New Phytologist

Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency

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Received: *30 January 2016*
Accepted: *12 May 2016*

New Phytologist (2016) **212:** 646–656
doi: 10.1111/nph.14057

Key words: 1,9-decanediol, ammonia monooxygenase (AMO), biological nitrification inhibition/inhibitor (BNI), nitrogen-use efficiency (NUE), rice (*Oryza sativa*), root exudate.

Summary

• Microbial nitrification in soils is a major contributor to nitrogen (N) loss in agricultural systems. Some plants can secrete organic substances that act as biological nitrification inhibitors (BNIs), and a small number of BNIs have been identified and characterized. However, virtually no research has focused on the important food crop, rice (*Oryza sativa*).

• Here, 19 rice varieties were explored for BNI potential on the key nitrifying bacterium *Nitrosomonas europaea*. Exudates from both *indica* and *japonica* genotypes were found to possess strong BNI potential. Older seedlings had higher BNI abilities than younger ones; Zhongjiu25 (ZJ25) and Wuyunjing7 (WYJ7) were the most effective genotypes among *indica* and *japonica* varieties, respectively.

• A new nitrification inhibitor, 1,9-decanediol, was identified, shown to block the ammonia monooxygenase (AMO) pathway of ammonia oxidation and to possess an 80% effective dose (ED_{80}) of 90 ng μl^{-1}. Plant N-use efficiency (NUE) was determined using a ^{15}N-labeling method. Correlation analyses indicated that both BNI abilities and 1,9-decanediol amounts of root exudates were positively correlated with plant ammonium-use efficiency and ammonium preference.

• These findings provide important new insights into the plant–bacterial interactions involved in the soil N cycle, and improve our understanding of the BNI capacity of rice in the context of NUE.

Introduction

Nitrification is a pivotal soil process, mediated by microorganisms, which converts reduced nitrogen (N) from ammonium (NH_4^+)/ammonia (NH_3) forms to nitrate (NO_3^-), via nitrite (NO_2^-), and is the major pathway through which N can be lost from terrestrial ecosystems (Banning *et al.*, 2015). The product of nitrification, NO_3^-, is highly mobile in soil matrices, and thus can be readily leached and cause groundwater pollution. Nitrification is tightly integrated with the reductive process of denitrification, in which NO_3^- is converted to gaseous N_2, and both nitrification and denitrification are responsible for the production of nitrous oxide, N_2O, one of the critical greenhouse gases and the dominant ozone-depleting substance emitted from soils. The loss of ammonium fertilizer post-application is mainly caused by nitrification and consequent denitrification (Abbasi & Adams, 1998). Thus, the suppression of nitrification and the maintenance of N fertilizer in the reduced form are critical steps to increase fertilizer-N retention in soils and to improve the N-use efficiency (NUE) of crops with a view to agricultural production and environmental protection.

Some synthetic inhibitors, e.g. nitrapyrin, dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP), are widely used to retard nitrification. In agricultural systems, these inhibitors can effectively reduce N loss and enhance NUE of crops; environmentally, they are able to control nitrate leaching to water bodies and N_2O emissions to the atmosphere (Zaman *et al.*, 2009; Abalos *et al.*, 2014; Huang *et al.*, 2014; Sun *et al.*, 2015). The use of these synthetic inhibitors, however, has been restricted because of their high cost, lack of availability, inconvenience of application and the potential for environmental contamination; in particular, water-soluble inhibitors can lead to significant surface and below-ground water contamination (Qiu *et al.*, 2015). The application of DCD, for instance, is limited because very high DCD concentrations are needed to achieve the desired results. Moreover, as DCD is water soluble, it itself easily leaches out of crop root zones, i.e. the very zones in which it is intended to inhibit nitrification (Cahalan *et al.*, 2015). Given these constraints presented by synthetic inhibitors, it is necessary to develop plant-derived nitrification inhibitors, referred to either as natural nitrification inhibitors (NNIs) (Upadhyay *et al.*, 2011) or biological nitrification inhibitors (BNIs) (Subbarao *et al.*, 2015). These are easily available and environmentally friendly. Some such compounds from plants occurring predominantly in natural ecosystems have been reported (Northup *et al.*, 1995). More recently, some BNIs from two plants known to display

nitrification inhibitory potential, *Brachiaria humidicola* and *Sorghum bicolor*, have been identified and characterized for their effects and inhibition modes on the ammonia-oxidizing bacterium *Nitrosomonas europaea* (Gopalakrishnan *et al.*, 2007; Subbarao *et al.*, 2008, 2009; Zakir *et al.*, 2008). The inhibitors were reported to have ideal properties *vis-à-vis* BNI and to block either the ammonia monooxygenase (AMO) pathway or both the AMO and hydroxylamine oxidoreductase (HAO) pathways engaged in ammonia oxidation. Such approaches clearly need to be extended to major food crops.

Rice (*Oryza sativa*) is grown world-wide and is the most important food crop for human nutrition, providing over 21% of the caloric needs of the world's population and 76% of that of the population of South-East Asia (Fitzgerald *et al.*, 2009). High rates of N fertilizer application are the norm in paddy fields where rice is grown, to sustain food production and to satisfy increasing demand (Zhao *et al.*, 2014). Although it is known that some 50% or more of applied N can be lost in volatilization (Vlek & Byrnes, 1986; Cassman *et al.*, 1993, 1998; Britto & Kronzucker, 2004), the importance of the nitrification process in paddy fields has been largely neglected (Kirk & Kronzucker, 2005), as a common view is that nitrification is an aerobic process, whereas paddy fields are flooded and thus present an anaerobic environment. However, nitrification still takes place in aerobic microsites, such as the soil–water interface and rice rhizosphere (Kirk & Kronzucker, 2005; Chen *et al.*, 2008; Shen *et al.*, 2012; Luo *et al.*, 2014). Mature rice plants have extensive aerenchyma tissues that allow oxygen to diffuse from shoot tissue down into roots and be released into the soil (Li *et al.*, 2007). Once oxygen is introduced, nitrification starts quickly in previous anoxic niches (Jensen *et al.*, 1993). Recently, Yang *et al.* (2015) have reported that nitrifying microorganisms might have adapted better than hitherto thought to environments with low oxygen concentrations, such as paddy soils during the flooding period, providing the possibility that nitrification may happen extensively in paddy fields (Kirk & Kronzucker, 2005; Li & Wang, 2013). It should also be noted that nitrification is commonly considered to be most pronounced in neutral to alkaline soils (pH > 5), and in high-fertility arable soils, but has nevertheless also been documented in largely acidic paddy soils (Bodelier & Frenzel, 1999; He *et al.*, 2007; Jiang *et al.*, 2011, 2015), where, furthermore, nitrification intensity varies strongly with the rice varieties planted (Li & Wang, 2013). Thus, the need to better understand nitrification in rice soils, and the potential importance of nitrification inhibition in rice systems, is clear. Tanaka *et al.* (2010) compared the nitrification inhibitory abilities of root exudates of several rice genotypes. However, until now, no specific compounds have been isolated from rice root exudates demonstrated to inhibit nitrification.

