

# Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*)

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## Summary

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- Nitrification results in poor nitrogen (N) recovery and negative environmental impacts in most agricultural systems. Some plant species release secondary metabolites from their roots that inhibit nitrification, a phenomenon known as biological nitrification inhibition (BNI). Here, we attempt to characterize BNI in sorghum (*Sorghum bicolor*).
- In solution culture, the effect of N nutrition and plant age was studied on BNI activity from roots. A bioluminescence assay using recombinant *Nitrosomonas europaea* was employed to determine the inhibitory effect of root exudates. One major active constituent was isolated by activity-guided HPLC fractionations. The structure was analysed using NMR and mass spectrometry. Properties and the 70% inhibitory concentration (IC<sub>70</sub>) of this compound were determined by *in vitro* assay.
- Sorghum had significant BNI capacity, releasing 20 allylthiourea units (ATU) g<sup>-1</sup> root DW d<sup>-1</sup>. Release of BNI compounds increased with growth stage and concentration of NH<sub>4</sub><sup>+</sup> supply. NH<sub>4</sub><sup>+</sup>-grown plants released several-fold higher BNI compounds than NO<sub>3</sub><sup>-</sup>-grown plants. The active constituent was identified as methyl 3-(4-hydroxyphenyl) propionate.
- BNI compound release from roots is a physiologically active process, stimulated by the presence of NH<sub>4</sub><sup>+</sup>. Methyl 3-(4-hydroxyphenyl) propionate is the first compound purified from the root exudates of any species; this is an important step towards better understanding BNI in sorghum.

**Key words:** biological nitrification inhibition, BNI compounds, methyl 3-(4-hydroxyphenyl) propionate, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, sorghum (*Sorghum bicolor*).

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## Introduction

Nitrification is an important biological process in nitrogen (N) cycling, whereby ammonium (NH<sub>4</sub><sup>+</sup>), either added to

soils as fertilizer or formed through decomposition of organic N compounds, is oxidized to nitrite, and subsequently nitrate, by nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*, respectively). Nitrogen use efficiency (NUE) for global cereal production is

c. 33%. The unaccounted 67% represents a \$US16.7 billion annual loss, assuming fertilizer–soil equilibrium (Raun & Johnson, 1999). Nitrification is one of the main causes of inefficient N use and N loss-associated environmental pollution as a result of  $\text{NO}_3^-$  leaching and denitrification. The nitrification process, resulting from subsequent denitrification, is the principal agricultural source of  $\text{N}_2\text{O}$  emissions.  $\text{N}_2\text{O}$  is presently the third most important gas contributing to global warming (Parker, 1972). If the nitrification process is inhibited, N recovery by crop uptake can be improved, and  $\text{NO}_3^-$  leaching and  $\text{N}_2\text{O}$  emission can be reduced (Slangen & Kerckhoff, 1984; Subbarao *et al.*, 2006a). Excessive nitrification can be reduced by the release of inhibitors from plant roots or by application of synthetic nitrification inhibitors (Smart & Bloom, 2001; Weiske *et al.*, 2001). Application of synthetic nitrification inhibitors is currently the only method being used proactively to reduce soil nitrification in some agricultural production systems (Slangen & Kerckhoff, 1984; Amberger, 1989; Zerulla *et al.*, 2001). The existence of plant-derived nitrification inhibitors has been known for several decades (Moore & Waid, 1971; Lata *et al.*, 1999, 2004); however, previous reports have been limited in their interpretation of the effect of root exudates on soil bacteria because of the unavailability of methodology that could directly demonstrate the inhibitory capacity. Recently, a bioassay system was developed that enabled quantification of the inhibitory effect from roots. The phenomenon was termed biological nitrification inhibition (BNI) (Subbarao *et al.*, 2006b). *In situ* production of nitrification inhibitors, by either plant roots or microorganisms within the rhizosphere of growing crops or pastures, is an appealing low-cost alternative to synthetic nitrification inhibitors (Fillery, 2007).

