Title
Biological Nitrification Inhibition (BNI) Potential in Sorghum

Authors
Guntur, Subbarao V
Zakir, Hossain AKM
Nakahara, K
et al.

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Introduction

The biological oxidation of ammonia is carried out by two groups of chemotrophic bacteria (*Nitrosomonas* sp. and *Nitrobacter* sp), a process termed 'nitrification', results in transformation of NH_4^+ (a relatively immobile nitrogen form) to NO_3^- (a nitrogen form that is highly mobile), making soil nitrogen susceptible to losses through multiple pathways - leaching (of NO_3^-) and gaseous losses (N_2, NO, N_2O) (Galloway et al. 2008). Due to these nitrogen losses, a major portion of the soil nitrogen and applied fertilizer nitrogen (>60%) is lost, in low nitrogen-use efficiency of agricultural systems (Subbarao et al. 2006a; Galloway et al. 2008; Schlesinger, 2009). Also, nitrogen pollution is the single most environmental concern from agricultural systems, contaminating ground and surface water (Galloway et al. 2008; Schlesinger, 2009). Denitrification of nitrate-N is the major source of nitrous oxide emissions from agricultural systems and contributes significantly to global warming (Kroeze, 1994). Blocking the function of nitrifying bacteria or slowing the nitrifiers' function (i.e. reducing the nitrification rates) can significantly reduce nitrogen losses associated with nitrification and extend the persistence of nitrogen as ammonium in the soil for uptake by plants, lead to improved nitrogen recovery and –use efficiency in agricultural systems (Subbarao et al. 2006a). Recently, it was shown that some plant species have the ability to release nitrification inhibitors from roots that suppress nitrifiers' function, a phenomenon termed 'biological nitrification inhibition' (BNI) (Subbarao et al. 2006b; Fillery, 2007). Using a luminescent recombinant *Nitrosomonas*, an assay has been developed to detect and quantify this inhibitory effect (BNI-activity, expressed in ATU, defined as the inhibitory effect from 0.22 μM allylthiourea in an assay containing 18.9 mM of NH_4^+) in plant soil systems (Iizumi et al. 1998; Subbarao et al. 2006b). With this newly developed methodology, it was shown that several tropical grasses and certain field crops including sorghum possessed the ability to release nitrification inhibitors from roots, termed BNI-capacity (Subbarao et al. 2007a,b,c; Fillery, 2007). We showed that sorghum roots release methyl 3-(4-hydroxyphenyl)propionate (MHPP), one of the active constituents with BNI activity of root exudates from sorghum (Zakir et al. 2008). Here we present our findings on further characterization of BNI function in sorghum and reveal the identity of the two nitrification inhibitors released from its roots. In addition, we present evidence to show wide genetic variability in the release capacity of one of the major nitrification inhibitors from sorghum roots and also report preliminary field-based results for the existence of BNI-capacity in wild sorghum.

Materials and Methods

1. ***Influence of growth stage on BNI activity release from sorghum roots***

   Raising seedlings of sorghum. Sorghum (*Sorghum bicolor* (L.) Moench), cv.ICSV 745 seeds were germinated in trays containing sand and moistened with distilled water. Plants were grown in a growth chamber with a day : night temperature of 30 : 28ºC, a

photosynthetic photon flux, averaging 300 μmol m^{-2} s^{-1} and a 14 : 10 h light : dark photoperiod. One-week-old seedlings were transferred to aerated nutrient solution in 75 L tanks on Styrofoam blocks with 12 holes and three plants per hole, supported with sponge. The composition of the nutrient solution is a modified Hoagland nutrient solution described earlier (Subbarao et al. 2006b). Nitrogen at 1 mM was added as $(NH_4)_2SO_4$ to the nutrient solution. The pH of the nutrient solution is adjusted to a range of 5.0 to 5.5 with 1N NaOH once a day. The nutrient solutions were replaced every 7 d.

