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REGULAR ARTICLE

Biological nitrification inhibition (BNI) activity in sorghum and its characterization

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Abstract
Aims The ability to suppress soil nitrification through the release of nitrification inhibitors from plant roots is termed 'biological nitrification inhibition' (BNI). Here, we aimed at the quantification and characterization of the BNI function in sorghum that included inhibitor production, their chemical identity, functionality and factors regulating their release.
Methods Sorghum was grown in solution culture and root exudate was collected using aerated NH_4Cl solutions. A bioluminescence assay using recombinant *Nitrosomonas europaea* was employed to determine the BNI activity. Activity-guided chromatographic fractionation was used to isolate biological nitrification inhibitors (BNIs). The chemical structure was analyzed using NMR and mass spectrometry; pH-stat systems were deployed to analyze the role of rhizosphere pH on BNIs release.
Results Sorghum roots released two categories of BNIs: hydrophilic- and hydrophobic-BNIs. The release rates for hydrophilic- and hydrophobic- BNIs ranged from 10 to 25 ATU g^{-1} root dwt. d^{-1}. Addition of hydrophilic BNIs (10 ATU g^{-1} soil) significantly inhibited soil nitrification (40 % inhibition) during a 30-d incubation test. Two BNI compounds isolated were: sakuranetin (ED_{80} 0.6 μM; isolated from hydrophilic-BNIs fraction) and sorgoleone (ED_{80} 13.0 μM; isolated from hydrophobic-BNIs fraction),

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which inhibited *Nitrosomonas* by blocking AMO and HAO enzymatic pathways. The BNIs release required the presence of NH_4^+ in the root environment and the stimulatory effect of NH_4^+ lasted 24 h. Unlike the hydrophobic-BNIs, the release of hydrophilic-BNIs declined at a rhizosphere pH >5.0; nearly 80 % of hydrophilic-BNI release was suppressed at pH ≥7.0. The released hydrophilic-BNIs were functionally stable within a pH range of 5.0 to 9.0. Sakuranetin showed a stronger inhibitory activity (ED_{50} 0.2 μM) than methyl 3-(4-hydroxyphenyl) propionate (MHPP) (ED_{50} 100 μM) (isolated from hydrophilic-BNIs fraction) in the in vitro culture-bioassay, but the activity was non-functional and ineffective in the soil-assay.
Conclusions There is an urgent need to identify sorghum genetic stocks with high potential to release functional-BNIs for suppressing nitrification and to improve nitrogen use efficiency in sorghum-based production systems.

Keywords Activity-guided isolation · Ammonia monooxygenase (AMO) · Biological nitrification inhibition (BNI) · BNI compounds · HAO (hydroxylamine oxidoreductase) · Hydrophilic and hydrophobic nitrification inhibitors · Sakuranetin · Sorghum · Sorgoleone

Introduction

Nitrification results in the transformation of relatively immobile ammonium (NH_4^+) to highly mobile nitrate (NO_3^-), which is susceptible to loss through leaching of NO_3^- and/or gaseous N emissions with potential adverse effects on the environment and human health (Galloway et al. 2008; Schlesinger 2009). The low agronomic N-use efficiency (NUE) reported in agricultural systems is largely the result of N loss associated with nitrification-denitrification (Sahrawat and Keeney 1985; Raun and Johnson 1999; Galloway et al. 2008; Schlesinger 2009). Blocking the function of nitrifying bacteria or slowing down soil nitrification can significantly reduce N loss associated with nitrification in situations where loss of N by leaching and denitrification is high following nitrification (Sahrawat and Keeney 1985; Subbarao et al. 2006a, 2012). Extending the time that ammonium remains in the soil, provides more opportunity for its uptake by plants which can improve N-recovery and -use efficiency in agricultural systems (Slangen and Kerkhoff 1984; Fillery 2007; Subbarao et al. 2012). We have used a sensitive recombinant luminescent *Nitrosomonas europaea* bioassay to detect biological nitrification inhibitors (BNIs) in plant-soil systems; and expressed the inhibitory activity in allylthiourea units (ATU) (Subbarao et al. 2006b). Using this methodology, we earlier established that certain plants release nitrification inhibitors or BNIs from their roots (Subbarao et al. 2006b, 2007a, b). The BNI capacity appears relatively widespread among tropical pastures. For example, the *Brachiaria* spp. had the highest capacity among the pasture grasses evaluated; and among field crops, sorghum showed a significant BNI capacity (Subbarao et al. 2007b). One of the nitrification inhibitors released from sorghum roots was identified as methyl 3-(4-hydroxyphenyl) propionate (MHPP) (Hossain et al. 2008). In addition, NH_4^+ was shown to stimulate BNIs synthesis and release in pasture grasses, sorghum and wheat (Subbarao et al. 2007a, b, c; Hossain et al. 2008). The present study aimed at the characterization of BNI function in sorghum that includes production of inhibitors, their identity, functionality and factors regulating the release.

Materials and methods

A number of experiments were conducted to characterize various aspects of BNI activity in sorghum.

Experiment 1

Assessment of BNIs release at various growth stages in sorghum

Sorghum [*Sorghum bicolor* (L.) Moench cv. Hybrid-sorgo] seeds were germinated in trays containing moistened sand, watered with distilled water. Plants were grown in a growth chamber with a day:night temperature regime of 30:28 °C, average photosynthetic photon flux of 300 $\mu mol\,m^{-2}\,s^{-1}$ and a 14:10 h light:dark photoperiod. Ten-day old seedlings were transferred to continuously aerated nutrient solution in 75 l tanks, and the plants were held in Styrofoam blocks with ten holes; three plants per hole were held in position supported with a sponge. The experiment consisted of four tanks (70 l volume; 70×90×60 cm). The composition of the nutrient solution is given in

Subbarao et al. (2006b). Nitrogen at 1 mMN was added as $(NH_4)_2SO_4$ to the nutrient solution. The pH of the nutrient solution was adjusted daily with 1 N NaOH to maintain a range of 5.0 to 5.5. The nutrient solution was replaced every 7 days.

Root exudate collection and determination of BNI activity of hydrophilic fraction of root exudate

In a first series of sequential sampling, root exudate was collected from intact plants at 24, 31, 38 and 45 DAS (days after sowing) to determine the BNI activity in the hydrophilic fraction. In the second series, root exudate was collected from plants at 40, 70, 100 and 130 DAS to determine and characterize the BNI-activity in hydrophilic and hydrophobic fractions of the root exudate and in the root tissue at various sorghum growth stages. For collecting root exudate, intact plants (a sample size of three plants with four replications) were removed from nutrient solution tanks, sequentially rinsed with deionized and distilled water, and immersed for 24 h in 2 l aerated solutions of 1 mM NH_4Cl with 200 μM $CaCl_2$ (pH 6.0). The BNIs released into water is referred to as hydrophilic-BNIs. For extracting BNIs in the hydrophilic fraction, root exudate was evaporated under vacuum at 40 °C to dryness using a rotary-evaporator (Buchi, V-850, Flawil, Switzerland); and this was followed by extraction of the residue with 20 ml methanol. The methanol extract was evaporated and the residue was extracted with 200 μl dimethyl sulphoxide (DMSO). The DMSO extract was used to determine the BNI-activity using a bioassay as described in the following section (Subbarao et al. 2006b).

Determination of BNI activity in the hydrophobic fraction with DCM (dichloromethane) root wash

Following collection of root exudate, roots were separated from shoots, and dipped in acidified DCM (v/v DCM: acetic acid 99:1) for 30 s to remove the hydrophobic compounds from the root surface. The DCM-root wash was then filtered and evaporated to dryness using a rotary-evaporator at 40 °C; the residue was extracted with 20 ml methanol, evaporated and re-extracted with 200 μl of DMSO, and this extract was used to determine the BNI activity in the hydrophobic fraction of the root exudate.