A range of field crops and pastures, including sorghum, have been evaluated for BNI capacity. Cultivated sorghum was found to have significant BNI capacity; while other important cereals such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*) and maize (*Zea mays*) did not show BNI capacity (Subbarao *et al.*, 2007a). Earlier reports also indicated the possibility of sorghum producing nitrification inhibitors from roots (Russell, 1914; Alsaadawi *et al.*, 1986). Sorghum has a strong phytotoxic impact on weeds (Einhelling & Rasmussen, 1989; Wetson, 1999). This toxicity results from the production and release of phenolic acids (Burgos-Leon *et al.*, 1980; Ben-Hammouda *et al.*, 1995), sorgoleone and xenognosin (Netzly & Butler, 1986; Czarnota *et al.*, 2003). Presently, no compounds have been isolated from sorghum root exudates that have been shown to inhibit nitrification.

Ammonium ( $\text{NH}_4^+$ ) has a stimulatory role in the release of BNI compounds from *Brachiaria humidicola* and *Leymus racemosus* roots (Subbarao *et al.*, 2007b,c); however, the exact roles of  $\text{NH}_4^+$  or the associated acidic pH that follows after its uptake (i.e. the secondary effect from ammonium uptake) in the release of BNI compounds from plant roots have not yet

been clarified. Supplying N as  $\text{NH}_4^+$  during exudate collection will result in pH decrease over the time period of collection, because of proton release to charge balance the ammonium uptake by the roots. Conversely, supplying N as the anion nitrate results in pH increase (Raven *et al.*, 1992). Decreased pH will affect root membrane permeability (Wollenweber, 1997), and this may cause increased release of compounds responsible for BNI. Therefore, in order to avoid fluctuations to these processes in a hydroponic study, we maintained constant pH in the nutrient solution during plant growth and root exudate collection period using a pH-stat system. Identifying the compound(s) responsible for BNI by sorghum could allow for more detailed physiological characterization of this trait in the future. Therefore, the objectives of our study were to evaluate the regulatory role of N forms (i.e.  $\text{NH}_4^+$  or  $\text{NO}_3^-$ ) in the root environment on the release of BNI compounds from roots, and to isolate, identify and characterize the compounds responsible for BNI function in terms of chemical properties, release stimulation and mode of inhibitory action.

## Materials and methods

### Expt 1: assessment of BNI activity in root exudates with increasing plant age

**Raising seedlings of sorghum** Seeds of sorghum (*Sorghum bicolor* (L.) Moench var. hybrid sorgo) were germinated in trays containing sand and watered with distilled water. Plants were grown in a growth chamber with a day : night temperature regime of 30 : 28°C, a photosynthetic photon flux, averaging c. 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and a 14 : 10 h light : dark photoperiod. One-week-old seedlings were transferred to continuously aerated nutrient solution in 75 l tanks on styrofoam blocks with 10 holes and two plants per hole, supported with sponge. The composition of the nutrient solution ( $\text{mg l}^{-1}$ ) was as follows:  $\text{KH}_2\text{PO}_4$ , 38.31;  $\text{K}_2\text{SO}_4$ , 31.02;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 36.93; FeEDTA, 15.1;  $\text{H}_3\text{BO}_3$ , 0.57;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.078;  $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$ , 2.35;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.126;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.220. Nitrogen at 1  $\text{mm l}^{-1}$  was added as  $(\text{NH}_4)_2\text{SO}_4$  to the nutrient solution. The pH of the nutrient solution was adjusted to 5.5 with 1 N NaOH twice a day. The nutrient solutions were replaced every 2 d.