Root exudates collection. Root exudates were collected from intact plants at 21, 29, 37, and 45 DAS (days after sowing). For collection of root exudates, intact plants (a sample size of three plants with four replications) were removed from the nutrient solution, the roots were rinsed with deionized water followed by distilled water, then immersed for 24 h in 2 L aerated solution of 1 mM NH_4Cl with 200 μM $CaCl_22H_2O$. After root exudate collection, the roots and shoots were separated and dried at 70ºC for 48 h in a forced air-circulating oven before determining their dry weight. For extraction of BNI compounds, root exudates are first evaporated to dryness using a rotary-evaporator (Buchi, V-850, Flawil, Switzerland) under vacuum at 45ºC, followed by extraction of the residue with 20 ml of methanol. The methanol extract was evaporated to dryness using rotary-evaporator at 35ºC and the residue was extracted with 120 μL of dimethyl sulphoxide (DMSO). The DMSO extract was then used to determine the BNI activity (Subbarao et al. 2006b).

Nitrification inhibition assay. The BNI activity of the root exudate sample (i.e. DMSO extract) was determined using a modified bioassay that employs recombinant luminescent *Nitrosomonas europaea* (Iizumi et al. 1998; Subbarao et al. 2006b). The BNI activity of the samples expressed in units defined in terms of the action of a standard inhibitor, allylthiourea (AT); the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one ATU (AT unit) of activity (Subbarao et al. 2006b).

2. Isolation of major BNI constituent from sorghum root exudates

Collection of root exudates with BNI activity. Seedlings of sorghum cv. Hybrid Sorgo were raised as described earlier, and plants were grown hydroponically for 50 d in a growth chamber, similar to the growing conditions described earlier. Root exudate was collected using 1 mM NH_4Cl with 200 μM $CaCl_22H_2O$. Constituents corresponding to nearly 5000 ATU were collected from about 1000 plants of 50 d old (about 250 L of root exudates) for the isolation of a major active compound.

Isolation of major BNI constituent. The crude methanol extracts containing BNI activity were diluted with water to reach a volume of 5% methanol before loading on to a reverse-phase column (25 cm x 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, and 30 fractions of 7 ml. All the

fractions were dried *in vacuo* at 35ºC and the residues were dissolved in MeOH (1 ml); aliquots of these fractions (50 µl) were dried again *in vacuo* at 35ºC and dissolved in 10 µl DMSO; 2 µl 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 x 100 mm or 10 mm x 100 mm) column (Tosoh, Tokyo, Japan), monitored at 280 nm. The column was eluted stepwise with aqueous acetonitrile (10 to 50%) and all the peaks and troughs were checked for BNI activity in the bioassay. Finally, the major active component from 50% methanol fraction was purified using an isocratic HPLC using 25% acetonitrile as mobile phase.

3. Isolation of major BNI constituent from DCM-root wash

Collection of constituents with BNI activity using DCM-root wash. Sorghum seeds (cv. Hybrid Sorgo) are soaked in water for 4 h, and then germinated in the dark at 28ºC on moistened filter paper discs placed in petri-plates. Root tissue was excised from 8 d old seedlings and immediately dipped in dichlomethane (DCM) with 1% acetic acid (v/v DCM: acetic acid 99:1) for 20 s.

Isolation of major BNI constituent. The DCM root-wash was then filtered, evaporated to dryness using rotary-evaporator at 40ºC. The residue was dissolved in acetonitrile. About 5000 ATU activity was obtained by washing 200 g of fresh roots with acidified DCM. The BNI compounds were purified using activity-guided HPLC fractionation. A major active constituent was eluted at 10 min with HPLC conditions of isocratic system of 63% acetonitrile and 37% of 0.5% formic acid with absorption at 280 nm.

4. *Instrumental analysis.*

The mass (MS) spectra of the isolated compounds were recorded on a GCMS-QP2010 spectrometer (Shimadze, Kyoto, Japan) by direct electron ionization (EI) at an ionization energy of 70 eV. The 1D-^{1}H and ^{13}C NMR and 2D-NMR spectra including DQF-COSY, HSQC, HMBC were recorded at 298 K on Avance 500 and Avance 800 spectrometers (Bruker Biospin, Karlsruhe, Germany). For NMR analysis, the active constituent from root exudates was dissolved in CD_3OD and that from DCM-root wash was dissolved in $CDCl_3$.

5. Genotypic variability for sorgoleone production capacity

A total of 38 advanced sorghum breeders lines that include several high-yielding cultivars were included for this study to determine genetic variation for sorgoleone-producing capacity of roots and to analyze the relationship between sorgoleone levels to the BNI activity of the root-DCM wash (which recovers most of the hydrophobic compounds

exuded from roots). Seedlings are raised as described earlier and root-DCM wash was collected from 10 d old seedlings (20 seedlings considered as an experimental unit; the experiment was replicated four times). BNI activity of the root-DCM wash was determined as described earlier. Sorgoleone levels in the root-DCM wash were determined using the HPLC as described above.

