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

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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 are 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 Wuyujing7 (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 nitrogen-use efficiency (NUE) was determined using a ^{15}N-labeling method. Correlation analyses indicate 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 insight into the plant–bacterial interactions involved in the soil N cycle, and improve our understanding of BNI capacity of rice in the context of NUE.

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

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 nitrogen, 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 due to nitrification and consequent denitrification (Abbasi & Adams, 1998). Thus, suppressing nitrification and maintaining N fertilizer in the reduced form is a critical step to increasing fertilizer-N retention in soils and to improving nitrogen-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 due to 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 due to the fact that very high DCD concentrations are needed to achieve desired results. Moreover, as DCD is water-soluble, it itself easily leaches out of crop root zones, i.e. the very zones where 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 can be 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*, were 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 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 worldwide 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 satisfy increasing demand (Zhao *et al*., 2014). While 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) 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 nitrification intensity furthermore 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 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 of ammonium rather than nitrate as a 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 for 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: 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 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 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 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 25°C : 28°C temperature regime, 65% humidity, 14 h : 10 h, light : dark photoperiod, 400 μmol m^{-2} s^{-1} light intensity 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

Due to 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 Milli-Q water. Specifically, the roots were immersed gently in water, while shoots were held and supported with sterilized sponges. Mechanical damage to roots could lead to significant alteration of both 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

filtrated using 0.45-μm filter membranes to remove microorganisms; filtrated samples were pre-treated immediately or stored in 4°C until evaporation within 3 d. The samples were evaporated to dryness using a rotary evaporator (Eyela, N-1100D-WD, Japan) at 40°C, then resuspended in HPLC methanol and evaporated to dryness again, during which a freeze drier was used to guarantee removal of water after evaporation. The residues remaining in the round-bottom flasks were redissolved in 10 ml methanol and stored at -20°C, and, before measurement, 1-ml samples were centrifuged to dryness and redissolved in 5 μl dimethyl sulphoxide (DMSO).

For the aseptic experiment, an *in-situ* cultivation and collection was 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 identification of BNI substances.

Determination of nitrification inhibitory effect of root exudates

Nitrosomonas europaea (ATCC 19718) was obtained from NITE, Biological Resource Center (NBRC), Japan. The strain was grown aerobically in HEPES medium, as recommended by the 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 HEPES medium using an incubation shaker (set at 200 rpm, 30°C). A 7-d-old culture mix was centrifuged and re-suspended in fresh HEPES medium, and adjusted to an OD_{600} of 1.0 using a spectrophotometer (SmartSpec plus, Bio-Rad).

In the assay, a mixture of 195 μl sterilized Milli-Q water, 5-μl root-exudate samples (in DMSO), 100 μl HEPES medium, and 200 μl resuspended cells, was added to a 1.5-ml tube and was incubated at 25°C for 2 h. The reaction was stopped by adding 20 μl 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. 2-ml root-exudate samples in methanol from each variety were evaporated to dryness under N and derivatized with 500 μl BSTFA in 60°C for 2 h. The mixture was evaporated to dryness again,

redissolved in 100 µl 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. 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 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 NIST library. 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), dicyandiamide (DCD), 2-amino-4-chloro-6-methylpyrimidine (AM), methyl 3-(4-hydroxyphenyl) propionate, linoleic acid, linolenic acid, and linolenic acid were obtained from ©Sigma-Aldrich (St Louis, MO, USA), 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 above, 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 1,9-decanediol affects, an experiment was performed incubating the bacteria in the presence and absence of hydroxylamine, as described by Subbarao *et al*. (2007). 100 µl 1 mM hydroxylamine was added into the assay mixture before incubation, and, except for 100 ng $µl^{-1}$ 1,9-decanediol, 0.026 ng $µl^{-1}$ of the standard inhibitor AT was used, which was reported to have an 80% inhibitory effect.

Determination of 1,9-decanediol in rice root exudates

Two-ml root-exudate samples (in methanol) of 6-wk-old seedlings of 19 rice varieties were evaporated to dryness under N and derivatized with 500 μl BSTFA in 60°C for 2 h. The mixture was then evaporated to dryness again, redissolved in 100 μl hexane, and subjected to GC. The 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 an FID detector. Splitless injection was performed at 250°C; the oven temperature was initially 80°C and was raised to 160°C at a rate of 20°C min^{-1} and 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 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 measurement of N absorption kinetics. Following 2-d 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 $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ were added. In addition, 6-wk-old seedlings of all rice varieties were treated with 0.5 mM $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$, respectively. After 3-h treatment, shoots and roots were washed, separated, freeze-dried, and then ground into powder and subjected to a Thermo Flash 2000 analyzer hyphenated with a Thermo Fisher Delta-V isotope Ratio MS, to determine total N and ^{15}N abundance.