Extraction of BNIs from root tissue

After washing of root tissue with DCM, half of the fresh root tissue was used for the extraction of BNIs; the other half of root tissue was oven-dried at 70 °C for the determination of dry matter. To extract BNIs, the fresh root tissue was macerated with methanol using a blender, followed by filtration. Methanol extract from the root tissue was evaporated to dryness using a rotary-evaporator at 40 °C; the residue was extracted with methanol, evaporated to dryness and the residue dissolved in 200 μl DMSO for the determination of BNI activity.

Nitrification inhibition bioassay

A bioluminescence assay using a recombinant strain of *Nitrosomonas europaea* was adopted to detect nitrification inhibitors released from plant roots (i.e. BNI activity) (Subbarao et al. 2006b). The recombinant strain of *N. europaea* carries an expression vector for the *Vibrio harveyi luxAB* genes, and produces a distinct two-peak luminescence pattern during a 30-s measurement period (Subbarao et al. 2006b). The functional relationship between bioluminescence emission and nitrite production in the assay has been shown to be linear using a synthetic nitrification inhibitor, allylthiourea (AT) (Subbarao et al. 2006b). The inhibition caused by 0.22 μM AT in this assay, about 80 % inhibition in bioluminescence and NO_2^- production, is defined as 1 ATU (allylthiourea unit) (Subbarao et al. 2006b). Using the response to a concentration gradient of AT (i.e. dose–response standard curve), the inhibitory effect of test samples (e.g. root exudates or plant tissue extracts) are expressed and compared in ATU units. The detailed methodology is described in Subbarao et al. (2006b).

Experiment 2a

Isolation of BNIs from the hydrophilic fraction of the exudate

Seedlings of sorghum cv. Hybridsorgo were raised and plants were grown for 50 d in a growth chamber as described in Experiment 1. Root exudate was collected in aerated 1 mM NH_4Cl solution with 200 μM $CaCl_2{\cdot}2H_2O$ and BNI activity was extracted from root exudate and determined using the bioassay as described earlier. Sorghum plants release hydrophilic-BNI of about

40 ATU plant^{-1}; about 500 plants were used to collect sufficient amount of BNI activity for the purification work. Based on our earlier experience, about 5,000 to 10,000 ATU activity is needed to support purification efforts to purify a minimum of 200 μg of purified inhibitor to determine the chemical structure and properties.

The crude methanol extract having BNI activity was diluted with water to achieve 5 % methanol concentration before loading on to a reverse-phase column (25 cm× 2.8 cm, Wakosil 40C18, Wako). The column was eluted with 210 ml each of 5, 10, 20, 30, 40, 50, 75 and 100 % MeOH in 30 fractions of 7 ml. All the fractions were dried under vacuum at 35 °C and the residues were dissolved in MeOH (1 ml); aliquots of these fractions (50 μl) were dried under vacuum at 35 °C and dissolved in 10 μl DMSO; and a 2 μl aliquot was used to determine BNI activity. The active fractions were further purified by HPLC on a Jasco Gulliver HPLC system consisting of a PU-1580 pump, UV-1570/1575 UV detector, and 807-IT integrator equipped with TSKgel Super-ODS (4.6 mm× 100 mm or 10 mm×100 mm) column (Tosoh, Tokyo, Japan) monitored at 280 nm. The column was eluted stepwise with aqueous acetonitrile (10 to 50 %) and all peaks and troughs were checked for BNI-activity in the bioassay. The active component from the 50 % methanol fraction was purified using an isocratic HPLC using 20 % acetonitrile as mobile phase.

Experiment 2b

Isolation of BNI-activity constituents from root-DCM wash, hereafter referred to as hydrophobic-BNIs

Sorghum seeds cv. Hybridsorgo soaked in water for 4 h were germinated in the dark at 28 °C on moistened filter paper discs placed in petri-plates. Root tissue was excised from 8-d old seedlings and immediately dipped in dichloromethane (DCM) with 1 % acetic acid (v/v DCM:acetic acid 99:1) for 30 s. The DCM root-wash was then filtered and stored at 5 °C.

Isolation of a BNI compound from hydrophobic-BNIs in the root-DCM wash

The root-DCM wash was evaporated to dryness using a rotary-evaporator at 40 °C. The residue was dissolved in acetonitrile; preliminary tests suggest that samples dissolved in acetonitrile produced chromatogram with a sharp major peak where the activity was detected; methanol is a broad-based organic solvent, solubilize several non-BNI compounds and interfere with the HPLC analysis and was the reason to choose acetonitrile as the solvent to dissolve the final sample for HPLC analysis. About 5000 ATU hydrophobic-BNIs were obtained by washing 200 g of fresh roots with acidified DCM. The BNIs were purified using activity-guided HPLC fractionation. A major active constituent with absorption at 280 nm was eluted at 10 min with isocratic HPLC conditions of 63 % acetonitrile and 37 % of 0.5 % formic acid.

Instrumental analysis

Mass spectra (MS) of the isolated compounds were recorded on a GCMS-QP2010 spectrometer (Shimadzu, Kyoto, Japan) by direct electron ionization (EI) at ionization energy of 70 eV. The NMR was recorded at 298 K on spectrometer (Bruker, Avance 500). For NMR analysis, the active constituents from root exudate were dissolved in CD_3OD or $CDCl_3$.

Experiment 3

Evaluation of BNI-constituents – methyl 3-(4-hydroxyphenyl) propionate (MHPP), sakuranetin and sorgoleone for their inhibitory effects on *Nitrosomonas*

Authentic sakuranetin was obtained from ©Extrasynthese and MHPP from ©Aldrich. Sorgoleone was purified from root-DCM wash using HPLC. The purified compound was subjected to EI-MS and/or NMR analysis. The EI-MS spectral data matched with that published for sorgoleone (Erikson et al. 2001). Sakuranetin, MHPP and sorgoleone were dissolved in DMSO to determine their inhibitory effects on *Nitrosomonas* using the bioassay described earlier.

Experiment 4

Mode of inhibitory action of MHPP, sakuranetin and sorgoleone on *Nitrosomonas*

Purified sorgoleone and two authentic BNI compounds (MHPP and sakuranetin) along with synthetic

nitrification inhibitors [allylthiourea (AT), nitrapyrin and dicyandiamide (DCD)] were evaluated to determine their modes of inhibitory action on *Nitrosomonas*. The mode of action was determined by incubating a pure culture of *N. europaea* in the presence or absence of hydroxylamine in the assay medium using a previously reported protocol (Subbarao et al. 2006b). Ammonia is first oxidized to hydroxylamine by ammonia monooxygenase (AMO), a copper containing enzyme that is a membrane-bound protein. Hydroxylamine is oxidized to nitrite by hydroxylamine oxidoreductase (HAO) (a cytosolic enzyme); this oxidation releases four electrons—two of which are returned to AMO to sustain ammonia oxidation and the remaining two are available for generation of NADPH or this reductive power is routed through luciferase protein for luminescence during the assay. Most commercially available nitrification inhibitors (such as nitrapyrin and DCD) target the AMO enzymatic pathway to inhibit *Nitrosomonas* function, but have no effect on HAO enzymatic pathway. This could be tested using the bioassay by incubating the *Nitrosomonas* culture with the inhibitor in the presence and absence of hydroxylamine to determine the inhibitor's effect on AMO pathway. If the inhibitor only affects the AMO pathway, then the inhibitory effect can be removed in the presence of hydroxylamine (i.e. byproduct of AMO activity) in the assay medium where *Nitrosomonas* can oxidize hydroxylamine and generate energy; i.e. the culture will regain its luminescence to support the metabolic functions of *Nitrosomonas*. If the inhibitor affects HAO enzyme, then *Nitrosomonas* can't regain its luminescence (i.e. biological activity) even in the presence of hydroxylamine in the assay. This is how the mode of inhibition of the BNIs are evaluated based on this principle. The present method assumes that if HAO is inhibited, then AMO is most likely also inhibited. However, if the inhibitor blocks only the HAO pathway without affecting the AMO pathway, then our assay method can't resolve such a situation; only way to resolve such cases are to analyze the sample mixture (i.e. culture incubated with inhibitor) for hydroxylamine levels, which is beyond the scope of this study.

