Nitrification inhibition activity, a novel trait in root exudates of rice

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Abstract

Background and aims
Nitrification is an important process in soil–plant systems for providing plant-available nitrate ($\text{NO}_3^-$). However, $\text{NO}_3^-$ is less stable in soils compared with ammonium ($\text{NH}_4^+$) and is more easily lost through leaching, runoff or denitrification. This study tested whether biological nitrification inhibition (BNI) activity is present in the root exudates of rice ($\text{Oryza sativa}$) and also the extent of variation between different genotypes.

Methodology
The BNI activity of root exudates was estimated by a bioluminescence assay using a recombinant $\text{Nitrosomonas europaea}$ strain. Afterwards, the effect of a single application of concentrated root exudates and that of exudates deposited in the rhizosphere soil was tested on BNI using soil incubation. Soil was added with ($\text{NH}_4$)$_2\text{SO}_4$ and water to reach 60 % of the water-holding capacity and incubated at 30 $\degree$C for different periods. Amounts of $\text{NH}_4^+$ and $\text{NO}_3^-$ were determined using a continuous-flow auto-analyser.

Principal results
In an initial screening experiment, BNI activity in the exudates of 36 different rice genotypes was evaluated using a bioassay based on a recombinant $\text{Nitrosomonas}$ strain. Significant genotypic variation was detected with the upland cultivar IAC25 demonstrating consistently high BNI activity, while modern lowland varieties like Nipponbare or IR64 exhibited lower activity. Subsequent experiments ruled out the possibility that BNI activity is simply due to non-specific (solute) leakage from roots. Soil incubation studies with concentrated root exudates of IAC25 showed significant reductions in $\text{NO}_3^-$ formation. This effect was confirmed by detecting lower $\text{NO}_3^-$ levels in incubation experiments using rhizosphere soil obtained from IAC25.

Conclusions
Our results provide first evidence that root exudates of rice can reduce nitrification rates in soil. Having shown this for a model crop, rice, offers possibilities for further exploitation of this phenomenon through molecular and genetic tools.

Introduction
The process of nitrification, in which ammonia ($\text{NH}_3$) is converted to nitrate ($\text{NO}_3^-$), is a key soil process that provides plant roots with the nitrogen (N) form that is preferentially taken up. However, when nitrification occurs rapidly, $\text{NO}_3^-$ supply may exceed plant demand. Such excess soil $\text{NO}_3^-$ is easily lost because $\text{NO}_3^-$ is less tightly bound to the soil compared with $\text{NH}_4^+$. Losses of the highly mobile $\text{NO}_3^-$ due to leaching,
runoff or denitrification can amount to 60% of applied fertilizer N in coarse-textured soils (Gaines and Gaines, 1994). Such a reduction in N use efficiency represents a large economic cost, estimated to be around US$15 billion annually (Subbarao et al., 2006). In addition, off-site movement of $\text{NO}_3^-$ can also cause environmental problems such as groundwater contamination and eutrophication of surface water. Nitrogen lost by gaseous emission, as the powerful greenhouse gas $\text{N}_2\text{O}$, contributes to the climate change phenomenon.

In general, the regulation of nitrification to a level in which nitrification rates are in synchrony with $\text{NO}_3^-$ uptake by plants may alleviate these problems and help achieve a more sustainable modern agriculture.

Nitrification can be generalized as a two-step process wherein the initial oxidation of $\text{NH}_3$ to $\text{NO}_2^-$, considered as the rate-limiting step, is followed by the oxidation of $\text{NO}_2^-$ to $\text{NO}_3^-$. Historically, the former step was believed to be carried out solely by chemolithoautotrophic ammonia-oxidizing bacteria (AOB), represented by the genus *Nitrosomonas* spp., and the latter step by chemolithoautotrophic nitrite-oxidizing bacteria, represented by the genus *Nitrobacter*. However, recent evidence, reviewed by Hayatsu et al. (2008), suggests that the process is much more complex, involving autotrophic *Archaea* nitrifiers, heterotrophic nitrifiers and anammox bacteria, which convert ammonium and nitrite into N gas under anaerobic conditions (Mulder et al., 1995). Adding to this complexity is the potential for plants to stimulate or inhibit each of these groups specifically or as a whole.

Numerous compounds that interfere with nitrification have been identified, but very few of these have been used successfully as synthetic nitrification inhibitors in agriculture and horticulture (Slangen and Kerkhoff, 1984; Zerulla et al., 2001). The additional cost and variability in the effectiveness of synthetic inhibitors because of site-specific factors, such as soil type and weather conditions (Goertz, 1994), have limited their wide adoption. Recent research suggests that plant root exudates can inhibit nitrification in soil, as shown for the tropical grass *Brachiaria humidicola* (Subbarao et al., 2009). This opens possibilities for using biological nitrification inhibition (BNI) as a low-cost in situ biological alternative. The BNI potential of root exudates has often been estimated by using a recombinant *Nitrosomonas europaea* strain (harbouring luxAB genes from *Vibrio harveyi*) in a bioluminescence assay (Iizumi et al., 1998; Ishikawa et al., 2003). This methodology has been used extensively in characterizations of BNI-related processes, including the identification of putative specific inhibitor compounds in roots of *B. humidicola* and sorghum (*Sorghum bicolor*) (Gopalakrishnan et al., 2007; Zakir et al., 2008; Subbarao et al., 2009).