Rice has a preference for ammonium rather than nitrate as an N nutrient (Balkos *et al.*, 2010). Urea-based N fertilizers, which are rapidly transformed to ammonia in soils through the process of ammonification, are the most commonly used source of N in paddy fields, owing to their high solubility and low cost. Ammonia/ammonium can then either be taken up by plants or microbes and enter these organisms' metabolism, or serve as a substrate for nitrification, with subsequent potential for N loss from the agroecosystem. As a result, the BNI phenomenon in rice, to the extent that it is present, is expected to reflect on NUE, in particular ammonium-use efficiency, of the rice crop. Moreover, previous research has demonstrated a close relationship between plant NUE and rhizosphere nitrification (Li *et al.*, 2008), which can be directly affected by the BNI ability of roots. However, no direct connection has been discovered as yet between the intrinsic NUE of rice varieties and their BNI abilities. Therefore, the objectives of our study were as follows: to screen rice varieties of both *indica* and *japonica* progeny with respect to BNI ability; to identify and characterize BNIs in rice root exudates; and to investigate the relationship between BNI abilities and NUEs of rice varieties.

Materials and Methods

Rice growth conditions

Seeds of rice (*Oryza sativa* L.) varieties were sterilized with 10% H_2O_2 for 30 min, rinsed and soaked with deionized water for 24 h. The seeds were then germinated on floating nets in a culture box containing 0.5 mM $CaCl_2$. After 4 d of incubation at 30°C in the dark, the germinated seeds were placed under light, and the $CaCl_2$ solution was replaced with half-strength modified Kimura B nutrient solution. The full-strength nutrient solution contained macronutrients, as follows: NH_4NO_3, 0.5 mM; KH_2PO_4, 0.18 mM; $MgSO_4{\cdot}7H_2O$, 0.54 mM; KCl, 0.18 mM; $CaCl_2$, 0.36 mM; and micronutrients, as follows: $CuSO_4{\cdot}5H_2O$, 0.2 μM; $MnCl_2{\cdot}4H_2O$, 0.5 μM; $ZnSO_4{\cdot}7H_2O$, 0.4 μM; H_3BO_3, 3 μM; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1 μM; Na_2EDTA-Fe, 20 μM. Subsequently, three 10-d-old seedlings at a time were bundled and transplanted into a larger culture box with an identical nutrient solution. Two weeks after sowing, the nutrient solution was changed to full strength and, 4 wk after sowing, to double full strength. The pH of the solution was 5.8, and 0.2 g l^{-1} MES was added to maintain the pH during cultivation. The nutrient solution was changed every 3 d, and the solution volume was restored daily with deionized water. The plants were grown in a controlled-environment chamber with a temperature regime of 25°C : 28°C, 65% humidity, 14 h : 10 h, light : dark photoperiod and light intensity of 400 μmol m^{-2} s^{-1} provided by high-pressure sodium lamps. Rice subspecies and varieties were as follows: *indica* varieties: ZJ25 (Zhongjiu25), YD6 (Yangdao6), IR26, Kasa (Kasalath), LH1 (Lvhan1), IR36; *japonica* varieties: WYJ7 (Wuyunjing7), GD4 (Guidan4), ZH6 (Zhenghan6), Koshi (Koshihikari), NJ46 (Nanjing46), XS123 (Xiushui123), XS63 (Xiushui63), ZD11 (Zhendao11), ZD10 (Zhendao10), WYJ23 (Wuyunjing23), Nipponbare, Minami (Minamihikari), WYJ3 (Wuyujing3).

Collection and preparation of root exudates for assay

As a result of their size differential, 120 3-wk-old seedlings and 30 6-wk-old seedlings were rinsed consecutively with deionized water before use. The seedlings were then transferred into dark flasks containing 1 l of Milli-Q water. Specifically, the roots were

immersed gently in water, whilst the shoots were held and supported with sterilized sponges. Mechanical damage to roots could lead to a significant alteration of both the exudate amount and composition; therefore, extreme attention was paid to the manipulation. Water was replenished after 12 h to avoid excessive evapotranspiration. After 24 h, both shoots and roots were washed, separated and freeze-dried for weighing. The collected exudates were filtered using 0.45-μm filter membranes to remove microorganisms; filtered samples were pretreated immediately or stored at 4°C until evaporation within 3 d. The samples were evaporated to dryness using a rotary evaporator (Eyela, N-1100D-WD, Tokyo, Japan) at 40°C, and then resuspended in HPLC-grade methanol and evaporated to dryness again, during which a freeze drier was used to guarantee the removal of water after evaporation. The residues remaining in the round-bottomed flasks were redissolved in 10 ml of methanol and stored at −20°C, and, before measurement, 1-ml samples were centrifuged to dryness and redissolved in 5 μl of dimethyl sulfoxide (DMSO).

For the aseptic experiment, an *in-situ* cultivation and collection were conducted using sterilized nutrient solution (Kuijken *et al.*, 2015), and the collected solution was subjected to a solid-phase extracting system (C18 SPE columns) to retain possible target substances in root exudates (Sun *et al.*, 2016); the exudate samples were later used for the identification of BNI substances.