**Root exudates collection** Root exudates were collected from intact plants at 7, 14, 21, 28, 35 and 42 dat (d after transplantation) to the nutrient solution culture. For collecting root exudates, intact plants (a sample size of two plants with three 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  $\text{mm NH}_4\text{Cl}$  (pH 6.1). After root exudate collection, roots and shoots were separated and dried at 70°C for 48 h in a forced air-circulating oven before determining dry weight. For extraction of BNI compounds, root exudates were evaporated

to dryness using a rotary evaporator (Buchi, V-850, Flawil, Switzerland) under vacuum at 40°C, followed by extraction with 20 ml of methanol. The methanol extract was then further evaporated to dryness using a rotary evaporator at 35°C and the residue was extracted with 20 µl of dimethyl sulphoxide (DMSO). The DMSO extract was then used to determine the BNI activity with a bioassay as described in the next section (Subbarao *et al.*, 2006b).

**Nitrification inhibition bioassay** The nitrification inhibition (NI) activity of the samples was determined using a modified bioassay that employs recombinant luminescent *N. europaea* (Iizumi *et al.*, 1998; Subbarao *et al.*, 2006b). The BNI activity of the samples is 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<sub>4</sub><sup>+</sup> is defined as one ATU (AT unit) of activity (Subbarao *et al.*, 2006b).

#### Expt 2: effect of different forms of nitrogen (NH<sub>4</sub><sup>+</sup> vs NO<sub>3</sub><sup>-</sup>) on the release of BNI compounds

This experiment was designed to clarify the role of different N-forms (i.e. NH<sub>4</sub><sup>+</sup> vs NO<sub>3</sub><sup>-</sup>) on BNI compound release from roots. The uptake of NH<sub>4</sub><sup>+</sup> results in the acidification of the rhizosphere (Maschner *et al.*, 1991), and the experiment was designed to separate the direct effect of NH<sub>4</sub><sup>+</sup> from the secondary effect of acidic pH on BNI compound release. Seedlings were grown in hydroponics as described in Expt 1; plants were raised with 1 mM N as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or KNO<sub>3</sub> for 28 d using two 45 l tanks connected to a pH-stat system (Nissin NPH-660NDE, Tokyo, Japan). The pH-stat system used proved to be very reliable and sensitive. By addition of an acid or base to the nutrient solutions, the set pH could be maintained within 0.01 pH units. The units contained one control machine, connected to a pump for controlling acid or base flow, and two electrodes, one for pH and another for temperature, connected with the main system. The nutrient solutions were replaced every 2 d. For collection of root exudates, intact plants (a sample size of two plants with three replications) were removed from the nutrient solution after 28 d, then the roots were rinsed with deionized followed by distilled water, then immersed for 24 h in a 2 l black bottle containing an aerated solution of either 1 mM NH<sub>4</sub>Cl or distilled water; the pH of the root exudate collection solutions were maintained using the pH-stat system. BNI activity in the root exudate samples was measured as described in Expt 1.

#### Expt 3: influence of NH<sub>4</sub><sup>+</sup> concentration in the root exudate collection solution on the release of BNI compounds from roots

Seedlings of sorghum were grown in hydroponics as described in Expt 1, with 1 mM N as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or KNO<sub>3</sub> for 28 d.

Root exudates were collected from intact plants after 28 d. For collecting root exudates, intact plants (a sample size of two plants with three replications) were removed from the nutrient solution, the roots were rinsed with deionized followed by distilled water, then immersed for 24 h in aerated 2 l NH<sub>4</sub>Cl treatment solutions (0.0, 0.1, 1.0 and 5.0 mM) with and without the use of the pH-stat system; after collection of root exudates, roots were separated from the shoot, dried at 70°C for 48 h in a forced air-circulating oven before determining dry weight. The pH changes were also calculated in the solution by taking the initial and final pH measurements for the exudate collections not using the pH-stat systems. Root exudate samples were processed for the determination of BNI activity in a similar way to Expt 1.

#### Expt 4: isolation and characterization of BNI compound (s) from the root exudates

**Chemicals** All the solvents and other chemicals used were purchased from Wako (Osaka, Japan) and Sigma (St Louis, MO, USA) Pure Chemical Industries Ltd unless otherwise stated.