Biological nitrification inhibition has first been described in detail in *B. humidicola*; however, this is a rather minor crop that is typically grown with little or no application of N fertilizers. It is therefore highly desirable to identify BNI in a crop that (i) is of major importance, (ii) receives large amounts of N fertilizer and thus contributes significantly to the environmental problems associated with nitrification and subsequent N losses in leaching and denitrification, and (iii) lends itself to detailed physiological and genetic studies due to the availability of a variety of genetic stock and molecular tools.

*Rice (Oryza sativa)* is not only the most important food crop in the world and the biggest ‘consumer’ of N fertilizers in Asia, but also its N fertilizer use efficiency is rather low, often not exceeding 35% (Dobermann et al., 2002). Equally important is the fact that rice has become the cereal model organism because of its relatively small genome size, the availability of several whole-genome sequences and relatively easy tools for genetic manipulation. If BNI activity could be detected in some rice genotypes, exploiting this novel biological function might be rapid with potential applications reaching beyond rice to other cereals. The objectives of this study, therefore, were to elucidate whether BNI activity is present in root exudates of rice, to further characterize this activity in contrasting genotypes and ultimately to confirm the inhibitory effect in soil.

**Materials and methods**

**General experiment procedures**

**Growth conditions** Seeds of rice genotypes were sterilized with a solution containing 2% NaOCl and 150 mM KH$_2$PO$_4$, for 5 min, rinsed with distilled water, and incubated at 30°C. The germinated seeds were then transferred to a net floating system containing 0.5 mM CaCl$_2$. Subsequently, 14-day-old seedlings were grouped in bundles of three to five plants and transplanted into a solution culture box containing half-strength modified Yoshida solution (nutrient concentrations at full strength: N 2.86 mM (as NH$_4$NO$_3$), P 0.05 mM, K 1 mM, Ca 1 mM, Mg 1 mM, Mn 9 $\mu$M, Mo 0.5 $\mu$M, B 18.5 $\mu$M, Cu 0.16 $\mu$M, Fe 36 $\mu$M and Zn 0.15 $\mu$M; Yoshida et al., 1976). After 10 days, the nutrient solution was changed to full strength. The pH was monitored regularly and adjusted to 5.5–5.8. In experiments with a long growth period, solutions were changed every 2 weeks.

**Collection of root exudates** Plants were transferred from the culture solution containers to jars, where their roots were rinsed consecutively with distilled water and trap...
solution (1 mM NH₄Cl and 0.1 mM CaCl₂). Finally, the root mass was carefully immersed in a 1-L dark bottle containing trap solution for 24 h to collect root exudates. The pH was monitored every 8 h during the 24-h collection period and readjusted to 5.6 ± 0.2 with HCl or NaOH if necessary. After 24 h, the root and shoot tissue were saved for dry matter determination. The trap solution was evaporated to dryness using a rotary evaporator (Buchi, V-850, Switzerland) at 40 °C, then re-suspended in 100 % methanol and re-evaporated twice. Finally, the concentrated sample was suspended in 1.0 mL of 100 % methanol and passed through a syringe-driven 0.22 µm membrane filter (Milllex, Millipore, USA). The filtrate was completely evaporated in a centrifuge evaporator, re-suspended in 50 or 100 µL of dimethyl sulfoxide (DMSO) and 2 µL used in the BNI bioassay. Controls for the trap solution were subjected to the same procedure as for the sample (drying and dissolving in DMSO).

**Assessment of BNI using a bioassay** The BNI was assessed using a recombinant *N. europaea* strain that carries a luciferase gene (luxAB derived from *V. harveyi*) and a Kan resistance gene that enables the growth of pure cells on media containing the antibiotic kanamycin. The recombinant strain produces bioluminescence under normal growing conditions due to expression of the luxAB gene. Reduced bioluminescence in whole cells that are challenged with known nitrification inhibitors and toxic compounds is possibly caused by the immediate decrease in reducing power in the cell due to the inactivation of ammonia monooxygenase, as well as by the interruption of other cellular metabolic pathways (Iizumi et al., 1998).

*Nitrosomonas europaea* was cultured in a mineral medium (referred to as P medium) with the following composition (in g L⁻¹): (NH₄)₂SO₄ 2.5, KH₂PO₄ 0.7, Na₂HPO₄ 13.5, NaHCO₃ 0.5 and MgSO₄·7H₂O 0.1, and (in mg L⁻¹) CaCl₂·2H₂O 0.005, Fe-EDTA 0.001 and kanamycin 0.025. After a 7-day culture period at 30 °C, cells were harvested by centrifugation, re-suspended in fresh P medium and adjusted to a final OD₆₀₀ of 1.3–1.4 in a spectrophotometer (SmartSpec Plus, BioRad).

In the bioassay, a mixture of 200 µL of bacterial cells, 2 µL of sample (root exudates dissolved in DMSO), 198 µL of distilled water and 100 µL of P medium was incubated at 25 °C for 30 min. A 100-µL aliquot of the mixture was added to tubes containing 2.5 µL of 10 % (v/v) n-decyl aldehyde, and the bioluminescence (light-emitting reaction of luciferase) of each sample was then measured using a Gloma 20/20 luminometer (Promega, USA). Four relative light unit (RLU) readings were obtained for each sample. Each sample had three independent replications. Biological nitrification inhibition activity in samples was compared with that of DMSO (the organic solvent used in sample preparation) and of the synthetic nitrification inhibitor allylthiourea (AT, at 0.22 µM), which causes around 80 % relative inhibition in the bioassay (Subbarao et al., 2007).