Determination of nitrification inhibitory effect of root exudates

Nitrosomonas europaea (ATCC 19718) was 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 (1 l): $(NH_4)_2SO_4$, 2.5 g; KH_2PO_4, 0.5 g; HEPES, 11.92 g; $NaHCO_3$, 0.5 g; $MgSO_4{\cdot}7H_2O$, 100 mg; $CaCl_2{\cdot}2H_2O$, 5 mg; Fe-EDTA, 75 mg; pH 7.8–8.0. Bacteria were cultured in 500-ml flasks containing 200 ml of HEPES medium using an incubation shaker (set at 200 rpm, 30°C). A 7-d-old culture mix was centrifuged and resuspended in fresh HEPES medium, and adjusted to an optical density at 600 nm (OD_{600}) of 1.0 using a spectrophotometer (SmartSpec plus; Bio-Rad).

In the assay, a mixture of 195 μl of sterilized Milli-Q water, 5 μl of root exudate samples (in DMSO), 100 μl of HEPES medium and 200 μl of resuspended cells was added to a 1.5-ml tube and incubated at 25°C for 2 h. The reaction was stopped by the addition of 20 μl of 0.1 mM allylthiourea (AT), a standard nitrification inhibitor. NO_2^- production was then determined using a modified Griess nitrite test method, with a spectrophotometer (Sastry *et al.*, 2002).

Identification of BNI substances

The representative varieties WYJ7 and WYJ3 were chosen for their difference in allelochemicals. Samples (2 ml) of root exudates in methanol from each variety were evaporated to dryness under N_2 and derivatized with 500 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Bellefonte, PA, USA) at 60°C for 2 h. The mixture was evaporated to dryness again, redissolved in 100 μl of hexane and subjected to GC-MS. The GC-MS analysis was carried out using an Agilent 6890 gas chromatograph equipped with a fused silica capillary column DB-5 (30 m × 0.25 mm × 0.25 μm) hyphenated to an Agilent 5975 mass spectrometer (Santa Clara, CA, USA). Splitless injection was performed at 280°C; the oven temperature was initially 60°C for 2 min and was then raised to 300°C at a rate of 10°C min^{-1} and held for 30 min. The carrier gas was helium, provided at a flow rate of 1.0 ml min^{-1}, and the sample size was 1.4 μl. 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 times and mass spectra with those reported in the National Institute of Standards and Technology (NIST) library (Gaithersburg, MD, USA) . Specific compounds identified were then tested using authentic compounds to confirm their characteristics in GC-MS and to examine their BNI effects.

Evaluation of 1,9-decanediol for its inhibitory effect

Authentic 1,9-decanediol was synthesized by WuXi AppTec (Shanghai, China). Authentic 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin), DCD, 2-amino-4-chloro-6-methylpyrimidine (AM), methyl 3-(4-hydroxyphenyl) propionate, linoleic acid and linolenic acid were obtained from Sigma-Aldrich, and methyl-*p*-coumarate was obtained from TCI (Tokyo, Japan). DCD was dissolved in sterilized Milli-Q water, and all the other authentic compounds were dissolved in DMSO. The dose–response curve of 1,9-decanediol (with concentrations of 0, 20, 40, 60, 80, 100, 150 and 200 ng μl^{-1}) was established using the method discussed in the BNI assay, and the effects of various nitrification inhibitors were compared using the same method and expressed as ED_{80} (80% effective dose).

Inhibition mode of 1,9-decanediol

To explore which specific process of ammonia oxidation is affected by 1,9-decanediol, an experiment was performed by incubating the bacteria in the presence and absence of hydroxylamine, as described by Subbarao *et al.* (2007). One hundred microliters of 1 mM hydroxylamine were added to the assay mixture before incubation and, as well as 100 ng μl^{-1} of 1,9-decanediol or 0.026 ng μl^{-1} of the standard inhibitor AT was used, the latter of which was reported to have an 80% inhibitory effect.

Determination of 1,9-decanediol in rice root exudates

Two milliliters of root exudate samples (in methanol) of 6-wk-old seedlings of 19 rice varieties were evaporated to dryness under N_2 and derivatized with 500 μl of BSTFA at 60°C for 2 h. The mixture was then evaporated to dryness again, redissolved in 100 μl of hexane and subjected to GC. GC analysis was performed on an Agilent 6850 gas chromatograph equipped with a

fused silica capillary column HP-5 (25 m × 0.2 mm × 0.33 μm) and a flame ionization detector (FID). Splitless injection was performed at 250°C; the oven temperature was initially 80°C and was increased to 160°C at a rate of 20°C min^{-1}, then to 200°C at a rate of 5°C min^{-1} and, finally, to 310°C at a rate of 20°C min^{-1}. The carrier gas was helium, provided at a flow rate of 1.0 ml min^{-1}, and the sample size was 2 μl. Authentic 1,9-decanediol was used to produce a standard curve.

Determination of NUE of rice varieties

NUE was measured using a ^{15}N labeling method. The representative varieties, WYJ7 and WYJ3, were selected for the measurement of N absorption kinetics. Following 2-d of N starvation, both 3-wk-old and 6-wk-old seedlings of WYJ7 and WYJ3 were transferred to nutrient solution in which 0, 0.05, 0.2, 0.5, 1 and 3 mM of $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ were added. In addition, 6-wk-old seedlings of all rice varieties were treated with 0.5 mM of $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$. After 3 h of treatment, shoots and roots were washed, separated, freeze-dried, ground into a powder and subjected to a Thermo Flash 2000 analyzer hyphenated with a Thermo Fisher (Waltham, MA, USA) Delta-V isotope ratio mass spectrometer to determine total N and ^{15}N abundance.

Statistical analyses

The data were subjected to SPSS 18.0 (SPSS, Chicago, IL, USA) and were analyzed using Duncan's multiple-range test, Student's *t*-test and Pearson correlation analysis, as appropriate. Significance levels and other data formats are as illustrated in the figure and table legends.

Results

Nitrification inhibitory effects of rice root exudates

Seven 3-wk-old seedlings and 15 6-wk-old seedlings of the 19 rice varieties showed significant nitrification inhibitory effects compared with 'Blank' control, regardless of *indica* or *japonica* subspecies (Fig. 1). With similar amounts of root dry weight used in exudate collection, 6-wk-old seedlings displayed stronger inhibitory effects than 3-wk-old seedlings, which was consistent for all varieties, including seven varieties that showed inhibitory effects in both growth periods. ZJ25 and WYJ7, at 6 wk of age, were the most effective varieties with regard to nitrification inhibition among *indica* and *japonica* varieties, respectively, with both exhibiting >40% inhibition. Some varieties, however, did not show any inhibitory effect at either developmental stage. One exceptional genotype was WYJ3, which showed nitrification-promoting effects at both developmental stages.