Statistical analyses

The data were subjected to SPSS 18.0 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 figure and table legends.

Results

Nitrification inhibitory effects of rice root exudates

Seven of 3-wk-old seedlings and 15 of 6-wk-old seedlings out of 19 rice varieties had significant nitrification inhibitory effects compared to '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 at both growth periods. ZJ25 and WYJ7, at 6 wk of age, were the most effective varieties regarding 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

Since WYJ7 had significant nitrification-inhibitory ability, while WYJ3 showed a promoting effect, we focused on these two varieties to isolate the specific inhibitors involved. Using 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 WYJ3 or 'Blank' samples (Supporting Information Figs S1, S2). Determination with authentic compounds (Table 1) showed 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}, while 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 biological nitrification inhibitor 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 results revealed a similar inhibitory effect of 1,9-decanediol to that of the well-known synthetic nitrification inhibitor AM, and the biological nitrification inhibitors linoleic acid and linolenic acid. Dicyandiamide (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; the subsequent reaction is 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 77–53% (Table 3). Similarly, the inhibition by 1,9-decanediol was alleviated by 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-decanedil was 0.25 ng μl^{-1}. Nine varieties of 6-wk-old seedlings secreted 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 out of five *indica* varieties and six out of 10 *japonica* varieties secreted 1,9-decanediol. The amount of 1,9-decanediol in 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 had 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, while WYJ3 had higher nitrate uptake in roots and accumulation in shoots (**Figs 2, 3**). Numerically, data were fitted to the Michaelis–Menten 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- and 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 out of five nitrification-inhibitory *indica* varieties (YD6, ZJ25 and IR26) displayed significantly higher (as per 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 (as per 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 inhibition effects, root ammonium uptake, root ammonium to nitrate uptake ratios, and 1,9-dacanediol amounts in root exudates of all 19 rice varieties were subjected to a Pearson correlation analysis (Table 5). Nitrification inhibition 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 way. Plant-derived biological nitrification inhibitors 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 have been completed, which may facilitate the development of genetic tools for improving 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 then included from Central and Southern China (ZJ25, LH1, ZH6), India (Kasa), Japan (Koshi, Nipponbare, Minami), and the Philippines (IR26, IR36). There is a growing demand for *japonica* rice in China (Hansen *et al*., 2002), and growth security as well as yields of *japonica* rices exceed those of *indica* varieties in the lower reaches of the Yangtze River, East China (Zhang *et al*., 2013). Thus, 13 out 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 our principal aims motivating the study of biological nitrification inhibition in rice.

Early examinations had shown the majority of varieties we selected (15 out of 19) to possess nitrification inhibitory abilities in their root exudates, although there were large differences in inhibition potency among genotypes. Tanaka *et al*. (2010), however, suggested less than 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 that study was NH_4Cl, thus producing, by necessity, a higher 'Blank' value (obscuring the cut-off of a 15% BNI effect), while 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 had suggested younger seedlings may have higher BNI abilities, normalized to root weight (Tanaka *et al*., 2010). This discrepancy might be due to the difference in rice varieties chosen, and in growth and collecting conditions. The relationship between BNI and growth stages is important, as it can guide the design of fertilization timing protocols, but field experiments will be 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 its isomers of the compound, however, are used in industrial production. 1,10-decanediol is an intermediate in pharmaceutical production (Li *et al*., 1999), and 1,2-decanediol can be used in cosmetics production (Gerhard *et al*., 2008). Other, similar, fatty alcohols are principally used 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 extract 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 (produced by Dow Chemical) 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, while not as strong as the allelochemicals exuded from some other grasses, 1,9-decanediol is a potent biological nitrification inhibitor.

Using Subbarao's method, we discovered the AMO process to be the inhibition 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, like 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 due to competition for the active site at the enzyme (McCarty, 1999), 1,9-decanediol emerges as one of them.

No 1,9-decanediol was detected in genotypes which possessed no nitrification-inhibiting abilities, and most inhibiting varieties had 1,9-decanediol in their root exudates. A few inhibiting varieties, however, had no 1,9-decanediol, and their effective allelochemicals remain to be identified. WYJ7,

which displayed the highest nitrification-inhibiting ability among *japonica* varieties, secreted the highest amount of 1,9-decanediol of all genotypes, underscoring the importance of the compound.

