that nitrification inhibition by syringic acid was due to a direct targeting of ammonia oxidizers. It should be pointed out that C/N ratio plays a pivotal role in regulating the N immobilization-nitrification balance in soils (Verhagen et al. 1992). Indirect effects of phenolics on nitrification have been mostly found at higher C/N ratios conditions (Clein and Schimel 1995; Schimel et al.1996), such as in N-limited forest ecosystems. While the initial NH_4^+ added in the present study is fully sufficient and nitrification activity of paddy soil was not strong in the 14-day incubation period, it is not unreasonable to conclude that the low C/N ratio by syringic acid had a limited effect on N immobilization.

We further observed that both AOB and AOA abundance in paddy soil was inhibited by syringic acid (Fig. 2d). Other BNIs, such as MHPP, 1,9-decanediol, and sorgoleone, had similarly inhibitory effects on both AOA and AOB (Nardi et al. 2013; Lu et al. 2019; Sarr et al. 2020). These findings suggest that BNIs have the potential for targeting both AOB and AOA. However, most SNIs (DCD, DMPP, and nitrapyrin) appear to have a limited capacity to inhibit archaeal ammonia oxidation (Shen et al. 2013), possibly due to the structural difference of the *amoB* subunit in these organisms. Given that AOA represent the dominant ammonia-oxidizing contributors in acidic environments (Zhang et al. 2012), there is a need to develop new effective inhibitors for ammonia archaea (Li et al. 2018; Lu et al. 2019). Since the inhibitory activity of syringic acid on AOA was here only tested in paddy soil at pH 6.2, its effect on ammonia oxidation should be further verified in highly acidic environments (pH < 6). Indeed, the effect of more general inhibitors targeting not only AOB but also AOA might be better and last longer, which could minimize the risk of developing resistance (Coskun et al. 2017b; Beeckman et al. 2018). That may be another advantage of BNIs over commercial SNIs.

Although a recent study by Kaur-Bhambra et al. (2021) showed that six BNIs produced from plant roots and shoots exhibited similar inhibitory effects on AOA, such effects were only verified using AOA and AOB pure cultures. It has been suggested that BNIs isolated from pure cultures may exhibit no nitrification inhibition activity in soils (Subbarao et al. 2013), and plants may secrete different BNIs to

adapt to their unique habitats (Zakir et al. 2008; Subbarao et al. 2013; Sun et al. 2016). Therefore, the identification of different BNIs will help in the tailored development of single applications or combinations of BNIs that are of use in real field settings, and, from a fundamental perspective, will help us better understand the ecological mechanisms of plant adaptation to their environments in the context of BNI.

We elucidate that the enzyme target for inhibition by syringic acid is AMO, using a *N. europaea* assay with either ammonia or hydroxylamine as the substrate. Most frequently used SNIs, such as nitrapyrin, DCD, and DMPP, were shown to exert inhibition of the AMO enzyme, but not of the HAO enzyme (Zakir et al. 2008; Subbarao et al. 2009). Some BNIs (sorgoleone, sakuranetin, and brachialactone) are believed to block both AMO and HAO activity (Subbarao et al. 2009, 2013), while other BNIs, such as MHPP and 1,9-decanediol, inhibit *Nitrosomonas* by blocking only the AMO pathway (Zakir et al. 2008; Sun et al. 2016), as does syringic acid. AMO has a wide substrate range, and the inhibitory effects of many compounds are due to competition for the active site (McCarty 1999). As a phenolic acid, syringic acid is likely to act as a competitive inhibitor of the AMO enzyme.

Synergism between rice BNIs

No BNI synergisms have been reported in the literature hitherto, while we, here, show clear synergism between syringic acid and 1,9-decanediol in terms of nitrification inhibition in *N. europaea* cultures. This might be attributed to the enhancement of 1,9-decanediol competition for the bacterial AMO enzyme sites in the presence of syringic acid. The cooperative inhibition between two rice BNIs was confirmed using a nitrifying strain of *N. stercoris*, indicating that this synergistic effect of syringic acid and 1,9-decanediol on microbial nitrification may be more general.

In addition to occurring in the purely cultured bacterial systems, the synergy of the two BNIs from rice was also demonstrated under soil conditions. As a phenolic acid and a fatty alcohol possessing different chemical structures, syringic acid and 1,9-decanediol will display different binding characteristics with soil particles, thus differentially affecting the transport and distribution of the two BNIs. In this context, the mixtures of different BNIs may act on a more diverse and broader cross section of ammonia-oxidizing microorganisms in soil than in single-BNI applications (Beeckman et al. 2018). However, the synergistic mechanisms of syringic acid and 1,9-decanediol on nitrification warrant further more detailed study.

It should be noted that requirement for a large dose and chemical instability are the most common limitations to nitrification inhibitor applications in the field. For example, the most frequently used NI DCD can be easily leached from N-application zones in the crop rhizosphere because it is highly water-soluble (McCarty and Bremner 1989). Compared with the recommended dosage of commercial SNIs (1–10% applied N), the effective doses of the BNIs 1,9-decanediol, MHPP, and sorgoleone were relatively high in incubation experiments (Nardi et al. 2013; Tesfamariam et al. 2014; Lu et al. 2019). We show that the combined effect of 100 mg kg^{-1} soil of syringic acid and 1,9-decanediol can be equivalent to that of 500 mg kg^{-1} soil for 1,9-decanediol alone (Lu et al. 2019), implying that syringic acid may reduce the dose requirement for 1,9-decanediol while preserving its role. Although this synergy has not as yet been verified among soil types, our study provides a novel means to reduce the amount and cost of individual BNIs. Such mixed inhibitor formulation containing two or more BNIs may hold promise for improving the efficacy and durability of single BNIs in different soil environments.

Conclusions

We identified the phenolic compound syringic acid from rice root exudates as a potential inhibitor for both nitrification and urea hydrolysis, and show that it targets both AOA and AOB abundance and bacterial AMO activity. Our results also demonstrate a synergistic effect of two rice BNIs, syringic acid and 1,9-decanediol, on nitrification. This concept could be applied to other BNIs in food crops, pastures, and trees, thus improving the effectiveness of these products. The present findings represent a significant step forward in our understanding of BNIs in rice and point at opportunities for the design of novel fertilizer formulations based on the synergistic concept of multiple BNIs, benefiting crop NUE and reducing N loss from agricultural systems. Our ongoing research is aimed at further characterizing the effect of syringic acid under different soil types and moisture conditions, as well as clarifying the synergistic mechanisms of syringic acid and 1,9-decanediol in inhibiting nitrification.

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Declarations

Conflict of interest The authors declare no competing interests.

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