**Plant materials** Seeds of sorghum were germinated and grown in hydroponics as described in Expt 1. Nitrogen at 1 mM was added as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to the nutrient solution. The pH of the nutrient solution was adjusted to 5.5 with 1 N NaOH or 1N HCl twice a day. The nutrient solutions were replaced every 2 d.

**Collection of nitrification inhibition activity (BNI) from roots** Root exudates were collected from intact plants after 28 d following transplantation to nutrient solution culture, in a similar way to Expt 1 using aerated solutions of 1 mM NH<sub>4</sub>Cl. Nearly 70 l of root exudate were collected for the concentration and extraction of BNI activity. The BNI activity from the root exudate was extracted as described earlier in Expt 1; this amounted to *c.* 1000 ATU.

**Isolation of BNI compounds** The crude methanol extracts (containing BNI activity, *c.* 1000 ATU) were diluted with water to reach a volume of 5% methanol before loading into 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, and 30 fractions of 7 ml each were collected using a fraction collector (CHF121SA, Advantec, Tokyo, Japan). All the above fractions were dried *in vacuo* at 35°C using a centrifugal evaporator and the residues were collected in MeOH (1 ml); aliquots of these fractions (50 µl) were dried again *in vacuo* at 35°C in a centrifugal evaporator using 0.5 ml Eppendorf 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 reciprocating 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) C18 column (Tosoh, Tokyo, Japan), monitored at 280 nm. The column was eluted with a linear gradient mobile-phase system of aqueous acetonitrile (10% to 30%) and all the peaks and troughs were checked for BNI activity in the bioassay. Finally the major active peak from 30% methanol fractions was isolated using an isocratic HPLC (15% acetonitrile) programme.

**Instrumental analysis** The mass (MS) spectra were recorded on a GCMS-QP2010 spectrometer (Shimadzu, Kyoto, Japan) by direct electron ionization (EI) at an ionization energy of 70 eV. The  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and heteronuclear multiple bond correlation (HMBC) spectra were recorded at 298 K on an Avance 800 spectrometer (Bruker Biospin, Karlsruhe, Germany) using the pulse sequences and software provided by the manufacturer.

**BNI activity of free acid and synthesized methyl 3-(4-hydroxyphenyl) propionate** 3-(4-hydroxyphenyl) propionic acid was purchased from Sigma in free acid form. Methyl esters of 3-(4-hydroxyphenyl) propionic acid were synthesized by acid esterification (Gopalakrishnan *et al.*, 2007). Briefly, 3-(4-hydroxyphenyl) propionic acid (0.5 g) was dissolved in 10 ml of acidified (0.1 N HCl) methanol. The solution was incubated at 37°C for 24 h. The volatiles were removed with a centrifugal evaporator. The reaction product was then purified using a HPLC, and the molecular mass was confirmed by EIMS. The BNI activity of 3-(4-hydroxyphenyl) propionic acid and its methyl esters were assayed using the bioassay system, and 70% inhibitory concentration ( $\text{IC}_{70}$ ; mean of three replications) was calculated using a dose–response curve.

**Expt 5: influence of nitrogen forms ( $\text{NH}_4^+$  vs  $\text{NO}_3^-$ ) on the release of methyl 3-(4-hydroxyphenyl) propionate from sorghum roots**

Seedlings were grown in hydroponics and exudates collected with three replications as described in Expt 2. For the extraction of methyl 3-(4-hydroxyphenyl) propionate, the root exudate was evaporated to dryness using a rotary evaporator under vacuum at 40°C, followed by extraction with 20 ml of methanol. The methanol-soluble fraction was then evaporated using a centrifugal evaporator at 35°C; the residue was then extracted again with 2 ml acetonitrile, and passed through a millipore filter (Millex-R-LG, Bedford, MA, USA) before injecting the sample into HPLC; the HPLC column was eluted with an isocratic phase system (15% acetonitrile) and monitoring was performed at a wavelength of 280 nm. The concentration of methyl 3-(4-hydroxyphenyl) propionate in the root exudate sample was determined from the HPLC peak area and calculated against a standard of pure methyl 3-(4-hydroxyphenyl) propionate (Sigma).