Identification of BNI substances

As WYJ7 showed significant nitrification inhibitory ability, whereas WYJ3 showed a promoting effect, we focused on these two varieties to isolate the specific inhibitors involved. Using

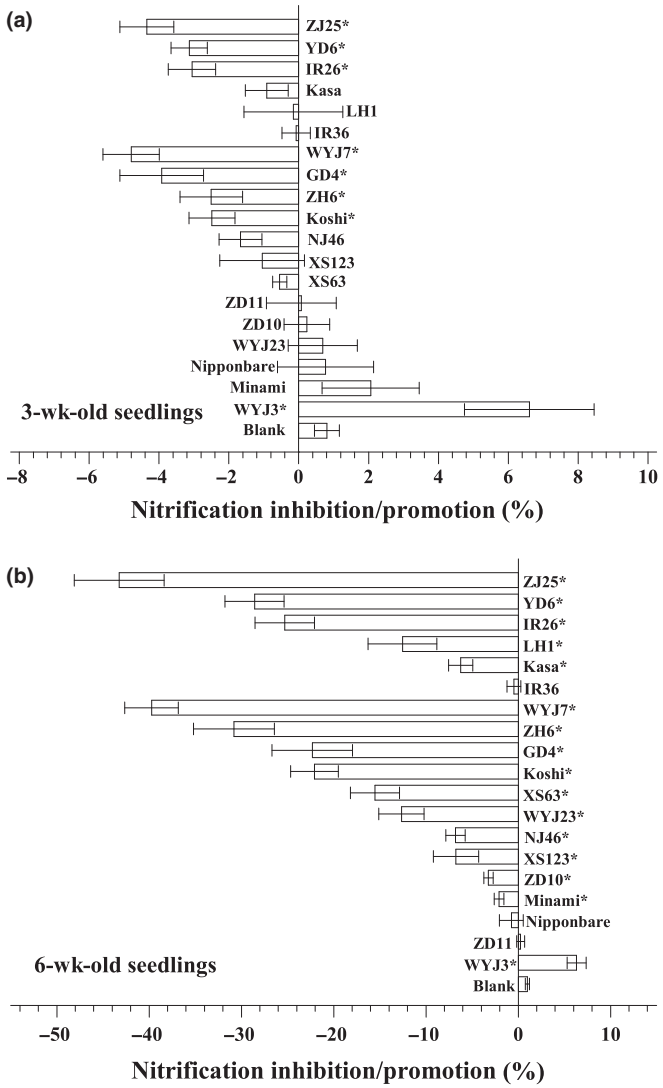

Fig. 1 Nitrification inhibitory effects of root exudates from (a) 3-wk-old rice seedlings and (b) 6-wk-old rice seedlings on *Nitrosomonas europaea*. Abbreviations of variety names are used as in the Materials and Methods section. Negative and positive values represent inhibitory and promoting effects, respectively. Significant difference from Blank (no plants in root exudate collection): *, $P < 0.05$. Data are shown as means ± SE ($n = 3$).

GC-MS analyses, 1,9-decanediol (15.29 min) and shikimic acid (15.90 min) were identified in WYJ7 root exudates and were not found in WYJ3 or 'Blank' samples (Supporting Information Figs S1, S2). Determination with authentic compounds (Table 1) showed that 1,9-decanediol had an inhibitory effect of 21.3% when provided at 20 ng μl^{-1} and of 97.6% when provided at 200 ng μl^{-1}, whereas no effect was detected with shikimic acid. Moreover, 1,9-decanediol was identified in root exudates collected under both natural and aseptic conditions (Figs S1a, S3a). These results indicate that 1,9-decanediol might be the target BNI exuded from rice roots.

Comparative evaluation of BNI substances

In order to evaluate the BNI potential of 1,9-decanediol, its ED_{80} value was compared with that of other inhibitors (Table 2). The

Table 1 Nitrification inhibitory effects of authentic 1,9-decanediol and shikimic acid

Compound	Nitrification inhibition rate (%)	
	20 ng μl^{-1}	200 ng μl^{-1}
1,9-Decanediol	21.29 ± 1.09	97.61 ± 0.22
Shikimic acid	−1.94 ± 0.42	−1.56 ± 0.52

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

Table 2 Comparison of the nitrification inhibitory effect of 1,9-decanediol and that of other synthetic and biological inhibitors

Compound	ED_{80} (ng μl^{-1})
1,9-Decanediol	90
Synthetic nitrification inhibitors	
Nitrapyrin	4
Dicyandiamide	250
2-Amino-4-chloro-6-methylpyrimidine	75
Biological nitrification inhibitors	
Methyl 3-(4-hydroxyphenyl) propionate	30
Methyl-*p*-coumarate	5
Linoleic acid	80
Linolenic acid	80
Methyl linoleate	200

ED_{80} refers to the effective dose (ng μl^{-1}) at which 80% inhibition was observed.

results revealed a similar inhibitory effect of 1,9-decanediol to that of the well-known synthetic nitrification inhibitor AM and the BNIs linoleic acid and linolenic acid. DCD and methyl linoleate were less effective than 1,9-decanediol. Other inhibitors, however, were superior to 1,9-decanediol, including the synthetic inhibitor nitrapyrin and the biological inhibitors methyl 3-(4-hydroxyphenyl) propionate and methyl-*p*-coumarate.

Mode of inhibition of 1,9-decanediol

Ammonia oxidation consists of two processes: one process conducted by the enzyme AMO, in which ammonia is oxidized to hydroxylamine; and a subsequent reaction, catalyzed by HAO, which oxidizes the intermediate hydroxylamine to nitrite (Costa *et al.*, 2006). The standard inhibitor AT is known to block the AMO pathway specifically. Inhibition by AT was alleviated by adding the intermediate hydroxylamine to the reaction mixture, in a range of 77% to 53% (Table 3). Similarly, the inhibition by 1,9-decanediol was alleviated by the addition of hydroxylamine, from 82% to 57%, indicating that 1,9-decanediol specifically blocks the AMO process.