6. Determining the BNI-capacity of wild sorghums under field conditions

Rhizoplane soil samples (defined as the soil in the vicinity of the roots) were collected from perennial wild sorghums [*S. nitidium* (IS-243513) and *S. arundinaceum* (IS-18828)] established in Alfisol fields of ICRISAT (International Crops Research Institute for the semi-Arid Tropics). Plants were two-year old at the time of soil sampling. Fertilizer-N was applied as urea (@60 kg N ha^{-1} y^{-1}). The air-dried rhizoplane soil samples (2 g soil is used as experimental unit in a glass vessel and the experiment was replicated three times) were used for the determination of nitrification rates in the soil. A control soil was collected from the adjacent field plots without a crop. For each incubation vessel containing 2 g of soil, 400 μg N as $(NH_4)_2SO_4$ was added; soil water status during the experiment was maintained at a level where 60% of the soil pore space was water-filled, considered as optimum for nitrification (WFPS) (Mosier et al. 1996); soil samples were incubated at 20ºC and 80% relative humidity in an incubator. The detailed protocol of the incubation study was described earlier (Subbarao et al. 2006b).

Results and Discussion

Influence of growth stage on the release of BNI activity from sorghum roots

The specific activity (i.e. ATU g^{-1} root dwt d^{-1}) in sorghum roots declined with age of the plants; the BNI activity decreased from 60 ATU at 3 week to 20 ATU at 45 days after germination, indicating that the young roots released higher BNI activity than did the older roots (data not presented). The total BNI activity (i.e. ATU $plant^{-1}$ d^{-1}) released from roots increased to nearly three-fold during this period, largely due to an increase in the root mass, about 14-fold increase during this period (data not presented). The BNI activity levels measured during this study were substantially higher than reported earlier (Subbarao, et al. 2007b), possibly due to different cultivar used in this study (ICSV 745 compared to Hybrid Sorgo used earlier) and differences in growing conditions and/or sampling time.

Isolation and characterization of constituent with BNI activity from root exudate

Crude extracts with BNI activity was fractionated by reverse-phase column chromatography. Activity-guided fractionations indicated that 10, 30 and 50% methanol effluents represent most inhibitory activity (Fig. 1). In an earlier study, we purified a BNI constituent from 30% methanol effluent and identified it as methyl 3-(4-hydroxyphenyl) propionate (MHPP) (Zakir et al. 2008). In this study, we purified the active constituent from 50% methanol effluent using activity-guided approach; this led to the isolation of one of the active components (Fig. 1). The purified compound was subjected to NMR analysis and the spectral data revealed the identity of the isolated compound as sakuranetin (The detailed physico-chemical properties and the NMR data is not presented here) (Fig. 1a). The BNI activity of the authentic (standard)

sakuranetin (obtained from ©Extrasynthese) was confirmed by the assay and the strength of the inhibitory effect on *Nitrosomonas* activity was linear in the concentration range from 0 to 2.0 μM.

Isolation and characterization of constituent with BNI activity from DCM-root wash

HPLC chromatogram of the crude DCM-root wash indicated a major peak (Fig. 2) and several minor peaks; only the major peak showed BNI activity in the assay, and the constituent was purified repeatedly using HPLC; the purified compound was subjected to ^{1}H and ^{13}C NMR analysis. The NMR spectral data matched with the published information on sorgoleone, 2-hydroxy-5-methoxy-3-[(8'Z,11'Z)-8',11',14'-pentadecatriene]-*p*-benzoquinone (Fig. 2a) (Erickson et al. 2001). The molecular weight was confirmed by EIMS data. Purified sorgoleone showed inhibitory effect on *Nitrosomonas* activity in the bioassay and the strength of the inhibitory effect was linear in the assay in the concentration range of 0 to 13 μM (data not presented).