Solutions of (200 μl) of water-soluble inhibitors, AT, DCD and nitrapyrin were added to 250 μl of bacterial culture; and the contents were incubated for 10 min, followed by addition of 200 μl of 1 mM hydroxylamine (to give 307 μM). The total volume of the assay was 650 μl. The mean of the 10 bioluminescence measurements made during the 10-min incubation period was taken as the activity level. All measurements were repeated three times and treated as replications for statistical analysis of the data. The effect of the addition of the AMO enzyme product (i.e. hydroxylamine) to the reaction mixture was evaluated. The inhibitory effects of purified sorgoleone, authentic MHPP and sakuranetin, and synthetic inhibitors—AT, nitrapyrin and DCD on *Nitrosomonas* activity were determined in the presence (i.e. inhibition of the HAO enzymatic pathway) or absence of hydroxylamine (i.e. inhibition of the AMO enzymatic pathway) as described earlier (Subbarao et al. 2006b).

Experiment 5

Determining longevity of the stimulatory effect of NH_4^+ on the BNIs release from sorghum

Our earlier research showed that sorghum plants released BNIs from roots mostly in the presence of NH_4^+ (i.e. root exudate collection solutions contained NH_4^+) (Hossain et al. 2008). However, the longevity of the stimulatory effect of NH_4^+ on the BNIs release in sorghum is not known. As a continued availability of NH_4^+ in the rhizosphere is unlikely in agricultural systems in the field, this experiment was conducted to obtain information on the longevity of the NH_4^+ influence on the BNIs release in sorghum. Sorghum plants were raised hydroponically with $(NH_4)_2SO_4$ (0.5 mM) as N source for 30 d and the growing conditions were similar to those in Experiment 1. Root exudate was collected from six plants (sample size) using aerated solutions of 1 mM NH_4Cl with 200 μM $CaCl_2$ for 8 h (9.00 am to 5.00 pm); plants were then returned to N-free nutrient solution tanks; root exudate was collected from the same plants on the following day (i.e. 2nd day of the root exudate collection) using NH_4^+-free aerated 200 μM $CaCl_2$ solution for 8 h, and this process was repeated for the third day (i.e. root exudate was collected in NH_4^+-free solution); the fourth day root exudate was collected using 1 mM NH_4Cl with 200 μM $CaCl_2$; the experiment was replicated three times. After the fourth-day of root exudate collection, roots were separated from shoots and dried at 70 °C and dry weights recorded. Root exudate was evaporated to dryness and BNIs were extracted and the activity was measured as described earlier.

Experiment 6

Influence of rhizosphere pH on the synthesis and release of BNIs

Our results previously showed that BNIs release was higher in sorghum plants raised with NH_4^+ as N source and when root exudate was collected using NH_4Cl instead of KNO_3 solution (Hossain et al. 2008). In this experiment, we further analyzed the influence of rhizosphere pH on BNIs release using a pH-stat system to decouple the pH change associated with NH_4^+ uptake/assimilation. Sorghum plants (cv. Hybridsorgo) were hydroponically grown for 10 d (17 DAS) under plant growing conditions and nutrient management described in Experiment 1; $(NH_4)_2SO_4$ is the sole N source at 0.5 mM for growing plants; pH of the nutrient solution was adjusted to 5.0 twice a day. Plants were allowed to grow in hydroponic systems for 10 d, and then used in this experiment. For collecting root exudate, 21 plants were used as the sample size for each of the pH treatment using aerated NH_4Cl (1 mM) solution for a 24 h period; root exudate collection solution pH was maintained using a pH-stat system (NPH-660 NDE, Nissin, Japan). There were 3 pH treatments of 3.0, 5.0, and 7.0 used for the collection of root exudate; the experiment was replicated thrice; root exudate was evaporated to dryness and the activity of hydrophilic-BNIs was determined as described earlier. After the collection of root exudate, roots were separated from shoots and fresh root tissue was immersed for 30 s in acidified DCM to remove the hydrophobic-BNIs from the root surface as described earlier. The root-DCM wash was filtered and evaporated to dryness, re-extracted with methanol and evaporated; the residue was dissolved in 200 μl DMSO for determination of the activity of hydrophobic-BNIs. After the roots were washed with DCM, half of the root tissue was used to extract BNIs from the root tissue following the protocol described in Experiment 1. The remaining half of the root tissue was oven-dried to determine dry weight.

Experiment 7

Determining pH stability of the BNI activity in the hydrophilic fraction

This experiment was conducted to evaluate the pH stability of the BNI activity exudate from sorghum roots. Sorghum plants (cv. Hybridsorgo) were grown hydroponically and root exudate was collected 30 DAS using aerated solutions of NH_4Cl (1 mM) for 24 h. The pH of the root exudate was recorded (which is about 3.1) and adjusted to the treatment pH of 3.0, 5.0, 7.0 or 9.0 with 1 N HCl or NaOH. For each of these pH treatments, a 500-mL sample of root exudate was used as the sample size for pH adjustment treatment; there were two samples (replications) for each pH treatment. One hour after pH adjustment at room temperature of 25 °C, the root exudate was evaporated to dryness, and BNI-activity in the sample was determined as described earlier.

Experiment 8

Inhibitory effects of BNIs (crude extract of BNIs from root exudate) and purified BNI compounds on soil nitrification

Several incubation experiments were conducted to characterize the inhibitory function of hydrophilic-BNIs released from sorghum roots and the isolated BNIs, MHPP and sakuranetin on soil nitrification.

8a. Inhibitory effects of released BNIs on soil nitrification

Root exudate was evaporated to dryness and extracted with methanol (i.e. RE-methanol extract) as described earlier. The inorganic salts in the RE-methanol extract were removed as described (Subbarao et al. 2006b; Gopalakrishnan et al. 2009). The methanol in the desalted RE-methanol extract was removed using rotary-evaporator, and the BNI activity in the water-based medium was quantified for use in the soil-incubation experiments. The soil used in the incubation experiments was a volcanic ash, Typic Hapludands [pH (H_2O) 6.0; clay 54.8 %, silt 26.3 %, sand 18.9 %, total carbon=29.2 $mg\,g^{-1}$ soil; total N= 2.5 $mg\,g^{-1}$ soil; and C/N ratio 11.7] collected from the JIRCAS (Japan International Research Center for Agricultural Sciences, Tsukuba, Japan) experimental farm. The soil was air dried and passed through a 2-mm sieve before use. Two g soil was weighed into a 10-ml glass bottle with 0.72 ml of $(NH_4)_2SO_4$ solution providing 200 $mg\,N\,kg^{-1}$ of soil, and this treatment was termed the control. The soil water status was

maintained at 60 % WFPS (i.e. 60 % of the pore space was water filled; 0.36 mL water g^{-1} soil in this case) considered optimum for soil nitrification (Mosier et al. 1996). There were 7 BNI-activity treatments (0, 5.0, 10.0, 15.0, 20.0, 25.0 or 30.0 ATU g^{-1} soil) along with 3 DCD synthetic nitrification inhibitor treatments (10, 20 or 50 μg g^{-1} soil), and the samples were incubated at 20 °C or 30 °C; the experiment was replicated four times.