**Expt 6: mode of inhibitory action of BNI compounds from the root exudates**

The mode of inhibitory action of root exudates and methyl 3-(4-hydroxyphenyl) propionate on *N. europaea* was evaluated by incubating the pure cultures of *N. europaea* in the presence or absence of hydroxylamine in the assay medium using a previously reported protocol (Subbarao *et al.*, 2006b). Hydroxylamine is the AMO (ammonia monooxygenase) enzymatic product resulting from ammonia oxidation, which is a substrate for the HAO (hydroxylamine oxidoreductase) enzyme; therefore hydroxylamine addition allows for determination of whether the HAO enzymatic pathway has also been blocked. The modes of inhibitory action of the known synthetic nitrification inhibitor dicyandiamide (DCD) were also tested. Root exudates and methyl 3-(4-hydroxyphenyl) propionate were dissolved in DMSO and 2 µl of each were used after adding 198 µl of distilled water in the 250 µl of bacterial culture. The sample was then incubated for 15 min before 20 µl of 10 mM hydroxylamine or distilled water was added. DCD, 200 µl (2200 µM), of the water-soluble inhibitor solution was added to the 250 µl of bacterial culture and it was incubated for 15 min before 20 µl of 10 mM hydroxylamine or distilled water (as a control) was added. The mean of the 3-bioluminescence measurements made during the 10 min incubation period was taken as the activity level. Every incubation was replicated three times. The effect of addition of the hydroxylamine to the reaction mixture was evaluated. The inhibition percentage of AMO and HAO by root exudates and methyl 3-(4-hydroxyphenyl) propionate were evaluated using DMSO as a control; for DCD, water was used as a control. Data were subjected to analysis of variance and least significant difference at  $P < 0.001$  was determined (Fisher LSD).

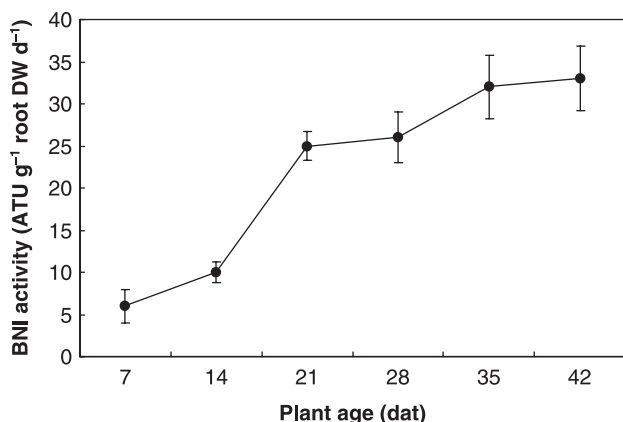
## Results

**Expt 1: release of BNI compounds from sorghum roots**

The inhibitory effect of root exudates was found to increase with plant age when expressed on a per unit root DW basis (Fig. 1). The results showed that the release of BNI compounds increased fivefold, from 6.22  $\text{ATUg}^{-1}$  root DW  $\text{d}^{-1}$  at 7 dat (dat to hydroponics) to 30.1  $\text{ATUg}^{-1}$  root DW  $\text{d}^{-1}$  at 35 dat. The pH of the growth solution decreased by 0.3 to 0.5 units at 14 dat and 0.7 to 1 unit at later growth stages for every 12 h.