1,9-Decanediol, thus, serves as a biological nitrification inhibitor, 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 that the denitrification stimulators oleamide and erucamide can be identified from duckweed root exudates under both conditions (Lu *et al*., 2014). But we are not able to confirm whether or not microorganisms may also produce this compound, or degrade it to some extent, which has beyond our 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 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 to determining 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 show group differences between *indica* and *japonica* subspecies. In China, *japonica* rice was traditionally grown and consumed primarily in the northern provinces, while *indica* rice was dominant in the south (Hansen *et al*., 2002) and, worldwide, *indica* varieties are grown in tropical and subtropical Asia, while *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 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 factors of (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 biological nitrification inhibitor 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 abilities by genetic approaches in the future.

The application of 1,9-decanedilol could increase NUE in fertilized paddy soils, significantly reducing run-off and leaching and pollution associated with surplus nitrate produced in the nitrification process. Additionally, 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 then decrease N uptake (Shimono & Bunce, 2009); total protein and N content in plants generally decline under elevated CO_2 atmospheres, 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. Since the assimilation of ammonium entails lower energy costs compared to 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 where 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 inhibitor 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.

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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 interpreting the results and writing the paper. **[Author, please note initials have been changed for consistency with the title page. Please check that this is ok.]**

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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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Fig. 1 Nitrification inhibition 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 inhibition and promotion effects, respectively. Significant difference from Blank (no plants in root exudates collection): *, $P < 0.05$. Data are shown as means ± SE ($n = 3$).

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 ($n = 3$).

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 ($n = 3$).

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 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.

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 nitrification-inhibitory effects of 1,9-decanediol and those 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.

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

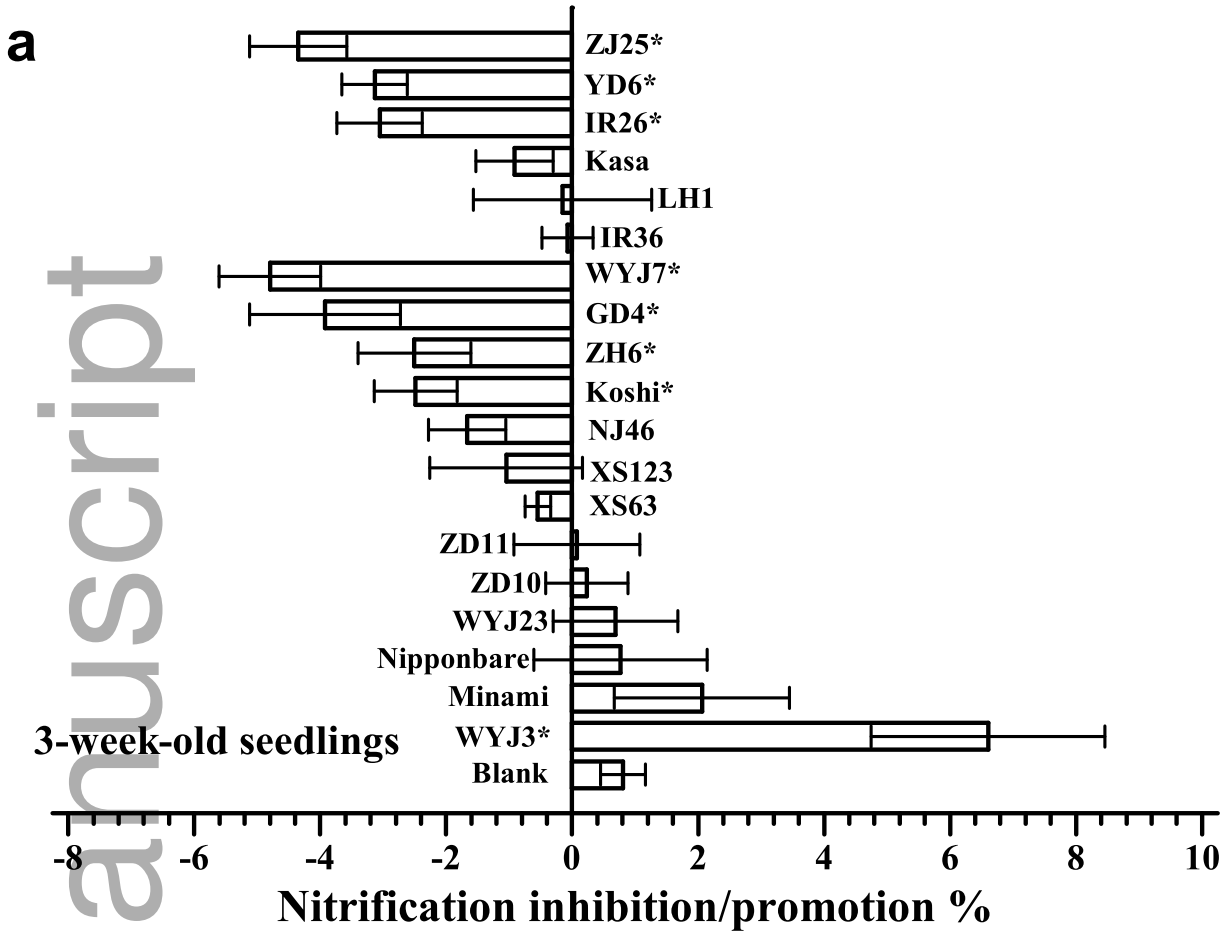
Abbreviations of variety names are used as in the Materials and Methods section. Data are presented as