For the soil-BNI activity treatments, aliquots of root exudate with treatment level BNI-activity was added to the soil along with NH_4^+-N and the experiment was replicated four times; for DCD treatments, DCD (dissolved in water) was added according to the treatment along with NH_4^+-N. The bottle mouth was sealed using parafilm; a pinhole was made in the parafilm using a needle with 0.35 mm diameter to facilitate aeration. Each bottle along with the soil and treatment solution was weighed. The samples were placed in a temperature- and humidity-controlled incubator (bench-top type temperature and humidity chamber, ESPEC Corp., Osaka, Japan) and the treatment temperature set at 20 °C or 30 °C depending on the treatment; humidity was maintained at 95 %. Bottles were weighed every 15 d and water status adjusted with distilled water. After 55 d of incubation, soil samples were extracted by shaking with 20 mL of 2 M KCl for 30 min, and the contents filtered through Whatman No. 1 filter paper. The filtrate was analyzed for NH_4^+ with an indophenol colorimetric method and NO_3^- was determined with sulfanilamide-a-naphthylamine method using an Auto Ion analyzer (model AAII, Brant +Luebbe, Germany) (Litchfield 1967). The inhibition of nitrification by a treatment was calculated as described earlier (Subbarao et al. 2006b):

8b. Inhibitory effects of BNI-compounds, MHPP and sakuranetin on soil nitrification

BNI activity in sorghum root exudates is likely due to a mixture of inhibitory compounds whose identity is yet to be established and remains to be characterized, thus can only be expressed as inhibitory activity in ATU; this applies to soil incubation studies when BNI activity is added to the soil. For pure compounds such as MHPP, the inhibitory effect in the assay was very weak, 60–70 % inhibition in the assay concentration range of 100 to 1,000 μM, making it difficult to express in ATU for soil incubation studies. Also, dicyandiamide is ineffective in the assay system (ED_{80}=2,200 μM) as the secondary compounds released from dicyandiamide hydrolysis are the main potent inhibitors of *Nitrosomonas* in the soil. These are the reasons for adding the pure BNI comounds (including dicyandiamide) to the soil on a concentration basis than on inhibitory activity basis. Pure compounds: MHPP (©Aldrich) and sakuranetin (©Extrasynthase) were added to the soil, and thoroughly mixed to prepare a stock soil with a concentration of 10,000 μg g^{-1} soil for each of these two BNI compounds. This stock soil was used to prepare treatment concentrations of 50, 100, 200, 400, 600, 800, 1,000 and 2,000 μg of MHPP or sakuranetin g^{-1} soil; DCD was used as a standard synthetic nitrification inhibitor at a concentration of 25 μg g^{-1} soil. The soils were incubated at 20 °C and 85 % humidity for 60 d; the experiment was replicated four times. The other details of this soil incubation study were the same as described in Subbarao et al. (2008).

Statistical analysis of data

The data were subjected to two-way ANOVA using SPSS 3.5 statistical software (SPSS Inc., Chicago, IL).

Results

Release of BNIs at various growth stages of sorghum (Experiment 1)

Sorghum roots released two categories of nitrification inhibitors: the first category of inhibitors were those released into water-based collection medium, hereafter referred to as hydrophilic-BNIs; the second category of nitrification inhibitors were those released by washing roots for 30 s with dichloromethane (DCM), which has high-affinity for hydrophobic compounds, hereafter referred to as hydrophobic-BNIs (Fig. 1). We are introducing this terminology for the first time to separate these two kinds of nitrification inhibitors released from sorghum roots, and the concept is not just confined to sorghum roots, but covers other crops as well (unpublished preliminary observations).

Release of Hydrophilic-BNIs (monitored during 24 to 45 DAS) increased significantly ($P< 0.05$) from

Fig. 1 Hydrophobic- and hydrophilic- nitrification inhibitors (BNIs) released from sorghum roots and its significance to BNI function

about 40 ATU to about 100 ATU per 3 plants; during this period, the specific hydrophilic-BNI activity (i.e. ATU g^{-1} root dry wt.) declined significantly ($P< 0.05$) from about 71 to 15 (data not presented). Thereafter, the total hydrophilic-BNI activity produced per plant declined significantly ($P< 0.05$) despite an 8-fold increase in root (from 0.62 to 5.5 g $plant^{-1}$) and shoot dry matter (from 3.95 to 30.5 g $plant^{-1}$) at 130 DAS (Fig. 2). There was no significant ($P< 0.05$) change in the hydrophobic-BNI production (per plant) during 40 to 130 DAS (Fig. 2). It appears that amounts of hydrophobic- and hydrophilic-BNIs released from sorghum roots are similar (Fig. 2); the only exception was that the hydrophilic BNIs release declined substantially at 130 DAS. The BNI activity in the root tissue increased significantly ($P< 0.05$) with plant age until physiological maturity (i.e. 130 DAS) (Fig. 2). The specific BNI-activities (i.e. ATU g^{-1} root dry matter) of roots declined significantly ($P< 0.05$) with plant age (Fig. 3). The root tissue-BNI activity was only 50 to 60 % higher than the activities of BNIs released from sorghum roots at any growth stage (Figs. 2 and 3).

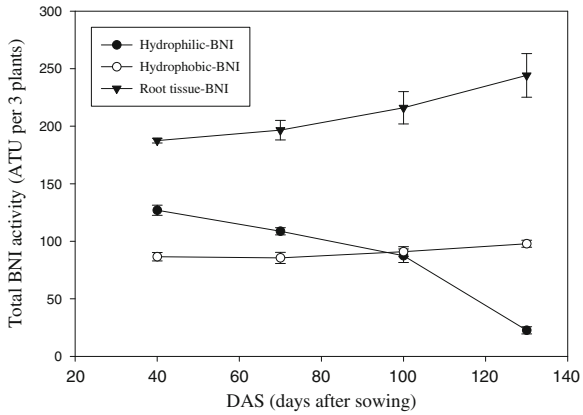

Fig. 2 Total BNI activity released in sorghum 40 to 130 DAS; *vertical bars* represent standard error of means (*n=4*)

Isolation and characterization of active constituent from hydrophilic-BNIs in root exudate (Experiment 2a)

Crude extract having BNI activity was fractionated by reverse-phase column chromatography. Activity-guided fractionations indicated that 10, 30 and 50 % methanol effluents represent most inhibitory activity; presently the 10 % methanol effluent is not purified; in an earlier study, we purified a BNI constituent from 30 % methanol effluent, which was identified as methyl 3-(4-hydroxyphenyl) propionate (MHPP) (Hossain et al. 2008). During this study, we purified the active constituent from 50 % methanol effluent using an activity-guided approach; this led to the isolation of another of the active components. The purified compound was subjected to EI-MS and NMR analyses and the spectral data revealed the identity of the isolated compound as sakuranetin (5,4′-dihydroxy-7-methoxyflavanone), a

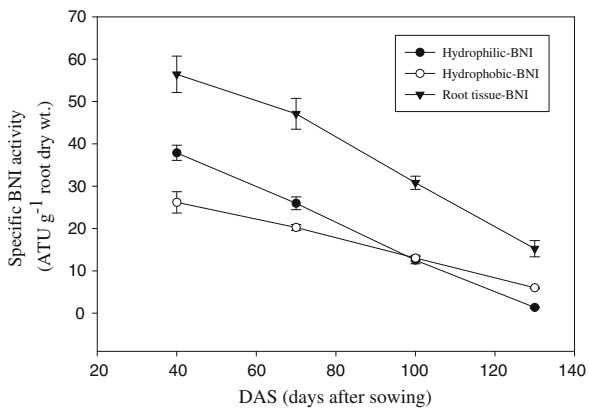

Fig. 3 Specific BNI activity released in sorghum at different growth stages; *vertical bars* represent standard error of means (*n=4*)

methoxy flavanone, classified as a pimarane-type diterpene phytoalexin (Fig. 4). The BNI activity of the authentic (standard) sakuranetin (obtained from @Extrasynthase) was confirmed by the bioassay, and the inhibitory effect on *Nitrosomonas* activity was linear in a concentration range from 0 to 2.0 μM.