The inhibition rate (IR) and the relative bioluminescence rate (RBR) were determined as follows:

\[
IR(\%) = \left(1 - \frac{RLU_{Sample}}{RLU_{Control}}\right) \times 100
\]

where RLU Sample is the value of the mixture as mentioned above, and RLU Control is the value obtained by using DMSO instead of the sample.

**Screening of genotypes (Experiments 1 and 2)** Thirty-six genotypes of rice (including wild relatives, traditional and cultivated) were screened for their nitrification inhibition (NI) potential (Experiment 1) in two consecutive years. Some background information on the 36 genotypes is given in Additional information Table S1.

Plants were cultivated hydroponically for 60 days in a greenhouse with three independent replicates per genotype. Root exudate collection and processing, and BNI estimation based on the bioassay were carried out as described in general experimental procedures. Subsequent experiments tested the effect of plant age and trap solution composition (1 mM NH₄Cl and 0.1 mM CaCl₂) on BNI activity in root exudates. Seedlings were grown for 21, 28, 35, 50 or 70 days, and exudates collected for 24 h (Experiment 2).

**Characterization of genotypes (Experiment 3)** In Experiment 3, seedlings were grown for 28 and 35 days, and root exudates were collected in trap solutions containing either NH₄⁺ or NO₃⁻ (1 mM NH₄Cl or 1 mM KNO₃, both with 0.1 mM CaCl₂). The effects of plant age and trap solution on BNI activity were then determined by the BNI bioassay as described above.

**Assessment of unspecific leakage in the exudates (Experiment 4)** In Experiment 4, the relationship between potential unspecific leakage and BNI was determined as affected by genotype (IAC25 vs. Nipponbare) and trap solution (1 mM NH₄Cl, 1 mM KNO₃ or distilled H₂O). Plants were grown for 30 days and the pH during exudate collection...
was controlled at 5.5 ± 0.1 using a pH stat system (NPH-660 NDE, Nissin, Japan). Root exudates were collected in a 3-L trap solution. One half of the total volume was evaporated to dryness and used for the BNI bioassay, while the other half was concentrated 100-fold (from 1.5 L to 15 mL) using rotary evaporators. At 100-fold concentration, no visible precipitation was detected in the sample.

To detect unspecific leakage, the composition of root exudates was analysed for the presence of low-molecular-weight organic acids (LMWOAs) and inorganic ions, and for electrical conductivity (EC). Malate and citrate were chosen as representatives for unspecific leakage of organic acids, and their concentration was measured following the protocol of the malate and citrate enzymatic bioanalysis (r-biopharma, Roche). Inorganic ion composition of root exudates was analysed by an inductively coupled plasma emission spectrometer (ICPE-9000, Shimadzu, Japan). The ion composition was first examined qualitatively to determine which ions were detectable, and then quantitatively to quantify their variation. Several ions were detected; however, as Ca, K and Na were part of the trap solution, only Mg was included in the result. Calibration curves were prepared in agreement with the estimated amount for each detected ion using ICPE standards (Wako Chemical, Japan).

Relative electrolyte leakage (REL) was determined as described by Tripathy et al. (2000) with some modifications. In brief, REL = (EC of trap solution after 24 h collection – initial EC from trap solution)/EC maximum (EC of autoclaved tissue: 120 °C for 20 min). Electrical conductivity values were measured using a hand-held EC meter (Yokogawa, Japan).

Assessment of BNI based on soil incubation (Experiment 5)

Two soils were used in this experiment. A brown lowland soil was collected in Saitama Prefecture, Japan, whereas the second soil was collected in central Italy (Lazio region). The properties of soils were as follows: for the Saitama soil, pH (H₂O) 5.6, EC 148 μS cm⁻¹, total C 0.95 %, total N 0.7 %, clay 26 % and sand 63 %; and for the Lazio soil, pH (H₂O) 6.3, EC 183 μS cm⁻¹, total C 0.8 %, total N 0.9 %, clay 28 %, silt 60 % and sand 12 %.

The net nitrification rate of the brown lowland soil was determined using an aerobic incubation procedure. Five grams of soil were placed in a 50-mL centrifuge tube and concentrated root exudates from Experiment 4 were added (1.2 mL) in conjunction with distilled water (0.3 mL) containing (NH₄)₂SO₄ (in a final concentration of 120 mg N kg⁻¹ of soil) in order to reach 60 % of the water-holding capacity (Mosier et al., 1990).

During collection of the root exudates, the pH of the trap solution was maintained at a constant level around 5.5 by a pH stat controller; thus the pH of the concentrated extract (compared with the original) did not change significantly. However, the EC of the exudate solution increased significantly from an original value of 0.2 to 1.2 mS cm⁻¹ after concentration. Therefore, solutions with EC values (based on NaCl) similar to that of the samples were used as a control for the EC effect. The initial soil weight was recorded and water loss was monitored by weight difference and readjusted by adding distilled water. Finally, the tubes were covered with perforated parafilm and incubated at 30 °C for different periods (referred to as standard incubation). Samples were extracted for each time point with 2 M KCl (soil:solution ratio 1:10). The soil–KCl solution was shaken for 1 h followed by filtration through filter paper, and the extracts were measured for NO₃⁻ using a continuous-flow auto-analyser (model Autoanalyzer 2; Bran + Lubbe, Hamburg, Germany).