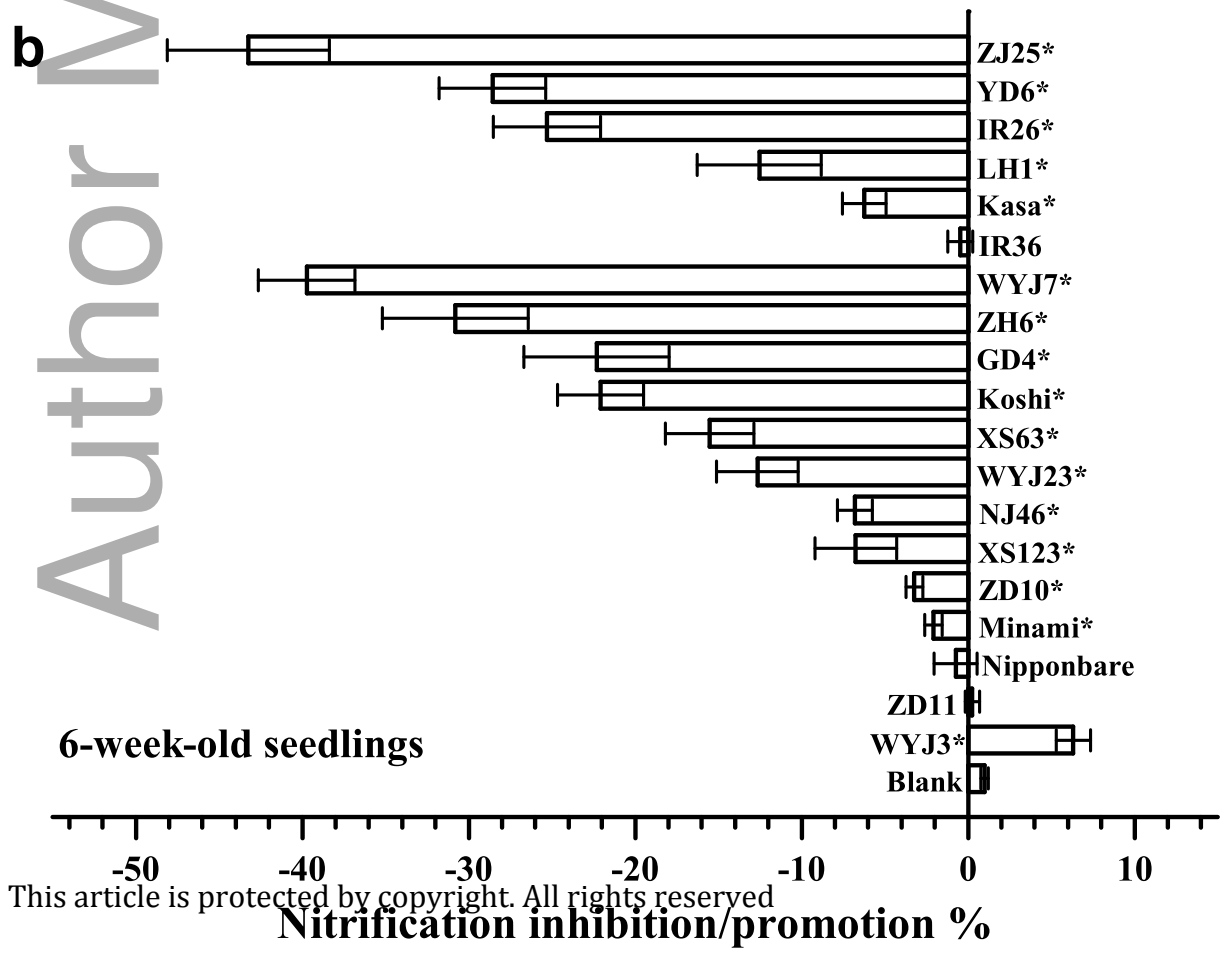
means ± SE (n = 3). nd, not detected.