Determination of 1,9-decanediol in root exudates of rice varieties

The detection limit of the GC analysis for 1,9-decanediol was 0.25 ng μl^{-1}. Nine varieties of 6-wk-old seedlings secreted

Table 3 The effect of hydroxylamine on the nitrification inhibition produced by 1,9-decanediol and allylthiourea

Treatment	Inhibition rate (%)	
	Without hydroxylamine	With hydroxylamine
1,9-Decanediol (100 ng μl^{-1})	82.49 ± 0.19	57.20 ± 1.22
Allylthiourea (0.026 ng μl^{-1})	77.32 ± 0.51	53.01 ± 0.20

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

Table 4 Amount of 1,9-decanediol in root exudates of various rice varieties

Variety	Secretion amount (ng g^{-1} root DW d^{-1})
ZJ25	124 ± 48
YD6	102 ± 36
IR26	49 ± 10
LH1	nd
Kasa	nd
IR36	nd
WYJ7	477 ± 133
ZH6	115 ± 46
GD4	112 ± 38
Koshi	73 ± 30
XS63	nd
WYJ23	nd
NJ46	189 ± 58
XS123	nd
ZD10	nd
Minami	34 ± 21
Nipponbare	nd
ZD11	nd
WYJ3	nd

Abbreviations of variety names are used as in the Materials and Methods section. Data are presented as means ± SE ($n = 3$). nd, not detected.

1,9-decanediol (Table 4); all seven varieties that showed inhibitory effects in 3-wk-old seedlings were included in the analysis. Among the 15 varieties whose root exudates showed inhibitory effects, three of five *indica* varieties and six of 10 *japonica* varieties secreted 1,9-decanediol. The amount of 1,9-decanediol in the root exudates of WYJ7 (477 ng g^{-1} root DW d^{-1}) was the highest of all varieties, followed by NJ46 and ZJ25. No 1,9-decanediol was detected in genotypes that displayed no inhibitory abilities, i.e. IR36, Nipponbare, ZD11 and WYJ3.

Nitrogen uptake kinetics of WYJ7 and WYJ3

As WYJ7 and WYJ3 showed opposite effects on nitrification, both 3-wk-old and 6-wk-old seedlings of the two varieties were treated with $^{15}NH_4^+$ or $^{15}NO_3^-$. WYJ7 had higher ammonium uptake in roots and accumulation in shoots, whereas WYJ3 had higher nitrate uptake in roots and accumulation in shoots (Figs 2, 3). Numerically, the data were fitted to the Michaelis–Menten

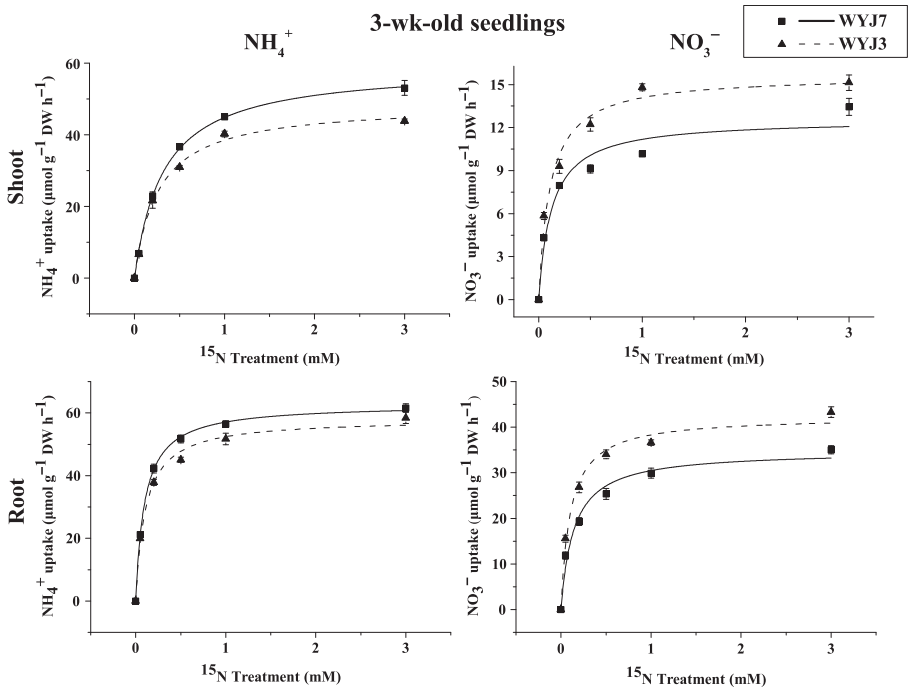

Fig. 2 $^{15}NH_4^+$ and $^{15}NO_3^-$ uptake of WYJ7 (Wuyunjing7) and WYJ3 (Wuyujing3) in 3-wk-old rice seedlings. Data are presented as means ± SE, and fitted to the Michaelis–Menten equation ($n = 3$).

equation, and K_m and V_{max} parameters were derived and are listed in Tables S1 and S2. There were no significant differences ($P < 0.05$) in K_m values for either 3- or 6-wk-old seedlings. V_{max}, ammonium in shoots and ammonium in roots were significantly higher ($P < 0.05$) in WYJ7, and those for nitrate were higher ($P < 0.05$) in WYJ3, for 3-wk-old seedlings; in 6-wk-old seedlings, however, only ammonium uptake and accumulation were significantly different between WYJ7 and WYJ3 ($P < 0.05$).

NUE of rice varieties

Using 6-wk-old seedlings, three of five nitrification inhibitory *indica* varieties (YD6, ZJ25 and IR26) displayed significantly higher (according to Duncan's test, at $P < 0.05$) root NH_4^+ uptake than IR36, which exhibited no nitrification inhibitory effect (Fig. 4a). All *japonica* varieties showed significantly higher (according to Duncan's test, at $P < 0.05$) root NH_4^+ uptake than Nipponbare, ZD11 and WYJ3, which showed no inhibitory or promoting effect. YD6 and WYJ7 were the most efficient genotypes in terms of ammonium absorption among *indica* and *japonica* subspecies, respectively. Root ammonium to nitrate uptake ratios were also explored (Fig. 4b). All *indica* varieties displaying nitrification inhibition had significantly higher root NH_4^+/NO_3^- uptake ratios than IR36, with ZJ25 at the top. All *japonica* varieties displaying nitrification inhibition, except for ZD10 and Minami, had significantly higher root NH_4^+/NO_3^- uptake ratios than Nipponbare, ZD11 and WYJ3, with Koshi ranking first, followed by ZH6 and NJ46.