Isolation and characterization of active constituent from DCM-root wash, i.e. hydrophobic-BNIs (Experiments, 2b; 3)

The HPLC chromatogram of crude DCM-root wash indicated a major peak and several minor peaks; however, only the major peak showed BNI activity in the bioassay. The constituent of this peak was purified repeatedly using HPLC, and the purified compound was subjected to NMR analysis. The NMR spectral data matched with that published for sorgoleone and its chemical structure is presented (Fig. 4) (Erikson et al. 2001). The molecular weight of the compound was confirmed by EI-MS data. Purified sorgoleone showed inhibitory effects on *Nitrosomonas* activity in the bioassay, and the strength of the inhibitory effect was linear in the concentration range from 0 to 13 μM (Fig. 5). The ED_{80} (effective dose for 80 % inhibition on *Nitrosomonas* function) for the isolated BNIs (MHPP, sakuranetin and sorgoleone) released from sorghum roots suggested that sakuranetin has the strongest inhibitory effect on *Nitrosomonas* function, followed by sorgoleone and MHPP (Table. 1; Fig. 5).

Mode of inhibitory action of BNI-activity on *Nitrosomonas* (Experiment 4)

Both the hydrophilic- and hydrophobic-BNIs (crude extracts) blocked the AMO (ammonia monooxygenase) and HAO (hydroxylamine oxidoreductase) enzymatic pathways in *Nitrosomonas* with similar effectiveness, as the addition of hydroxylamine to the assay medium did not alleviate the inhibitory effects (Table 2). However, this is based on the assumption that if an inhibitor blocks HAO, then most likely the AMO enzymatic pathway has also been blocked. Among the isolated BNIs, MHPP has inhibitory effect only on the AMO pathway, but did not affect the HAO enzymatic pathway. Sakuranetin and sorgoleone blocked both the AMO and HAO enzymatic pathways (Table 2). The three synthetic nitrification inhibitors (allylthiourea, nitrapyrin and dicyandiamide) blocked only the AMO

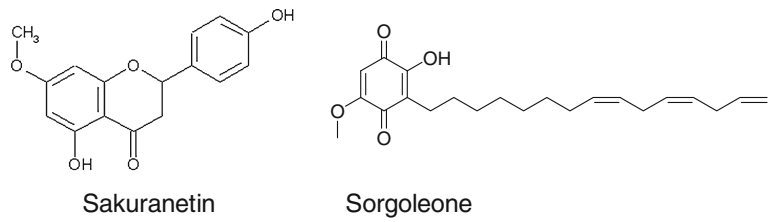

Fig. 4 Chemical structures of sakuranetin (MW 286.3) and sorgoleone (2-hydroxy-5-methoxy-3[(Z,Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone; MW 358.0)

enzymatic pathway in *Nitrosomonas* (Table 2), confirming our earlier results (Subbarao et al. 2008). These results are also in agreement with those published for synthetic nitrification inhibitors (McCarty 1999).

Longevity of the stimulatory effect of NH_4^+ on BNIs release in sorghum (Experiment 5)

We earlier showed that BNIs synthesis and release in sorghum was stimulated by the presence of NH_4^+ in the rhizosphere (Hossain et al. 2008). In this study, we monitored the activity of BNIs released after the removal of NH_4^+ during a subsequent 48 h period to understand the longevity of the stimulatory effect of NH_4^+ on BNIs release from sorghum. In the absence of NH_4^+, the BNI activity declined significantly ($P< 0.05$) (by nearly 50 %) within the first 24 h and by the next day (i.e. the 3rd day of the experiment, expressed as 3rd day in Fig. 6), the activity of BNIs released from sorghum roots was negligible (Fig. 6), indicating that the stimulatory effect from NH_4^+ lasted for about 24 h, although at a reduced rate of BNIs release; when NH_4^+ is reintroduced into root exudate collection solutions on 4th day, BNI activity of the root exudate has been restored to some extent (Fig. 6).

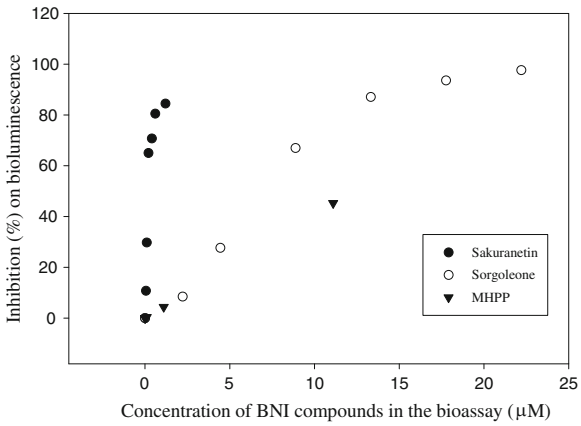

Fig. 5 Relative effectiveness of sakuranetin, sorgoleone and MHPP in inhibiting *Nitrosomonas* activity in an in vitro assay

Rhizosphere pH influence on BNIs synthesis, release, and stability of the released BNI activity (Experiments, 6; 7)

By using a pH-stat system, we separated the pH effects associated with NH_4^+ uptake and assimilation in the rhizosphere on BNIs synthesis and release in sorghum. When root exudate is collected using aerated solutions of 1 mM NH_4Cl for a 24 h period, the initial solution pH of 6.2 declined to about 3.1 within a 6 h period (based on our observations). With the pH-stat system, we could evaluate under identical conditions of NH_4^+ availability, the effects of a range of rhizosphere pH on BNIs release. The release of hydrophilic-BNI was significantly ($P< 0.05$) suppressed (>80 %) only at pH of 7.0; however, hydrophobic-BNI release and root tissue-BNI levels were not significantly ($P< 0.05$) affected by the pH treatments of the exudate solutions (i.e. 3 to 7) (Fig. 7). Hydrophilic-BNIs, once released from roots were stable to rhizosphere pH changes. Released hydrophilic-BNI activity retained its inhibitory function when subjected to pH treatments ranging from 3.0 to 9.0 (data not presented).

Table 1 Relative effectiveness of isolated BNIs from sorghum root exudate as compared with standard chemical nitrification inhibitors on *Nitrosomonas* in an in vitro bioassay

Compound	ED_{80} [effective dose (μM) for 80 % inhibition on *Nitrosomonas* function (i.e. bioluminescence) in an in vitro bioassay system]
Synthetic nitrification inhibitors	
©Allylthiourea	0.22
©Nitrapyrin	17.32
©Dicyandiamide	2200.00
Isolated BNIs released from sorghum roots	
Sakuranetin	0.6
Sorgoleone	12.0
MHPP	>120.0

Table 2 Nitrification inhibitory capacity of BNIs released from sorghum roots as compared with synthetic chemical inhibitors on the AMO and HAO enzymatic pathways of *Nitrosomonas*

Compound	Concentration (μM) in in vitro assay	Inhibition (%)	
		AMO pathway	HAO pathway
BNI activity/BNIs from sorghum root exudate			
Hydrophilic BNI activity		84.1±0.44	87.7±1.45
Hydrophobic BNI activity		71.7±1.20	75.7±0.88
MHPP	90.0	57.7±1.45	7.3±1.45
Sakuranetin	1.30	76.0±0.58	71.0±0.58
Sorgoleone	9.2	73.7±0.88	76.2±0.93
Synthetic nitrification inhibitors			
Allylthiourea	0.2	81.0±0.58	−0.3±2.73
Nitrapyrin	3.0	76.0±0.58	9.0±0.58
Dicyandiamide	2000	84.0±0.58	6.7±0.88