Genetic variability in sorgoleone production capacity of sorghum roots

Substantial genetic variability for sorgoleone production capacity was found in sorghum germplasm, similar to the BNI activity of the root-DCM wash (data not presented). The BNI activity ranged from 0.0 (i.e. no activity in IS-720) to 22.0 (ATU in PVK 801). Sorgoleone production from roots ranged from 0.0 (in IS-720) to 150 μg g^{-1} fresh root wt (data not presented). The BNI activity of the root-DCM wash showed a significant positive linear relationship ($r^2 = 0.64^{**}$; n = 38) with sorgoleone levels. Based on the analysis of 38 sorghum genotypes (Fig. 3), a major portion of the BNI activity from root-DCM wash (i.e. the hydrophobic compounds exuded from sorghum roots) appear to be due to the presence of sorgoleone. Nearly 40% of the variability in BNI activity in the root-DCM wash however, is not due to sorgoleone, but other hydrophobic compounds, yet to be identified along with their relative contribution to the BNI activity. Sorghum is also known to release several analogues/cogeners of sorgoleone (Barbosa et al. 2001; Kagan et al. 2003) and their inhibitory effects on *Nitrosomonas* activity is unknown at present, hence will be the subject of future investigations.

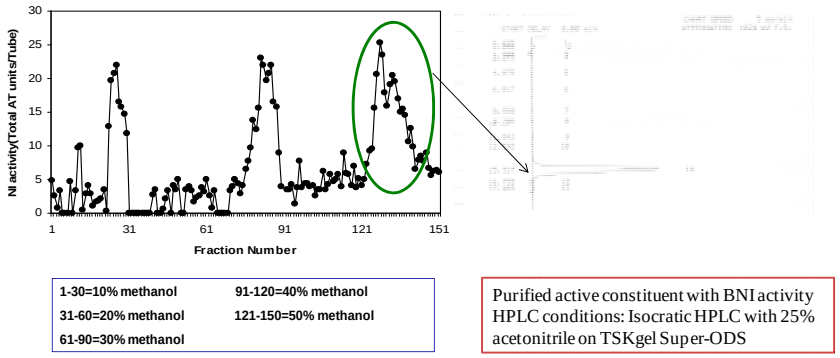
BNI-capacity of wild-sorghums under field conditions

One of the wild sorghum sp. *S. nitidium* (a collection from Australia) showed no inhibitory effect in its rhizoplane soils as all the added ammonium nitrogen was nitrified at the end of 60 d incubation period (Fig. 4). In contrast, wild sorghum sp. *S. arundinaceum* (a collection from Tanzania), only 33% of the added ammonium N was nitrified, indicating substantial reduction in the soil nitrification potential (Fig. 4); this is the first direct field-based evidence for BNI function in the wild-sorghum species, which can be exploited using genetic means, especially in case sufficient stable BNI-capacity is not found in cultivated sorghums. However, a thorough characterization of the BNI capacity of *S. arundinaceum* along with the identification of the inhibitors released is needed before using this wild sp. as a source for genetic improvement in the BNI capacity of the cultivated sorghum.

Conclusions and Perspectives

Our results indicate substantial levels of BNI activity released from sorghum roots and the BNI-capacity of the roots decline with age. One of the active components of BNI activity is isolated and identified as sakuranetin. This is the first report of sakuranetin to our knowledge to be released from sorghum roots. Also, BNI activity of sakuranetin is novel to our knowledge and the inhibitory strength is several-fold higher than that of MHPP (ED_{50} = 1.5 μM for sakuratin *vs*. ED_{50} = 20 μM for MHPP). Also, during this study we demonstrated that sorghum roots release substantial amounts of hydrophobic compounds that contribute to the BNI-activity of its roots; the major constituent identified as sorgoleone. Sorgoleone contributes to about 80% of the hydrophobic component of BNI-activity (in sorghum cv. Hybrid Sorgo). Though sorgoleone is known to be released from sorghum roots (Netzly and Butler, 1986; Dayan et al. 2003), this is the first report indicating it's -inhibitory effect on *Nitrosomonas* activity. Sorgoleone was initially thought to be the germination stimulant for witchweed (*Striga asiatica*) and suspected to be the key mechanistic basis for resistance to Striga infection (Netzly et al. 1988), but later it was found that other compounds (such as strigalactone) play more important role in those functions (Chang et al. 1986; Netzly et al. 1988; Fate et al. 1990; Erickson et al. 2001). Our results suggest a wide range of genetic variability for sorgoleone release-capacity in sorghum, and this variability has a -positive association with the hydrophobic component of BNI activity from sorghum roots. The existence of significant and substantial genetic variability is a pre-requisite for genetic improvement of this trait (i.e. sorgoleone-release capacity of roots) and our results indicate potential for selecting high-sorgoleone producing genetic stocks as a means to improve the BNI-capacity of sorghum roots.