The data on the nitrification inhibitory effects, root ammonium uptake, root ammonium to nitrate uptake ratios and 1,9-decanediol amounts in root exudates of all 19 rice varieties were subjected to a Pearson correlation analysis (Table 5). Nitrification inhibitory effects and root ammonium to nitrate uptake ratios displayed the highest correlation coefficient (0.753), revealing maximal linearity between the two factors. Four factors were correlated pairwise at the 0.01 significance level, indicating that each had a significantly positive relationship.

Discussion

Nitrification inhibition can enhance fertility and primary production in agroecosystems in a sustainable manner. Plant-derived BNIs are substances that are cost-effective, environmentally friendly and can be functionally highly effective at controlling soil nitrification (Subbarao *et al.*, 2008). Cereal crops, especially rice, are not very efficient at absorbing soil N, as the absorption ratio, in relation to the amount of N applied as fertilizer, is typically only 30–40% (Wang *et al.*, 2012). Thus, BNI in rice provides an important approach towards improving NUE and reducing N loss from paddy fields. We also focused on rice because it has the smallest genome size among cereal crops, and whole-genome sequencing in both *indica* and *japonica* subspecies has been completed, which may facilitate the development of genetic tools for the improvement of NUE via an optimization of nitrification inhibition in the future.

BNI abilities of rice varieties

The original selection of rice varieties was from the Taihu Lake region, East China, and 10 varieties (YD6, WYJ7, GD4, NJ46, XS123, XS63, ZD11, ZD10, WYJ23 and WYJ3) originated from this area. To expand the study and enhance its general utility, other varieties were included from central and southern China (ZJ25, LH1, ZH6), India (Kasa), Japan (Koshi, Nipponbare, Minami) and the Philippines (IR26, IR36). There is a

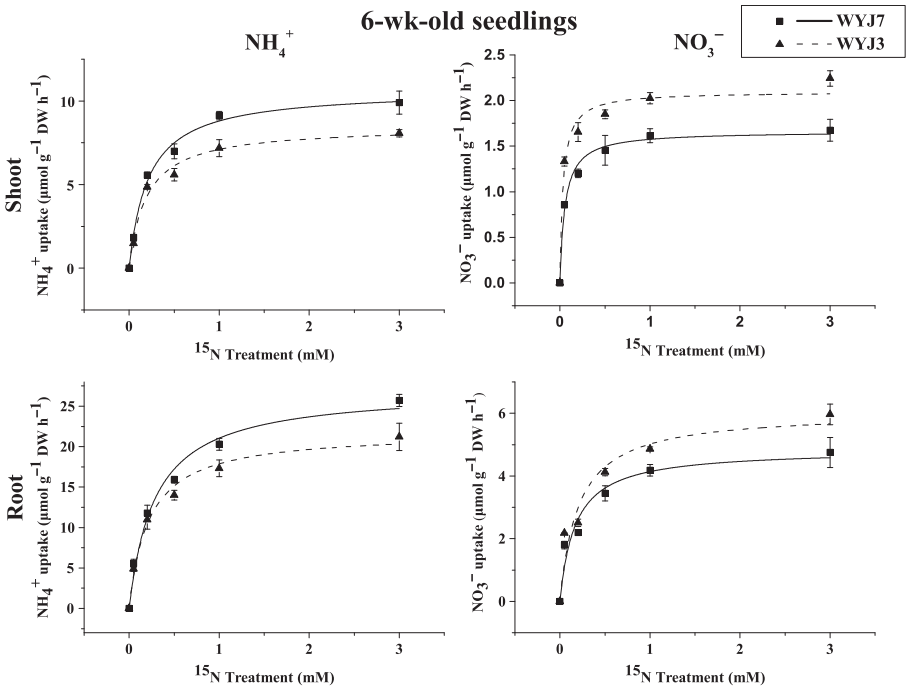

Fig. 3 $^{15}NH_4^+$ and $^{15}NO_3^-$ uptake of WYJ7 (Wuyunjing7) and WYJ3 (Wuyujing3) in 6-wk-old rice seedlings. Data are presented as means ± SE, and fitted to the Michaelis–Menten equation ($n = 3$).

growing demand for *japonica* rice in China (Hansen *et al.*, 2002), and growth security as well as yields of *japonica* rice exceed those of *indica* varieties in the lower reaches of the Yangtze River, East China (Zhang *et al.*, 2013). Thus, 13 of 19 varieties chosen were *japonica* varieties. Some varieties have been reported to have higher NUEs (YD6, Kasa, WYJ7, GD4, Koshi, WYJ23) and others lower ones (IR36, Nipponbare, WYJ3), according to previous studies (Li *et al.*, 2007; Namai *et al.*, 2009; Zhang *et al.*, 2009; Shi *et al.*, 2010; Chen *et al.*, 2015). NUE was critical, as the enhancement of NUE is one of the principal aims motivating the study of BNI in rice.

Early examinations have shown that the majority of varieties selected here (15 of 19) possess nitrification inhibitory abilities in their root exudates, although there were large differences in inhibitory potency among genotypes. Tanaka *et al.* (2010), however, suggested that less than one-half of the rice varieties examined by their group had BNI abilities; this may be attributable to the fact that the trap solution used in their study was NH_4Cl, thus producing, by necessity, a higher 'Blank' value (obscuring the cut-off of a 15% BNI effect), whereas we used Milli-Q water, which produces a much lower 'Blank' control. ZJ25 and WYJ7 were most effective among *indica* and *japonica* varieties, respectively. Six-week-old seedlings were more effective than 3-wk-old seedlings, whereas previous research on rice has suggested that younger seedlings may have higher BNI abilities, normalized to root weight (Tanaka *et al.*, 2010). This discrepancy might be caused by the difference in rice varieties chosen and in the growth and collection conditions. The relationship between BNI and growth stage is important as it can guide the design of fertilization timing protocols, but field experiments are needed to confirm this in a realistic setting. Both *indica* and *japonica* subspecies showed BNI abilities, in accordance with Tanaka's results, indicating that both *indica* and *japonica* should be kept in focus when breeding varieties. Extraordinarily, root exudates of WYJ3 were found to promote nitrification, and this discovery differs from the work of others (Subbarao *et al.*, 2007; Tanaka *et al.*, 2010). None of these previous studies showed rice to possess nitrification-promoting characteristics. This may be attributable to the choice of different varieties for the respective studies. By comparing the allelochemicals in the root exudates with the inhibitory varieties, 1,9-decanediol was identified as the chief nitrification inhibitory allelochemical.