Nitrification inhibitory effects from released BNIs (i.e. crude extract of BNIs in root exudate) and purified BNIs (Experiments, 8a; 8b)

The BNIs added to the soil showed significant ($P< 0.05$) inhibitory effects on soil nitrification during 30 d of incubation (Fig. 8). Nearly 40 % inhibition of soil nitrification was observed when BNIs added to the soil reach 10 ATU g^{-1} soil, but subsequent increases in BNI activity added to the soil (up to 30 ATU g^{-1} soil) led to only a marginal increase in the inhibitory effect on nitrification; the inhibitory effects on soil nitrification were similar at 20 °C and 30 °C (Fig. 8). The synthetic nitrification inhibitor, DCD was most effective (≥80 %) in suppressing soil nitrification in the concentration range of 10 to 50 μg g^{-1} soil at 20 °C incubation temperature; however, at 30 °C, DCD was relatively less effective at 10 and 20 μg g^{-1}. At 50 μg g^{-1} soil, DCD gave ≥80 % inhibition on soil nitrification at both the incubation temperatures (data not shown). The isolated BNIs, MHPP and sakuranetin showed a contrasting response in suppressing soil nitrification, different from their suppressing effect in the *Nitrosomonas* culture-assay. Sakuranetin showed a stronger inhibitory effect than MHPP in the culture-assay, but has no inhibitory effect on soil nitrification (Fig. 9); MHPP showed significant ($P< 0.05$) inhibitory effects on soil nitrification; nearly 50 % inhibition on nitrification was observed at 200 μg g^{-1} soil and >1,000 μg g^{-1} soil is required to suppress nitrification up to 80 % (Fig. 9).

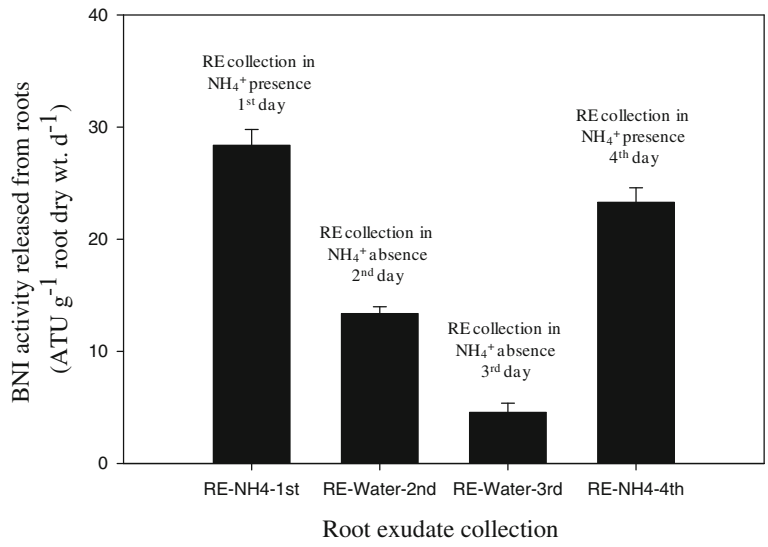

Fig. 6 Influence of NH_4^+ in the root exudate collection solutions on BNI activity release from sorghum roots; *vertical bars* represent standard error of means ($n=3$)

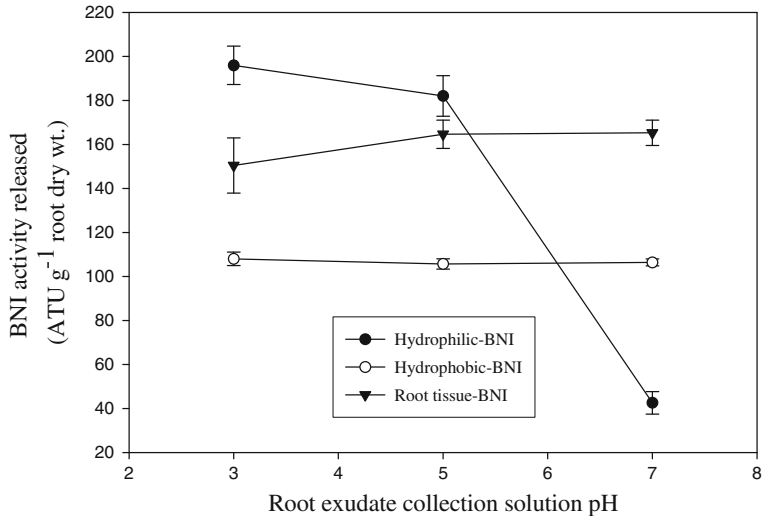

Fig. 7 Influence root exudate collection solution pH on the BNI release in sorghum; *vertical bars* represent standard error of means (*n=3*)

Discussion

BNIs release during various growth stages of sorghum

Sorghum roots released substantial amounts of BNIs and the release rates observed for the hydrophilic-BNIs (ranged from 10 to 25 ATU g^{-1} root dwt. d^{-1}) were similar to those observed earlier in *Brachiaria* sp. (Subbarao et al. 2006b, 2007b, 2009a, b). On a per plant basis, BNIs release reached a peak at about 50 DAS, and did not increase further despite an 8-fold increase in root growth during 50 to 130 DAS (physiological maturity). The combined activity of hydrophilic- plus hydrophobic-BNIs released on a daily basis is nearly equal to that has been observed in the root tissue, suggesting that most of the released BNI activity is synthesized from current photosynthesis; BNIs release thus could fluctuate depending on growing conditions. This is in contrast to the large reserve capacity (nearly 10-times) found in tropical grasses of the *Brachiaria* sp. where only 10 % of the BNIs found in the root tissue is released on a daily basis (Subbarao et al. 2007a, b).

We developed a methodology to measure BNI activities of the hydrophilic- and hydrophobic- fractions from sorghum roots. Based on the data presented, BNI activities of the two fractions released are similar. Due to their differential mobility and solubility in water, it is expected that hydrophobic BNIs may remain close to the root as

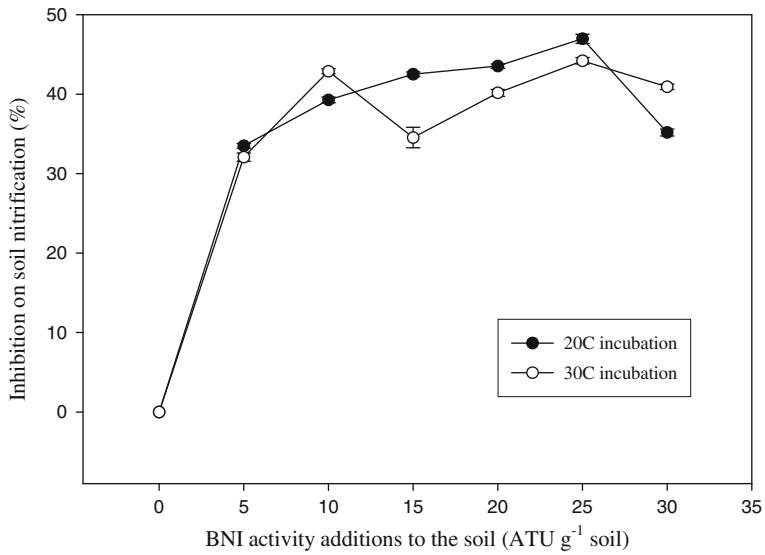

Fig. 8 Relationship between BNI activity (hydrophilic) added to the soil and inhibition on nitrification during a 30 d incubation period; *vertical bars* represent standard error of means (*n=4*)