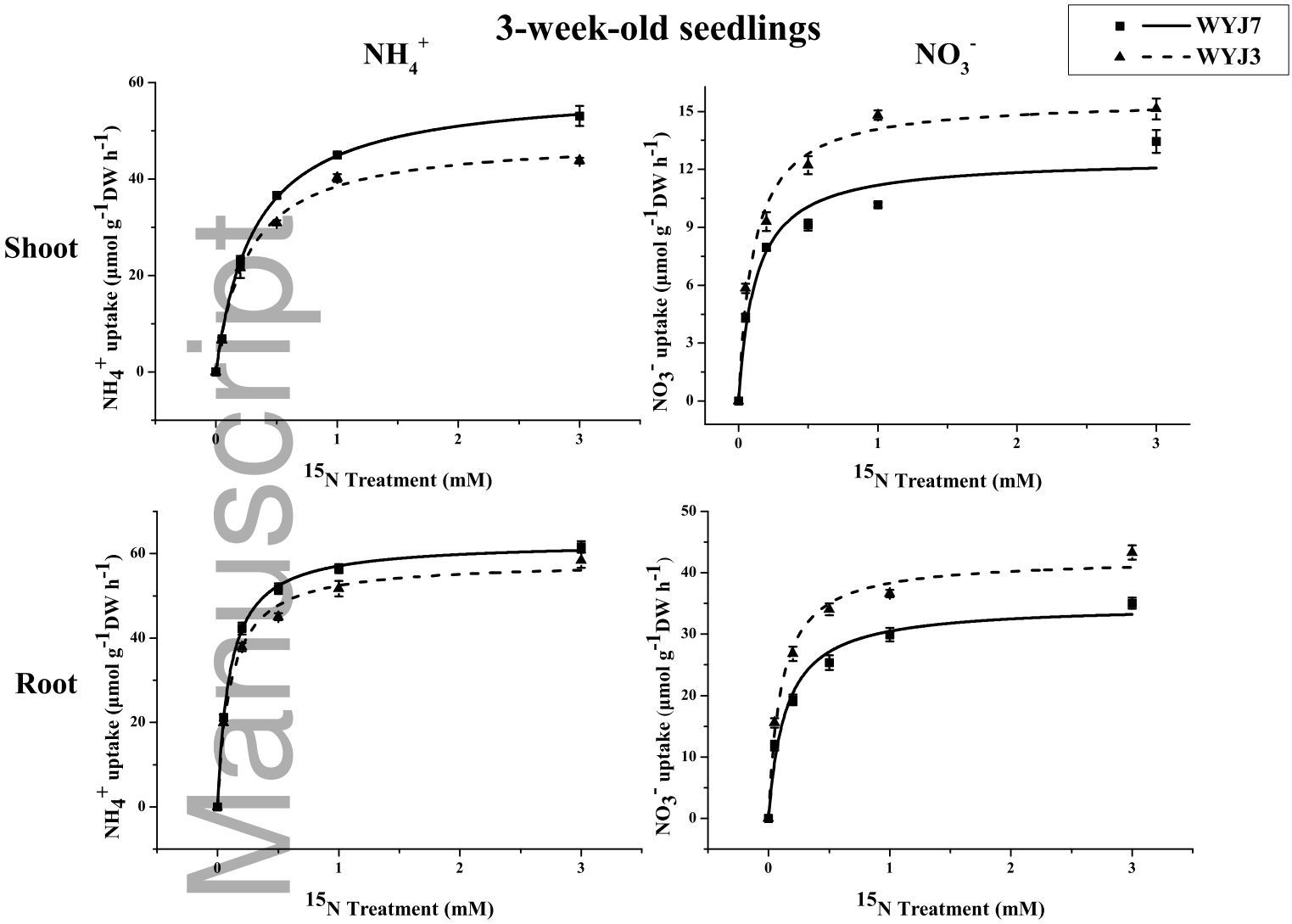
Table 5 Correlation value as per Pearson analysis of four factors: nitrification inhibition effect, root ammonium uptake, root ammonium to nitrate uptake ratio, and 1,9-dacanediol amount in root exudates

Item	Pearson correlation coefficient			
	Inhibition effect	Root NH_4^+ uptake	Root NH_4^+/NO_3^- uptake	1,9-Decanediol
Inhibition 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.