Potential nitrification

The shaken slurry method of Hart et al. (1994) was used to measure nitrification in soil amended with exudates. We used this procedure to test the activity of nitrifying bacteria in soil after root exudates were added. Ten grams of fresh soil were mixed with 98.5 mL of a buffer solution and 1.5 mL of concentrated exudates in a 250-mL Erlenmeyer flask to make slurry. One hundred millilitres of buffer solution without exudates were used as a control. In all treatments, the final concentration of buffer solution was 1.5 mM NH₄⁺ and 1 mM PO₄³⁻. Slurry was shaken on an orbital shaker at 180 r.p.m. to maintain aerobic conditions. After 2, 6 and 24 h, an aliquot of 10 mL was taken from each flask, centrifuged for 5 min at 10 000 r.p.m. and then analysed for NO₃⁻ as described above. The assay was replicated three times. This procedure was carried out using both the Saitama brown lowland soil and the Lazio soil; however, potential nitrification was much lower than expected based on standard incubation experiments done with Saitama brown lowland soil (see above), and results are therefore only reported for the Lazio soil. Probably, the pH of the incubation buffer was not suitable for the Saitama soil (pH 5.6), causing inhibition of the nitrifying bacteria activity.

Net nitrification rate in rhizosphere soil

Seeds of the two genotypes were sown in small pots containing brown lowland soil (350 g). Previously, the soil was supplied with 120 mg N kg⁻¹ as (NH₄)₂SO₄. There were three plants per pot and each treatment had five independent replications. Watering was done...
conservatively to avoid anaerobiosis throughout the growth period. After a 40-day growth period, the root system of plants had penetrated the entire soil volume in the container and the experiment was terminated. Soil from the top and external side of the block was removed and discarded, and the remaining block soil was then stripped off from the root, referred to as rhizosphere soil. Root tissue was also collected and the dry matter recorded. Subsequently, soil was incubated using the standard incubation procedure as described above, but without the addition of concentrated root exudates.

Statistical analysis

Data were analysed by ANOVA and mean values were compared using the least significance test (LSD). Experiments for genotype screening and characterization had three replications, while soil incubation had five independent replications.

Results

Screening of genotypes (Experiments 1 and 2)

The screening of 36 rice genotypes for BNI activity in their root exudates (Experiment 1) revealed substantial genotypic variation. Based on the bioassay of transgenic Nitrosomonas, estimates of BNI activity ranged from < 10% inhibition by genotypes Lemont, IR49830-7 and IR64 to > 50% inhibition by IAC25 (Fig. 1). Because the non-plant ‘blank’ containing 0.5 mM NH₄Cl (equivalent to the average concentration of NH₄⁺ remaining in the trap solution after 24 h) resulted in BNI of ~15%, we conclude that about half of the tested genotypes lack significant BNI activity. Among the group of genotypes with BNI activity significantly higher than the blank, traditional and upland varieties were over-represented while modern varieties developed for irrigated lowland rice production were under-represented.

Wild relatives of cultivated O. sativa were present with three accessions (one each of O. nivara, O. brachyanta and O. glumaepatula), but none showed particularly high BNI activity. Several genotypes with known high allelopathic activity (Fujii et al., 2005) were included in the screening to test whether allelopathy and BNI are related. Although some allelopathic genotypes such as Awa-Akamai and Midori-mai (both ancient Japanese rice cultivars) showed high BNI activity, others such as Soujiya Akamai and Dular had average activity. In general, the degree of variability for bioassay results was rather high among replications. One of the most stable genotypes in this regard was IAC25 and as it also showed the highest overall BNI activity, this Brazilian improved upland cultivar was chosen for further experiments. As contrasting genotypes with low BNI activity, we selected Nipponbare for the availability of its genome sequence and IR64 since it is probably the most widely grown rice cultivar.
Testing BNI activity in exudates collected from plants of increasing age (21, 28, 35, 50 and 70 days; Experiment 2) showed a trend of decreasing BNI activity in older roots (Table 1). Roots of 21-day-old plants had the highest activity per root dry weight (specific BNI), followed by 28-day-old plants. In general, the inhibitory activity decreased as root mass per plant increased.

**Characterization of genotypes (Experiment 3)**

In *B. humidicola*, the highest BNI activity was detected when exudates were collected in the presence of NH$_4^+$, while exudates collected in NO$_3^-$ showed lower activity (Subbarao et al., 2007). To test whether such an effect of trap solution exists in rice, the effect of 1 mM NH$_4^+$ or 1 mM NO$_3^-$ in the trap solution was evaluated using the genotype IAC25 at two different ages (28 and 35 days old; Experiment 3). The presence of 1 mM NH$_4^+$ in the trap solution significantly increased the BNI activity in root exudates compared with the NO$_3^-$ treatment (Table 2). There was no significant effect of plant age or interaction effect with trap solution.