1,9-Decanediol, a newly discovered nitrification inhibitor in rice root exudates

No specific BNIs in rice have been reported in the literature hitherto, and this is the first report on the biological activity of 1,9-decanediol. This compound is an organic synthesis intermediate in the reduction of 1,2-epoxides (Dragovich *et al.*, 1995), and isomers of the compound are used in industrial production. 1,10-Decanediol is an intermediate in pharmaceutical production (Li *et al.*, 1999), and 1,2-decanediol is used in cosmetics production (Gerhard *et al.*, 2008). Other, similar, fatty alcohols are principally employed to synthesize surfactants used in industry (Elsner *et al.*, 2012; Zheng *et al.*, 2012). As such, it is hoped that the economic cost of producing this kind of fatty alcohol would be lower than that for other BNIs previously reported. 1,9-Decanediol has a similar nitrification inhibitory effect to the synthetic inhibitor AM, and the biological inhibitors linoleic acid and linolenic acid, examined in extracts from shoot tissues of the pasture grass *Brachiaria humidicola* (Subbarao *et al.*, 2008). It is superior in efficacy to the widely used inhibitor DCD and the biological inhibitor methyl linoleate (Subbarao *et al.*, 2008), but less effective than the synthetic inhibitor nitrapyrin (Dow Chemical, Midland, MI, USA) and the biological inhibitors methyl 3-

(4-hydroxyphenyl) propionate from root exudates of *Sorghum* (Zakir *et al.*, 2008) and methyl-*p*-coumarate from root tissue extracts of *Brachiaria humidicola* (Gopalakrishnan *et al.*, 2007). Thus, although not as strong as the allelochemicals exuded from some other grasses, 1,9-decanediol is a potent BNI.

Using Subbarao's method, we discovered that the AMO process is the inhibitory target of 1,9-decanediol. Most of the synthetic inhibitors, such as nitrapyrin, DCD and DMPP, inhibit the *Nitrosomonas* genus by suppressing the AMO pathway, but have no effect on the HAO pathway. Some BNIs inhibit *Nitrosomonas* by blockage of both the AMO and HAO pathways (Subbarao *et al.*, 2015). Others, such as methyl 3-(4-hydroxyphenyl) propionate (Zakir *et al.*, 2008), inhibit only the AMO pathway, as does 1,9-decanediol. AMO has a broad substrate range and the inhibitory effects of many compounds are the result of competition for the active site of the enzyme (McCarty, 1999); 1,9-decanediol has emerged as one of these competitors.

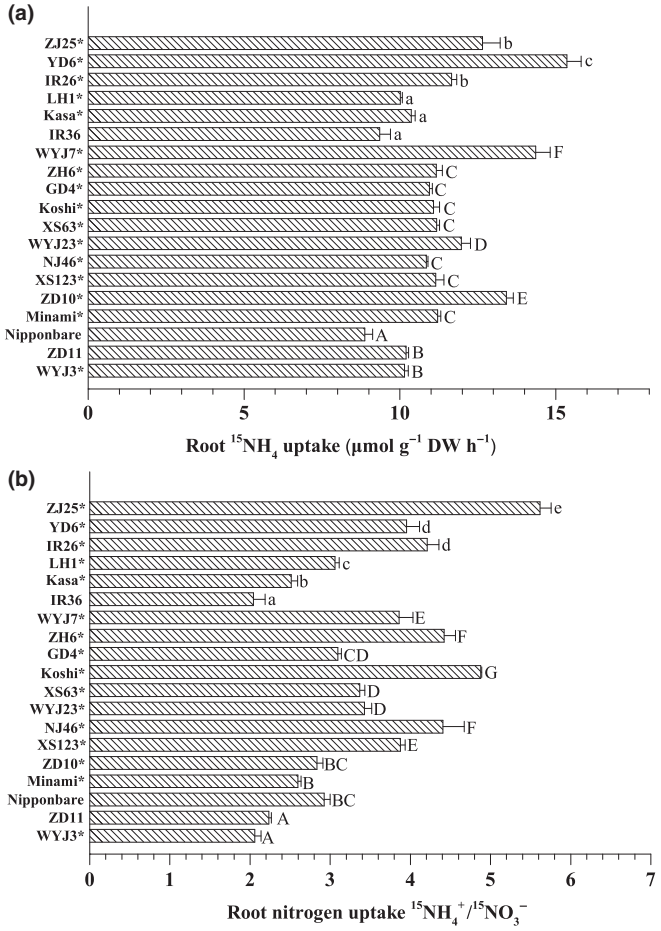

Fig. 4 (a) Root $^{15}NH_4^+$ uptake of 19 rice varieties in 6-wk-old seedlings. (b) Ratio of root $^{15}NH_4^+$ to $^{15}NO_3^-$ uptake of 19 rice varieties in 6-wk-old seedlings. Abbreviations of variety names are used as in the Materials and Methods section. Data are presented as means ± SE ($n = 3$). Lower-case and upper-case letters represent differences (Duncan's test, at $P < 0.05$) of *indica* and *japonica* rice varieties, respectively. *Significant nitrification inhibitory/promoting effects in root exudates of 6-wk-old seedlings.

No 1,9-decanediol was detected in genotypes which possessed no nitrification inhibitory ability, and most inhibitory varieties had 1,9-decanediol in their root exudates. A few inhibitory varieties, however, had no 1,9-decanediol, and their effective allelochemicals remain to be identified. WYJ7, which displayed the highest nitrification inhibitory ability among *japonica* varieties, secreted the greatest amount of 1,9-decanediol of all genotypes, underscoring the importance of the compound.

1,9-Decanediol thus serves as a BNI exuded from rice roots. According to the data obtained under both natural and aseptic conditions, we could clearly determine that 1,9-decanediol derives from rice root exudates, which is consistent with our previous study indicating that the denitrification stimulators oleamide and erucamide can be identified from duckweed root exudates under both conditions (Lu *et al.*, 2014). However, we are unable to confirm whether or not microorganisms may also produce this compound, or degrade it to some extent, which is beyond the research in this study. When using water as the trap solution in natural collection and nutrient solution in aseptic collection, similar results were obtained.