In addition, our preliminary field studies indicate the potential for wild sorghum sp. such as *S. arundinaceum* acting as a genetic source for high-BNI capacity. Given the current interest in commercial and large-scale exploitation of sorghum as a bio-ethanol crop and with the anticipated increase in nitrogen inputs as an industrial crop (Reddy et al. 2005), the BNI function could play an important role in improving the nitrogen-use efficiency in these production systems. Concerns were raised recently about the carbon-neutrality and environmental advantages from bio-fuel/bio-ethanol crops due to high N_2O emissions (linked with nitrification and denitrification processes) and CO_2 emissions (associated with land-use change), negating the carbon benefits associated with bio-fuel crops (Searchinger et al. 2008; Fargione et al. 2008; Hill et al. 2009). The BNI function in sorghum thus, could become a critical trait targeted for genetic improvement, as part of an integrated strategy towards development of sorghum varieties with adequate BNI-capacity to suppress soil nitrification and utilize nitrogen in NH_4^+ form. Such genetic capacity to suppress soil nitrification is a pre-requisite towards the development of low-N_2O emitting production systems for both economic and ecological sustenance.



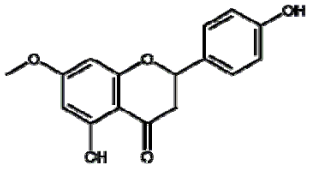


Fig. 1. Purification of BNI active constituent from root exudates and chemical structure of sakuranetin, the active constituent.

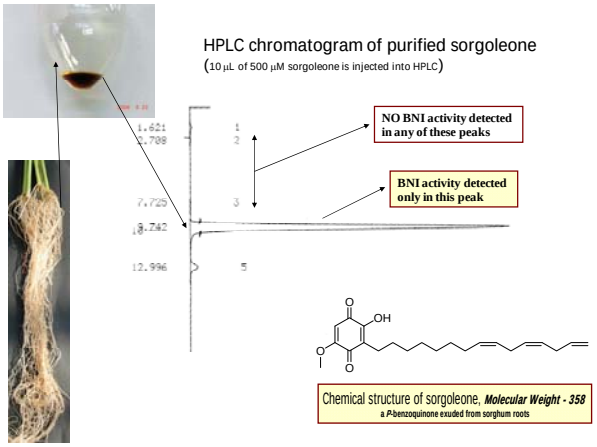


Fig. 2. Purification of BNI active constituent from DCM-root-wash and chemical structure of sorgoleone, the active constituent.

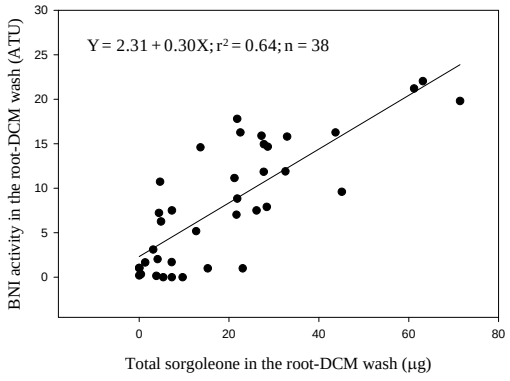


Fig. 3. Relationship between BNI activity of the root-DCM wash and sorgoleone levels among 38 sorghum genotypes (each data point is the mean of four replications from each genotype)

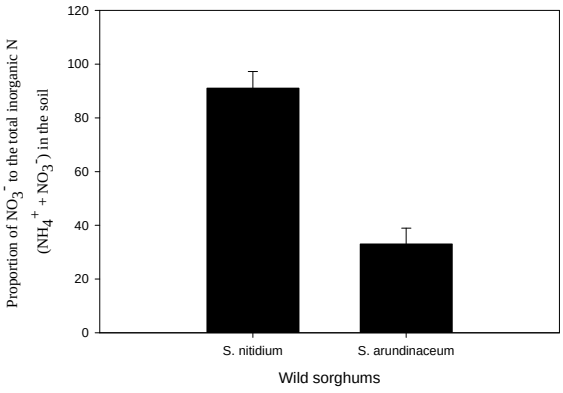


Fig. 4. Contrasting nitrification properties of rhizoplane soils collected from wild sorghum species grown in Alfisol fields after two years of establishment.

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