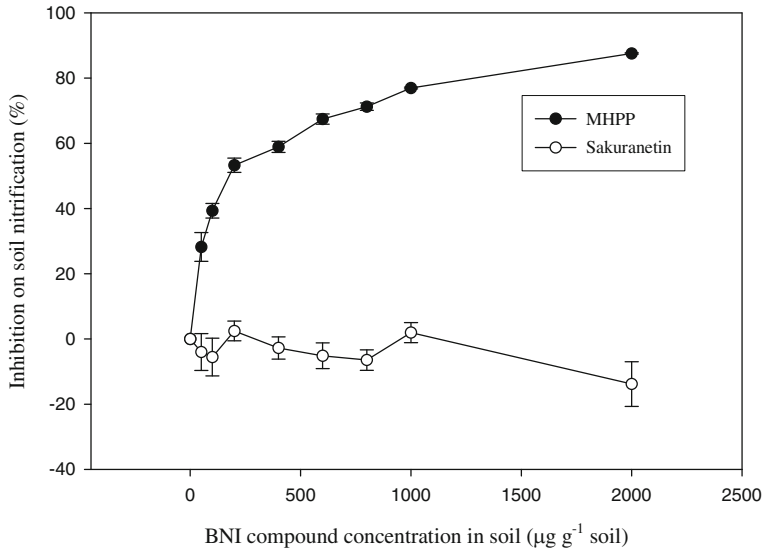

Fig. 9 Influence of pure BNI compounds: MHPP and sakuranetin addition on nitrification in soil during a 60-d incubation period; *vertical bars* represent standard error of means (*n=4*)

they could be strongly sorbed on the soil particles, increasing their persistence; their movement in soil is likely to be *via* diffusion across the concentration gradient and are likely to be confined to the rhizosphere (Dayan et al. 2010). In contrast, the hydrophilic-BNIs may move further from the point of release due to their solubility in water, and this may improve their capacity to control nitrification beyond the rhizosphere. The distribution of hydrophobic- and hydrophilic-BNIs in the rhizosphere, however, likely differ and may have a complementary functional roles, such as differential inhibitory effects on AOB (ammonia oxidizing bacteria, *Nitrosomonas*) vs. AOA (ammonia oxidizing archea). This could be an important topic for future research. Production of hydrophilic- and hydrophobic-BNIs are not just confined to sorghum roots, but are produced by other crops as well (unpublished observations).

The production and release of hydrophilic- and hydrophobic- BNIs appears to be of similar magnitude across the growth phases of sorghum. Based on the BNI activity observed (Figs. 2 and 3), we estimated the amounts of BNIs (hydrophilic plus hydrophobic) released from sorghum during a 130 d growing period (i.e. nearly physiological maturity) expressed as the equivalent of ©nitrapyrin (the commercial nitrification inhibitor) application (Table 3). BNI activity of the root exudate is expressed in ATU as allylthiourea is a salt and very stable at room temperature thus convenient to use as a standard (nitrapyrin which is volatile at room temperature thus difficult to use it as a

Table 3 Estimates of the BNI (hydrophilic and hydrophobic) activity release in sorghum cv. Hybridsorgo from 25 to 130 DAS (close to grain filling stage)

DAS	[a]BNI activity release d^{-1} $plant^{-1}$ (hydrophilic + hydrophobic)	Total BNI activity released during this period (ATU $plant^{-1}$)	[b]Inhibitory effect equivalent to nitrapyrin (μg)	[c]Amount of soil (g) where nitrification can be reduced up to 40 %
25 to 40	10+10	300	180	30
40 to 70	40+30	2100	1260	240
70 to 100	30+30	1800	1080	420
100 to 130	18+30	1440	864	564
Total (25 to 130)		5640	3384	

[a] Based on the data presented in Fig. 2

[b] One ATU in bioassay is equivalent to the inhibitory effect from 0.60 μg nitrapyrin

[c] About 10 ATU g^{-1} soil is needed to have about 40 % inhibition on soil nitrification based on the data presented in Fig. 8

standard for bioassay; allylthiourea standard solution once prepared can be used for several years and the inhibitory effect is very stable). Nitrification inhibitory effect from sorghum roots is quantified as equivalent to nitrapyrin application (Table 3) as nitrapyrin is extensively used in production agriculture as nitrification inhibitor; this makes it easy for researchers to comprehend the significance of BNI function when expressed as nitrapyrin equivalent. Similar approach was adopted when *Brachiaria* sp. were characterized for BNI function (Subbarao et al. 2009a). The amount of soil (in g) in which the released BNIs (based on data presented in Fig. 8) can potentially suppress nitrification, assuming that the released activity is stable and functionally effective, is presented (Table 3). Based on these estimates, it is evident that the nitrification inhibitors released per plant during a 130 d growth period could possibly reduce nitrification to ≤40 % in about 500 g soil (Table 3).

Isolation and identification of new BNIs

During this study, one of the components of hydrophilic-BNIs was isolated and identified as sakuranetin. Earlier we isolated MHPP, which contributes to BNI as a component of hydrophilic- BNIs in sorghum (Hossain et al. 2008). The inhibitory strength of sakuranetin is higher than that of MHPP (ED_{50} for sakuranetin is 1.5 μM vs. 100 μM for MHPP). Sakuranetin, a flavanone compound, is a phytoalexin synthesized in rice leaves for defense against rice blast and other fungal diseases (Atkinson and Blakeman 1982; Kodama et al. 1992). This is the first report of sakuranetin release from sorghum roots. Also, during this study we showed that sorghum roots release substantial amounts of hydrophobic BNI-compounds that contribute to the BNI-capacity in sorghum. Sorgoleone is the major constituent (>85 %) of the hydrophobic root exudate in sorghum (Einhellig and Souza 1992; Czarnota et al. 2001; Dayan et al. 2009), identified by the only major peak that has inhibitory function in the bioassay. With an ED_{50} of 8.0 μM and ED_{80} of 13.0 μM, sorgoleone is much stronger than MHPP, but weaker than sakuranetin in inhibiting nitrification (Fig. 7). Sorgoleone release from sorghum roots has been studied for its herbicidal and weeds suppressing functions (Netzly and Butler 1986; Czarnota et al. 2001; Dayan et al. 2009, 2010). Initially, sorgoleone was thought to be the major germination stimulant for seeds of parasitic weeds (*Striga* sp.) and hypothesized to be a key compound for the mechanistic basis for resistance to *Striga* infection (Netzly et al. 1988). Subsequently, it was discovered that dihydrosorgoleone, one of the analogues of sorgoleone has this function (Chang et al. 1986; Netzly et al. 1988; Erikson et al. 2001), while sorgolactones functions in stimulating *Striga* seed germination (Bouwmeester et al. 2007). Indeed five sorgoleone analogues are reported to be released from sorghum roots at various stages of growth (Barbosa et al. 2001), but no information is available on their BNI function.