**Assessment of unspecific leakage in the exudates (Experiment 4)**

To test the hypothesis that BNI activity is a consequence of specific root exudation rather than passive leakage, the composition of root exudates with regard to non-BNI leachates was determined (Experiment 4). Root exudates of genotypes IAC25 and Nipponbare were collected for 24 h using trap solutions containing NH$_4^+$, NO$_3^-$ or distilled water (all contained 0.1 mM CaCl$_2$). Chemical analysis of the exudates showed that an average of 48 % of the initial NH$_4^+$ remained, while an average of 69 % NO$_3^-$ remained in the respective trap solutions (data not shown). The BNI activity in exudates was higher in NH$_4^+$ compared with NO$_3^-$ and higher in IAC25 compared with Nipponbare. The highest BNI activity of 54.5 % was detected in IAC25 exudates collected in NH$_4^+$ trap solution (Table 3), with no evidence to suggest that this high BNI activity was related to non-specific leakage. Relative electrolyte leakage was generally low, ranging from 1.9 % for IAC25 in NO$_3^-$ to 4.8 % for Nipponbare in NH$_4^+$. Exceptions were exudates collected in distilled water, which showed an REL of up to 17.7 %, yet these samples had low BNI. No significant differences were observed between treatments for the unspecific efflux of LMWOAs such as malate and citrate, and for magnesium. Correlation analysis of the measured parameters thus revealed a significant negative correlation between REL and BNI percentage (Table 4). Although malate exudation positively correlated with the genotype IAC25 at two different ages (28 and 35 days old; Experiment 3). The presence of 1 mM NH$_4^+$ in the trap solution significantly increased the BNI activity in root exudates compared with the NO$_3^-$ treatment (Table 2). There was no significant effect of plant age or interaction effect with trap solution.

| Days after sowing | NI activity (AT units L$^{-1}$) | Root dry matter (RDM) (g) | Specific NI activity (NI g$^{-1}$ RDM) | Number of plants/collection |
|-------------------|-------------------------------|---------------------------|--------------------------------------|-----------------------------|
| 21                | 36.4 (6.6$^b$)                | 0.55 (0.18)               | 65.8 (7.6)                           | 100                         |
| 28                | 58.4 (7.8)                    | 1.58 (0.32)               | 36.8 (4.1)                           | 88                          |
| 35                | 21.7 (3.1)                    | 1.12 (0.21)               | 21.4 (2.8)                           | 15                          |
| 50                | 19.2 (3.0)                    | 0.81 (0.11)               | 23.7 (2.9)                           | 4–6                         |
| 70                | 13.3 (2.8)                    | 1.10 (0.12)               | 13.0 (2.1)                           | 4                           |

$^a$AT unit refers to 80 % relative inhibition in the NI bioassay caused by AT (at 0.22 μM), as explained in Materials and methods.

$^b$Values in parentheses represent standard deviation.

| Plant age (A) | Trap solution (T) | Percentage of inhibition$^a$ | NI activity | Specific NI activity (NI g$^{-1}$ RDM) | Significance |
|---------------|-------------------|-----------------------------|-------------|---------------------------------------|--------------|
| 28            | NH$_4^+$          | 58.46                       | 23.47       | 19.47                                 | ** ns ns ns  |
|               | NO$_3^-$          | 28.57                       | 13.50       | 9.51                                  | ** ** ** **  |
| 35            | NH$_4^+$          | 46.20                       | 19.38       | 15.39                                 | ** ns ns ns  |
|               | NO$_3^-$          | 30.54                       | 14.17       | 10.17                                 | ** ** ns ns  |

$^a$Based on the relative bioluminescence rate of sample and control, as explained in Materials and methods.

$^*$ refers to significance at $P \leq 0.01$. 

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Pariasca Tanaka et al. — Nitrification inhibition activity in root exudates of rice

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citrate and magnesium, no significant correlations were observed between these specific chemical exudates and the broader REL and BNI characteristics.

Assessment of BNI based on soil incubation (Experiment 5)

Having shown repeatedly that rice root exudates can inhibit nitrification in the bioassay, the logical next question was whether such BNI activity can also be shown in soil. For that purpose, soil samples were either incubated with concentrated root exudates, or rice rhizosphere soil collected and incubated in the presence of $\text{NH}_4^+$ (Experiment 5). Soil incubated with exudates from IAC25 had lower nitrate concentrations after 7 days of incubation compared with soil incubated with water (Fig. 2). However, this BNI effect of IAC25 exudates was no longer detected after 35 days, when $\geq 80\%$ of the initial $\text{NH}_4^+$ had been converted to $\text{NO}_3^-$ in all samples. One potentially confounding factor of using concentrated root exudates is the high concentration of salts they contain and which may have an unspecific BNI effect (concentrated IAC25 exudates had an EC of 1.2 mS cm$^{-1}$, in part due to remnant $\text{NH}_4\text{Cl}$ left in the trap solution). This hypothesis was tested by incubating with solutions containing a range of ECs. Some BNI effect of EC was detected after 7 days at 1.0 and 2.0 mS cm$^{-1}$; however, the effect of IAC25 exudates was significantly stronger, indicating that a specific BNI effect was present in these exudates.

A subsequent experiment used a different ‘slurry’ incubation method to test BNI ability in root exudates of IAC25 and Nipponbare. After 6 h of incubation, 10.4 mg $\text{NO}_3^-$ g$^{-1}$ soil were detected in Lazio soil incubated with IAC25 exudates or NaCl solutions of different ionic strength. Values at $t = 0$ refer to the initial nitrate percentage in the soil before addition of exudates/solutions. Different letters indicate significant differences within sampling times (LSD at $P \leq 0.05$).

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**Table 3** Effect of trap solution on the NI activity, LMWOAs and ion concentration of root exudates of two rice genotypes after 24 h collection (Experiment 4)

| Trap Solution   | NH$_4$ | NO$_3$ | Distilled Water |
|-----------------|--------|--------|----------------|
| Nipponbare IAC25 | 46.1   | 38.5   | 38.8           |
| Sig.$^a$        | 54.6   | 42.6   | 40.5           |
| Sig.$^a$        | **     | *      | ns             |
| REL (%)         | 4.8    | 2.3    | 17.7           |
| Malate (nmol h$^{-1}$ g$^{-1}$) | 20.8 | 20.2 | 15.9 |
| Citrate (nmol h$^{-1}$ g$^{-1}$) | 93.7 | 75.1 | 81.5 |
| Mg (mg L$^{-1}$) | 6.7    | 4.0    | 4.5            |

$^a$Significant difference between genotype means ($n = 3$) within trap solution, based on LSD. $^*$ and $^{**}$ refer to significance at $P \leq 0.05$ and 0.01, respectively.