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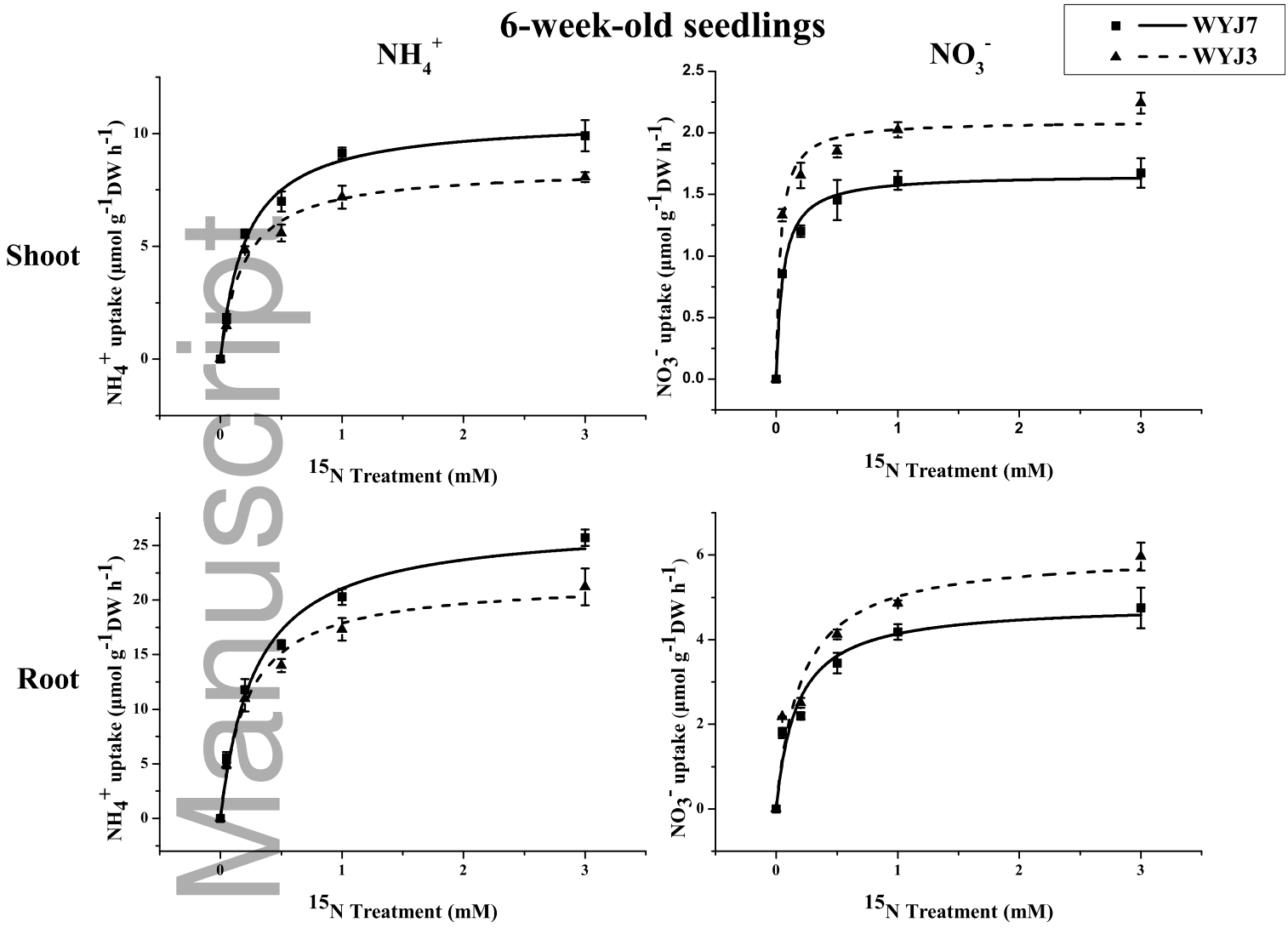

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