Nitrification inhibition, BNIs and NUE

One of the primary components of NUE is the efficiency of N uptake, which is interpreted as the N uptake efficiency (Hirel *et al.*, 2007). Therefore, NUE was assessed here using a ^{15}N-labeling method, in accordance with the fact that N uptake varies strongly between varieties and between different growth stages. Such real-time data are essential for the determination of the relationship between NUE and nitrification inhibitory effects.

Indica and *japonica* varieties were analyzed separately, as most *indica* genotypes have higher nitrate absorption activity (Hu *et al.*, 2015). Our results of ammonium uptake, however, did not

Table 5 Correlation value according to Pearson analysis of four factors: nitrification inhibitory effect, root ammonium uptake, root ammonium to nitrate uptake ratio and 1,9-decanediol amount in root exudates

	Pearson correlation coefficient			
Item	Inhibitory effect	Root NH_4^+ uptake	Root NH_4^+/NO_3^- uptake	1,9-Decanediol
Inhibitory effect	1	0.599**	0.753**	0.594**
Root NH_4^+ uptake	0.599**	1	0.441**	0.456**
Root NH_4^+/NO_3^- uptake	0.753**	0.441**	1	0.380**
1,9-Decanediol	0.594**	0.456**	0.380**	1

**Significance at the 0.01 level.

show group differences between *indica* and *japonica* subspecies. In China, *japonica* rice was traditionally grown and consumed primarily in the northern provinces, whereas *indica* rice was dominant in the south (Hansen *et al.*, 2002), and, world-wide, *indica* varieties are grown in tropical and subtropical Asia, whereas *japonica* varieties are primarily grown in temperate regions, such as Japan and northern China (Muthayya *et al.*, 2014). YD6 and WYJ7 are the most efficient genotypes in terms of root ammonium uptake among *indica* and *japonica* varieties, respectively. When examining the root ammonium to nitrate uptake ratio, the varieties that stood out were ZJ25 and Koshi. Given that ZJ25 and WYJ7 were also the most effective varieties with respect to nitrification inhibition, a close relationship between nitrification inhibitory ability and intrinsic NUE emerges.

Further analysis by the Pearson correlation method revealed that any two of the factors (a) nitrification inhibition ability, (b) root ammonium uptake, (c) root ammonium to nitrate uptake ratio and (d) 1,9-decanediol amount in root exudates were positively correlated, suggesting that nitrification inhibition by root exudates is closely coupled to ammonium-use efficiency and ammonium preference, and that 1,9-decanediol is a crucial BNI in rice root exudates which, in turn, is closely linked to NUE. Numerically, nitrification inhibition and root ammonium to nitrate uptake ratio were correlated most strongly. Thus, nitrification inhibitory rice varieties might possess a higher innate ammonium preference, manifesting in higher ammonium uptake as well as higher NUE. Some genetic regions have been linked to BNI activity (Subbarao *et al.*, 2015). Thus, it will be imperative to explore the relationship between intrinsic NUE in rice varieties and nitrification inhibitory ability by genetic approaches in the future.

The application of 1,9-decanediol could increase NUE in fertilized paddy soils, significantly reducing run-off and leaching, and pollution associated with surplus nitrate produced in the nitrification process. In addition, the high ammonium-use efficiencies of the varieties tested in our study might be of significance in the context of adaptation to climate change. Atmospheric CO_2 has increased from 280 ppm to 379 ppm since the Industrial Revolution; elevated CO_2 reduces stomatal opening and transpiration, and the decreased transpiration can decrease N uptake (Shimono & Bunce, 2009); total protein and N content in plants generally decline under elevated CO_2, especially when nitrate is the N source, as nitrate assimilation is slower under high CO_2, and deleterious consequences on food quality have been recorded (Bloom *et al.*, 2014). Thus, food nutritional quality is threatened under increased CO_2 in nitrate-based environments. As the assimilation of ammonium entails lower energy costs compared with the assimilation of nitrate (Chen *et al.*, 2013), crops with ammonium preference are superior in this important regard, and the enhancement of crop ammonium-use efficiency, even though N-source preference is a complex trait (Britto & Kronzucker, 2002, 2013), may carry benefits in the context of adaptation to atmospheric change, especially so in the tropical and subtropical regions of the developing world in which billions of people depend on rice crops for their sustenance.

Conclusions

The release of nitrification inhibitors from rice roots represents a hitherto overlooked phenomenon in the regulation of the N cycle in paddy fields. The identification and characterization of a fatty alcohol compound from rice root exudates as a nitrification inhibitor provides a specific case towards a better understanding of BNI in rice. BNI and the exudation quantity of nitrification inhibitors in rice are positively correlated with rice ammonium uptake and preference, bringing into focus BNI benefits for rice N use which can be exploited by breeding programs to develop improved cultivars. This work will pave the way for future strategies to improve crop NUE and reduce N loss from the field, benefiting both the efficiency of agricultural production and the environment.

Acknowledgements

This work was funded by grants from the Strategic Priority Research Program (B) – 'Soil–microbial system function and regulation' of the Chinese Academy of Sciences (XDB15030100), the National Natural Science Foundation of China (31501836) and the Jiangsu Municipal Natural Science Foundation (BK20151053).

Author contributions

L.S., Y.L. and W.S. designed the experiments. L.S. carried out the experiments and performed the analyses. F.Y. helped with the cultivation of bacteria. L.S., Y.L., H.J.K. and W.S. substantially contributed to the interpretation of the results and writing of the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Chromatogram of root exudates of WYJ7 (Wuyunjing7) and WYJ3 (Wuyujing3).

Fig. S2 Mass spectroscopy data of 1,9-decanediol and shikimic acid.

Fig. S3 Chromatogram of root exudates of WYJ7 (Wuyunjing7) collected under aseptic conditions and mass spectroscopy data of 1,9-decanediol.

Table S1 Michaelis–Menten equation parameters, K_m and V_{max}, of WYJ7 (Wuyunjing7) and WYJ3 (Wuyujing3) in 3-wk-old seedlings

Table S2 Michaelis–Menten equation parameters, K_m and V_{max}, of WYJ7 (Wuyunjing7) and WYJ3 (Wuyujing3) in 6-wk-old seedlings

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