**Table 4** Correlation coefficients for the association between NI and REL (%), and leakage of malate, citrate and Mg (Experiment 4)

| NI (%) | REL (%) | Malate | Citrate | Mg |
|--------|---------|--------|---------|----|
| NI (%) | $-0.45^*$ | 0.16   | 0.14    | 0.04 |
| REL    | 0.25    | $-0.18$ | 0.23    |    |
| Malate | 0.63$^*$ | 0.48$^*$ |        |    |
| Citrate|         |        | 0.18    |    |
| Mg     |         |        |         |    |

$^*$Significant at $P \leq 0.05$.

**Fig. 2** Proportion of total N in nitrate form in soil samples incubated for 7 and 35 days (Experiment 5). Soil samples had been mixed with root exudates of rice genotype IAC25 or with NaCl solutions of different ionic strength. Values at $t = 0$ refer to the initial nitrate percentage in the soil before addition of exudates/solutions. Different letters indicate significant differences within sampling times (LSD at $P \leq 0.05$).
with a water control (Fig. 3) compared with 8.3 and 6.9 mg NO$_3^-$ g$^{-1}$ in soil incubated with exudates from Nipponbare and IAC25, respectively. After 24 h of incubation, the NO$_3^-$ concentrations had doubled to 20 mg NO$_3^-$ g$^{-1}$ soil in the water control but remained significantly lower for exudates of IAC25, confirming the potential of IAC25 for BNI using a completely different methodology.

Having shown that exudates of IAC25 do have BNI activity in a soil-based assay as well, the final objective of this study was to test whether such BNI activity can also be detected in rhizosphere soil collected after 50 days of plant growth. As an unplanted soil does not represent a good control due to very different patterns of moisture and nutrient fluctuations, the low-BNI genotype Nipponbare was used in comparison with the high-BNI genotype IAC25. After 7 days of incubation, a small but significant reduction in NO$_3^-$ formation was seen in IAC25 relative to Nipponbare and this difference increased after 14 days (Fig. 4). Total N and C were determined in both rhizosphere samples but no difference between genotypes was detected, with the average total C and N content of rhizosphere soil collected from IAC25 and Nipponbare being 6.97 and 0.77 g C kg$^{-1}$ and 0.78 g N kg$^{-1}$, respectively.

**Discussion**

Recently, Subbarao and co-workers identified potential BNI in the root exudates of a number of plant species using a bioassay targeting *Nitrosomonas* (Subbarao et al., 2007). Subsequent studies demonstrated the potential for the pasture grass *B. humidicola* to inhibit nitrification on a significant scale under field conditions (Subbarao et al., 2009). In our study using rice, the screening of a broad range of genotypes for their NI activity using the *Nitrosomonas* bioassay resulted in the identification of genotypes with contrasting BNI activity in their root exudates. Higher BNI activity was more likely to be found in traditional and/or upland varieties, whereas modern high-yielding lowland varieties such as IR64 or Nipponbare tended to have lower activity.

No compelling evidence was found to support the hypothesis that BNI is a non-specific allelopathy

**Fig. 3** Nitrate accumulated in soil incubated for several hours with root exudates of two rice genotypes (Experiment 5). Soil was incubated following the nitrification potential method. Control refers to soil incubated without exudates. Different letters indicate significant differences within sampling times (LSD at $P \leq 0.05$).

**Fig. 4** Percent of total N in nitrate form in rhizosphere soil of two rice genotypes after incubation for 7 and 14 days (Experiment 5). The initial nitrate in the untreated soil was 20 % (as indicated in Fig. 2). Different letters indicate significant differences within sampling time (LSD at $P \leq 0.05$).
However, the direct inhibition of rice is not a consequence of arbitrary leakage. This study is a first indication that the BNI induced by IAC25 and others. Subsequent experiments on a subset of genotypes differing in BNI activity further characterized their potential under various conditions. More mature plants showed lower BNI per root weight than young plants, indicating that BNI is influenced by plant growth stage, as reported by Zakir et al. (2008) for sorghum. This trend is likely to reflect a relative decrease in the proportion of physiologically active root tissue in maturing root systems. It therefore appears likely that the release of BNI substances from roots continues throughout the plant growth stages studied here.

In order to treat BNI as a plant-induced process, it is important to confirm that the activity seen in exudates of rice is not simply the result of non-specific leakage. The negative correlation of BNI with REL observed in this study is a first indication that the BNI induced by rice is not a consequence of arbitrary leakage. However, the direct inhibition of Nitrosomonas by high salt concentrations in the trap solution could confound the interpretation of these results. We have tried to adjust EC values for the amount of NH$_4^+$ or NO$_3^-$ remaining in the trap solution after the 24 h collection period, but have not been able to account for any increase in EC contributed by companion ions or ions exuded to maintain charge balances upon uptake of respective N forms. A more reliable estimate for the presence of leakage may therefore be an unusually high concentration of the LMWOA malate and citrate. Both were detected in the exudates of IAC25 and other genotypes, but at concentrations considered normal or low (Hoffland et al., 2006; Widodo et al., 2010). Furthermore, genotypic differences for LMWOA concentrations as well as correlations between BNI and malate and citrate were not significant. The same was observed for concentrations of Mg, a cation that had not been supplied in the trap solution and may therefore be an additional indicator of leakage. It can thus be concluded that the BNI activity detected here was not the result of non-specific leakage.