Mode of inhibitory action

Ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) play critical role in the oxidation of NH_4^+ to NO_2^- in *Nitrosomonas* spp. (Bock et al. 1991). Crude extracts with hydrophobic- and hydrophilic- BNIs inhibited *Nitrosomonas* function by blocking both AMO and HAO enzymatic pathways. One of the hydrophilic-BNIs, MHPP blocked only the AMO pathway, but another constituent, sakuranetin blocked both AMO and HAO pathways in *Nitrosomonas*. Sorgoleone, the major constituent of hydrophobic-BNIs, blocked both AMO and HAO pathways. The unique chemical structure of sorgoleone, which has a hydroquinone head and a fatty acid tail with a terminal double bond, has the potential to disrupt the electron flow in *Nitrosomonas* and hence its biological activity (Rottenberg and Hashimoto 1986). The reducing power generated from the oxidation of hydroxylamine by HAO is thought to pass through cytochrome *c-554* to both cytochrome aa_3 oxidase and ubiquinone (McTavish et al. 1995), which is subsequently used for the reduction of $NHD(P)^+$, and for the maintenance of the AMO reaction (Hooper et al. 1997). Sorgoleone is known to interfere with the photosynthetic electron transport in chloroplasts (Dayan et al. 2009) and thus there is a likelihood of interference from sorgoleone with the electron transfer pathways of the cytochrome chain in the inner membrane of *Nitrosomonas*, disrupt the generation of $NHD(P)H_2$ and loss of light emission; this is independent of the direct effect on enzymatic pathways (i.e., AMO and HAO). Recently, linoleic acid, a major BNI compound present in the leaf tissue of *B. humidicola*, and brachialactone, a powerful nitrification inhibitor (ED_{80}=10.6 *m*M) released from its roots, were shown to block both AMO and HAO enzymatic pathways in a similar manner to that done by the BNIs of crude sorghum root exudate (both hydrophilic- and hydrophobic-), suggesting that a

single inhibitor can influence both the enzymatic pathways in *Nitrosomonas* (Subbarao et al. 2008, 2009a). High concentrations of monoterpenes in conifer forest systems have been reported to suppress nitrifier activity by blocking the AMO pathway (Ward et al. 1997). A few compounds such as phenyl, methyl, or hydroxyethyl hydrazine and hydrogen peroxide are known to inhibit the HAO enzymatic pathway in *Nitrosomonas* (Logan and Hooper 1995). Commercial nitrification inhibitors such as ©nitrapyrin and dicyandiamide suppress nitrifier activity by targeting primarily the AMO pathway (Hyman et al. 1988; McCarty 1999), which could be vulnerable to genetic changes in nitrifier populations or to natural genetic diversity in ammonia-oxidizers (AOs) (Norton et al. 2002; Belser 1979). It is thus likely that BNIs released from sorghum roots that comprise a cocktail of nitrification inhibitors with diverse modes of inhibitory action might indeed provide more durable inhibitory effects on nitrifier population to restrict soil nitrification.

Longevity of the stimulatory effect of NH_4^+ on BNIs release and the role of rhizosphere pH in the release of BNIs and their stability

Since the availability of NH_4^+ in the rhizosphere from fertilizer application or organic matter mineralization fluctuates over time, it is important that stimulatory effect of NH_4^+ in the rhizosphere lasts sufficiently long to ensure a continuing release of BNIs from sorghum roots. It appears that the stimulatory effect of NH_4^+ on the BNIs release lasts for about 24 h at a declining rate of release. The availability or presence of NH_4^+ in the rhizosphere is thus, critical for the sustained release of BNIs from sorghum roots. The molecular mechanisms involved and the functional links of NH_4^+ uptake, H^+ pumping activity with BNIs release are discussed in another paper (Zhu et al. 2012). Identification of sorghum cultivars/genetic-stocks that can release substantial amounts of BNIs in the presence of NH_4^+ for a short period or that retain the stimulatory effect long after the removal of NH_4^+ from the rhizosphere, would be desirable to ensure a sustained release of BNIs and facilitate reaching the critical BNI level (i.e. ≥10 ATU g^{-1} soil) in the rhizosphere. Preliminary results indicate the existence of such variability among sorghum germplasm lines and cultivars (G.V. Subbarao, unpublished results).

Rhizosphere pH appears to have a major effect on the release of hydrophilic-BNIs from sorghum roots. Threshold rhizosphere pH appeared to be ≤5.0, and at higher pH (i.e. >5.0), hydrophilic-BNIs release was severely affected; there was 80 % decline in the release of hydrophilic-BNIs at rhizosphere pH of 7.0 or higher. Hydrophobic-BNIs release appeared to be relatively less severely affected by the changes in rhizosphere pH. Also, once the BNIs are released from roots, their nitrification inhibitory function appears to be relatively stable over a pH range of 3.0 to 9.0. This is in contrast to BNIs released from *B. humidicola*, reported to be sensitive to rhizosphere pH ≥4.6, with a total loss of inhibitory function at pH ≥8.0 (Subbarao et al. 2007a). Hydrophilic-BNIs release from sorghum is highly sensitive to rhizosphere pH of 5.0 or higher. The heavy black soils (Vertisols), which generally have soil pH of ≥7.0, have a large buffering capacity (Burford and Sahrawat 1989) that resists changes in rhizosphere pH. Sorghum grown on soils with pH in the alkaline range might not release BNIs and hence such soil types might not be suitable for the expression of BNI function in sorghum. Perhaps, light-textured soils with a low-buffering capacity and moderate acidity (pH 5.0) might be better suited for the expression and exploitation of the BNI function in sorghum.

Contribution of the isolated compounds with BNI activity to inhibition of soil nitrification

The hydrophilic-BNIs in sorghum root exudate contributes moderately (≤40 % inhibition) to suppression of soil nitrification. A minimum inhibitory activity of about 10 ATU g^{-1} soil or higher is needed to suppress soil nitrification and further increase of BNI activity did not improve inhibitory effects on soil nitrification. These results suggest that compared with the BNI activity from *Brachiaria* sp., which suppressed nitrification by ≥70 % at ≥15 ATU g^{-1} soil (Subbarao et al. 2006b), the BNI activity from sorghum appears to have a moderate inhibitory effect on soil nitrification. The inhibitory effect on soil nitrification appears to be stable in the temperature range of 20 to 30 °C. In contrast, dicyandiamide, the commonly used synthetic nitrification inhibitor, is more effective at 20 °C than at 30 °C.

Soil -physical, -chemical and -biological processes can modulate the effectiveness of BNIs (Goring 1962a,

b; Sahrawat 1996; Subbarao et al. 2012). The isolated BNI compounds, MHPP and sakuranetin had distinctly different trends in inhibiting nitrification in soil as compared to their performance in the culture-bioassay. MHPP showed a relatively weak inhibitory activity in the culture-bioassay, but showed a moderate and stable inhibitory effect on soil nitrification. In contrast, sakuranetin showed strong inhibitory activity in the culture-assay, but its inhibitory function was lost in the soil-assay. Sakuranetin released from sorghum roots thus may not contribute to its potential BNI capacity, showing that not all compounds with BNI activity detected in an in vitro culture-bioassay are effective in the soil. Determination of BNI activity in the root exudate thus, provides an initial assessment of the BNI-potential of a cultivar/genetic stock or plant species. We suggest that BNI activity effectiveness should be confirmed using a soil system as part of the characterization of BNI -function and -capacity of sorghum genetic-stock/cultivar. It is likely that the amount of functional:non-functional components with BNI activity may vary among sorghum germplasm lines. It is thus prudent to select genetic stocks that not only have high potential to release BNIs, but the released BNIs should be effective and functionally stable in controlling the soil nitrifier activity.

Conclusions

Two categories of nitrification inhibitors are released from sorghum roots: hydrophilic- and hydrophobic-BNIs. Sakuranetin and sorgoleone are two newly identified nitrification inhibitors with BNI activity of multi-mode action on *Nitrosomonas*. Unlike hydrophobic-BNIs, the release of hydrophilic-BNIs are highly sensitive to rhizosphere pH >5.0. Apart from MHPP and sakuranetin, the hydrophilic-BNIs may consist of a cocktail of nitrification inhibitors, whose chemical identities are yet to be established. Though the BNIs release rates in sorghum were similar to that observed from *Brachiaria* pastures, the potential impact on soil nitrification could be limited due to the short growth duration of sorghum (120−150 d) compared to that of perennial *Brachiaria* pastures (5 to 10 y), and the presence of non-functional components of BNIs. There is thus an urgent need to identify sorghum genetic stocks with high potential to release functional BNIs, perhaps orders of magnitude higher than that detected in the current cultivars, in order to suppress nitrifier activity and nitrification in sorghum-based production systems.

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