**Expt 2: influence of  $\text{NH}_4^+$  or  $\text{NO}_3^-$  supply on the release of BNI compounds**

Sorghum grew well with both N forms (i.e.  $\text{NH}_4^+$  or  $\text{NO}_3^-$ ) when pH of the nutrient solutions was maintained using the pH-stat system, and total dry matter production was similar for both the N treatments (data not shown). Release of BNI compounds from roots was stimulated by the presence of



**Fig. 1** Biological nitrification inhibition (BNI) activity exuded from the roots of sorghum (*Sorghum bicolor*) at different plant ages. Plants were supplied with  $\text{NH}_4^+$  as the sole nitrogen source. Root exudates were collected with 1 mM  $\text{NH}_4\text{Cl}$ . Error bars indicate SE ( $n = 3$ ). dat, d after transplantation; ATU, allylthiourea units.

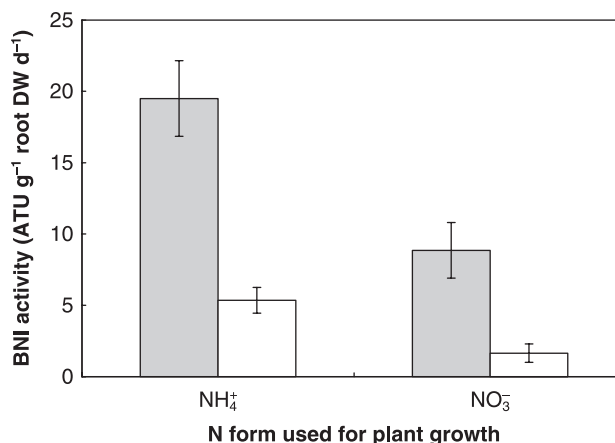
$\text{NH}_4^+$  in the root exudate collection solution for both  $\text{NH}_4^+$ - and  $\text{NO}_3^-$ -raised plants; however, the BNI activity in the root exudate was threefold higher from  $\text{NH}_4^+$ -grown plants compared with  $\text{NO}_3^-$ -grown plants (18.75 vs 6.78 ATU  $\text{g}^{-1}$  root DW  $\text{d}^{-1}$ ) (Fig. 2). In the absence of  $\text{NH}_4^+$  (i.e. root exudates collected using distilled water) BNI activity was detected in the root exudates of  $\text{NH}_4^+$ -grown plants (5 ATU  $\text{g}^{-1}$  root DW  $\text{d}^{-1}$ ), and was only just above detection limits for  $\text{NO}_3^-$ -grown plants (1.65 ATU  $\text{g}^{-1}$  root DW  $\text{d}^{-1}$ ).

#### Expt 3: influence of $\text{NH}_4^+$ concentrations on the release of BNI compounds

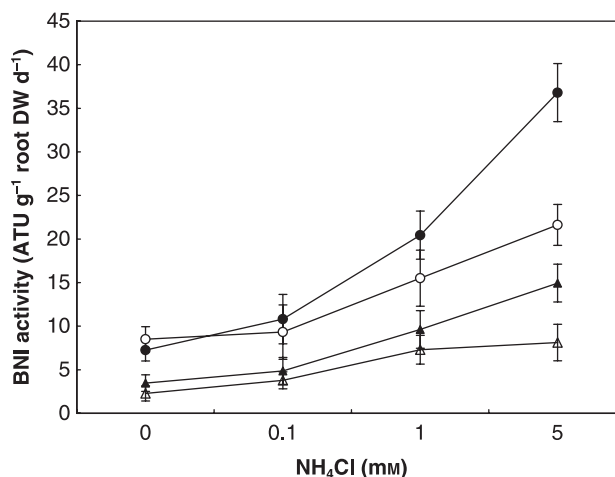
Release of BNI compounds increased with increasing concentration of  $\text{NH}_4^+$  in the collection medium, and this increase was greater when the plants were raised with N supplied as  $\text{NH}_4^+$  (Fig. 3). When BNI activity was collected using 1 or 5 mM  $\text{NH}_4\text{Cl}$ , plants raised with N supplied as  $\text{NH}_4^+$  showed higher BNI activity (c. 3.5-fold) than  $\text{NO}_3^-$ -supplied plants. When the pH was controlled using the pH-stat system, release of BNI activity was lower than when pH was not controlled for both  $\text{NH}_4^+$ - and  $\text{NO}_3^-$ -raised plants (Fig 3). For  $\text{NH}_4^+$ -grown plants, without pH-stat, the root exudate solution pH was c. 1.5 units lower at 1.0 mM and 2.2 units lower at 5 mM  $\text{NH}_4\text{Cl}$  treatment after the 24 h collection period; for  $\text{NO}_3^-$ -grown plants, the figures were 0.9 and 1.3 units lower, respectively.