An important aspect of this study was the inclusion of soil incubation experiments to validate the preliminary screening of root exudates in the bioassay. A single application of concentrated root exudates to a brown lowland soil showed a transient inhibition of nitrification: BNI was apparent in soil 7 days after application of rice root exudates, but by 35 days there was no significant difference between the applications of exudates or control solutions. The decrease in BNI after 7 days may be due to the progressive degradation in BNI-related compounds by soil microorganisms. Therefore, it would be interesting to see whether continuous application of a lower concentration of root exudates has a sustained inhibitory effect.

The BNI effect of exudates was confirmed using a slurry incubation experiment, where 1.5 mL of concentrated exudate also induced BNI, though only in Lazio soil. A more suitable buffer for Saitama soil would be necessary, as mentioned in Materials and methods. The trends regarding genotypic effects for BNI identified in the screening study were also apparent in rhizosphere soil, with nitrification being slower in rhizosphere soil collected from IAC25 compared with Nipponbare. At this point, the exact mechanisms by which rice root exudates inhibit nitrification are not known. In studies on BNI activity of B. humidicola, Ishikawa et al. (2003) detected a decrease in AOB population size in an Andosol soil, whereas Gopalakrishnan et al. (2009) found no differences in AOB counts. To shed more light on the effects of BNI in soil and whether other AOBs such as Nitrospira and Nitrosococcus (Hayasu et al., 2008) are affected similarly to Nitrosomonas, it would be important to validate the effect of BNI exudates on AOB population sizes by molecular methods, particularly because AOBs are generally recalcitrant to cultivation in artificial medium.

Nitrification rates in paddy soils are lower compared with typical upland soil due to the anaerobic soil environment, which largely restricts nitrification to the top soil layer and to parts of the rhizosphere where oxygen leakage from roots supports non-specific aerobic microbial processes, including nitrification (Arth et al., 1998; Briones et al., 2002). Further reductions in nitrification may therefore not be necessary since NO$_3^-$ uptake by rice appears to be beneficial, particularly if high yields are to be attained (Kronzucker et al., 2000). Nevertheless, N losses due to NO$_3^-$ leaching and denitrification remain a concern in paddy rice production (Akiyama et al., 2005; Ju et al., 2009). This would indicate a need for optimization of nitrification rates, and BNI could represent one tool in achieving this. It should be stressed at this point that the target of BNI in rice would not be to completely eliminate nitrification, but ideally to achieve a balance between NO$_3^-$ supply via nitrification and plant NO$_3^-$ uptake. That NO$_3^-$ leaching can be reduced via application of synthetic nitrification inhibitors in lowland rice (Li et al., 2009) suggests that an analogous biological inhibition of nitrification would also be beneficial to rice growers and the global environment.
Conclusions and forward look
The overall aim was to establish whether BNI can be detected in rice at a sufficiently high level to warrant further studies that can take advantage of the molecular tools and genetic stock available for this cereal model crop. With specific BNI activities ranging from 13 to 65 AT units g\(^{-1}\) root dry weight (Table 1), we have shown that some rice genotypes can have BNI activities that compare with B. humidicola, or sorghum (Zakir et al., 2008), even though reported peak activities were highest in B. humidicola (Subbarao et al., 2007). That BNI activity could be confirmed in soil incubation studies further suggests that this phenomenon should be exploited further in rice. To identify compounds responsible for the BNI activity detected in rice exudates and to employ molecular methods to investigate whether such exudates specifically affect relative population size or activity of soil nitrifiers will be key next steps.

Additional information
The following additional information is available in the online version of this article.

Table S1. Wild relative, traditional and cultivated genotypes of rice used for inhibition activity screening.

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Contributions by the authors
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Conflict of interest statement
None declared.

References
Akiyama H, Yagi K, Yan X. 2005. Direct N\(_2\)O emissions from rice paddy fields: summary of available data. Global Biogeochemical Cycles 19: GB1005, doi:10.1029/2004GB002378.

Arth I, Frenzel P, Conrad R. 1998. Denitrification coupled to nitrification in the rhizosphere of rice. Soil Biology & Biochemistry 30: 509–515.

Briones AM, Okabe S, Umemiya Y, Ramsing NB, Reichardt W, Okuyama H. 2002. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Applied and Environmental Microbiology 68: 3067–3075.

Dobermann A, Witt C, Dowd D, Gines GC, Nagarajan R, Satawathananont S, Son TT, Tan PS, Wang GH, Chien NV, Thao VTK, Phung CV, Stalin P, Muthukrishnan P, Ravi V, Babu M, Chatuporn S, Kongchum M, Sun Q, Fu R, Simbohom GC, Adviento MAA. 2002. Site-specific nutrient management for intensive rice cropping systems in Asia. Field Crops Research 74: 37–66.

Fuji Y, Araya H, Hiaruade S, Ebana K. 2005. Screening of allelopathic activity from rice cultivars by bioassay and field test. In: Tariyama K, Hoonong L, Hardy B, eds. Rice is life: scientific perspectives for the 21st century. Proceedings of the World Rice Research Conference, Japan. Session 16: pest management with minimal environmental stress. 485–487.

Gaines TP, Gaines ST. 1994. Soil texture effect on nitrate leaching in soil percolates. Communications in Soil Science and Plant Analysis 25: 2561–2570.

Goertz HM. 1994. Controlled release technology (Agricultural). In: Kirk-Othmer Encyclopedia of chemical technology, Vol. 7 Controlled release technology. New York: John Wiley & Sons, 251–274.

Gopalakrishnan S, Subbarao GV, Nakahara K, Yoshitani T, Ito O, Maeda I, Ono H, Yoshida M. 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agriculture and Food Chemistry 55: 1385–1388.

Gopalakrishnan S, Watanabe T, Pearse SJ, Ito O, Hassain ZAKM, Subbarao GV. 2009. Biological nitrification inhibition by Brachiaria humidicola roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Science & Plant Nutrition 55: 725–733.

Hart SC, Stark JM, Davidson EA, Firestone MK. 1994. Nitrogen mineralization, immobilization, and nitrification. In: Weaver RW, Angel JS, Bottomley BS, eds. Methods of soil analysis. Part 2. Microbiological and biochemical properties. Madison: Soil Science Society of America, 985–1018.

Hayatsu M, Tago K, Saito M. 2008. Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Science & Plant Nutrition 54: 33–45.

Hoffland E, Wei C, Wissuwa M. 2006. Organic anion exudation by lowland rice (Oryza sativa L.) at zinc and phosphorus deficiency. Plant and Soil 283: 155–162.

Izumi T, Mizumo M, Nakamura KA. 1998. Bioluminescence assay using Nitrosomonas europaea for rapid and sensitive detection of nitrification inhibitors. Applied and Environmental Microbiology 64: 3656–3662.

Ishikawa T, Subbarao GV, Ito O, Okada K. 2003. Suppression of nitrification and nitrous oxide emission by the tropical grass Brachiaria humidicola. Plant and Soil 255: 413–419.

Ju XT, Xing GX, Chen XP, Zhang SL, Zhang LJ, Kiu XJ, Cui ZL, Yin B, Christie P, Zhu ZL, Zhang FS. 2009. Reducing environmental risk by improving N management in intensive Chinese agricultural systems. Proceedings of the National Academy of Sciences of the USA 106: 3041–3046.

Kronzucker HJ, Glass ADM, Siddiqi MY, Kirk GJD. 2000. Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: implication for rice cultivation and yield potential. New Phytologist 145: 471–476.

Li H, Chen Y, Liang X, Lian Y, Li W. 2009. Mineral-nitrogen leaching and ammonia volatilization from a rice-rapeseed system as affected by 3,4-dimethylpyrazole phosphate. Journal of Environmental Quality 38: 2131–2137.
Mosier AR, Mohanty SK, Bhadrachalam A, Chakravorti SP. 1990. Evolution of dinitrogen and nitrous oxide from the soil to the atmosphere through rice plants. *Biology and Fertility of Soils* 9: 61–67.

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG. 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology* 16: 177–184.

Slagen J, Kerkhoff P. 1984. Nitrification inhibitors in agriculture and horticulture: a literature review. *Fertilizer Research* 5: 1–76.

Subbarao GV, Ito O, Sahrawat KL, Berry WL, Nakahara K, Ishikawa T, Watanabe T, Suenaga K, Rondon M, Rao IM. 2006. Scope and strategies for regulation of nitrification in agricultural systems – challenges and opportunities. *Critical Reviews in Plant Sciences* 25: 303–335.

Subbarao GV, Wang HY, Ito O, Nakahara K, Berry WL. 2007. NH₄⁺ triggers the synthesis and release of biological nitrification inhibition compounds in *Brachiara humidicola* roots. *Plant and Soil* 290: 245–257.

Subbarao GV, Nakahara K, Hurtado MP, Ono H, Moreta DE, Salcedo AF, Yoshihashi AT, Ishikawa T, Ishitani M, Ohnishi-Kameyama M, Yoshida M, Rondon M, Rao IM, Lascano CE, Berry WL, Ito O. 2009. Evidence for biological nitrification inhibition in *Brachiaria* pastures. *Proceedings of the National Academy of Sciences of the USA* 106: 17302–17307.

Tripathy JN, Zhang J, Robin S, Nguyen ThT, Nguyen HT. 2000. QTLs for cell-membrane stability mapped in rice (*Oryza sativa* L.) under drought stress. *Theoretical and Applied Genetics* 100: 1197–1202.

Widodo, Broadley MR, Rose T, Frei M, Pariasca-Tanaka J, Yoshihashi T, Thomson M, Hammond JP, Apile A, Close TJ, Ismail AM, Wissuwa M. 2010. Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. *New Phytologist* 186: 400–414.

Yoshida SD, Forno A, Cock JK, Gomez KA. 1976. Laboratory manual for physiological studies of rice. Manila, Philippines: International Rice Research Institute (IRRI).

Zakir HAKM, Subbarao GV, Pearse SJ, Gopalakrishnan S, Ito O, Ishikawa T, Kawano N, Nakahara K, Yoshihashi T, Ono H, Yoshida M. 2008. Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). *New Phytologist* 180: 442–451.

Zerulla W, Barth T, Dressel J, Erhardt K, Von-Locquenghien KH, Pasda G, Radle M, Wissemieher H. 2001. 3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. *Biology and Fertility of Soils* 34: 79–84.