#### Expt 4: isolation and characterization of BNI compounds from root exudates

The plants used for the isolation of BNI compounds were found to release, on average,  $20 \pm 1.2$  ATU  $\text{g}^{-1}$  root DW  $\text{d}^{-1}$ . The crude extracts with the activity of BNI compounds were

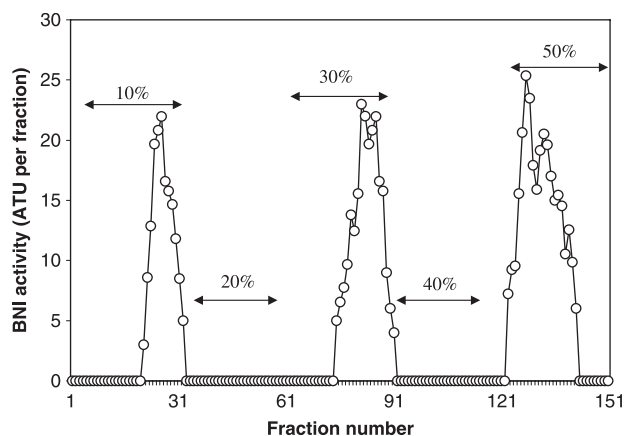


**Fig. 2** Biological nitrification inhibition (BNI) activity exuded from the roots of sorghum (*Sorghum bicolor*) supplied with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  as the sole nitrogen source. Root exudates were collected with 1 mM  $\text{NH}_4\text{Cl}$  (closed bars) or distilled water (open bars) using a pH-stat system. Error bars indicate SE ( $n = 3$ ). Differences between N form used for growth, and exudation collection medium ( $\text{NH}_4\text{Cl}$  and distilled water) were both significant ( $P \leq 0.01$ ). ATU, allylthiourea units.

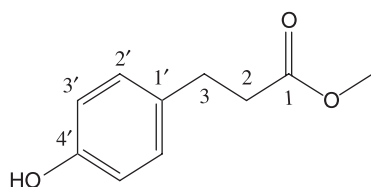


**Fig. 3** Biological nitrification inhibition (BNI) activity exuded from the roots of sorghum (*Sorghum bicolor*) supplied with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  as the sole nitrogen source. Root exudates were collected with different concentrations (0.0, 0.1, 1.0 and 5.0 mM) of  $\text{NH}_4\text{Cl}$  using pH-stat (open circles,  $\text{NH}_4^+$ -grown plants; open triangles,  $\text{NO}_3^-$ -grown plants) and without pH-stat (closed circles,  $\text{NH}_4^+$ -grown plants; closed triangles,  $\text{NO}_3^-$ -grown plants) conditions. Error bars indicate SE ( $n = 3$ ). ATU, allylthiourea units.

further fractionated by reverse-phase column chromatography. Activity-guided fractionations indicated that 10, 30 and 50% methanol effluents contained BNI activity (Fig. 4). Other fractions (20, 40, 75 and 100%) did not show BNI activity. The active constituent of the 30% fraction was successfully purified and results are presented here. Others active fractions, 10 and 50%, have not been purified yet.



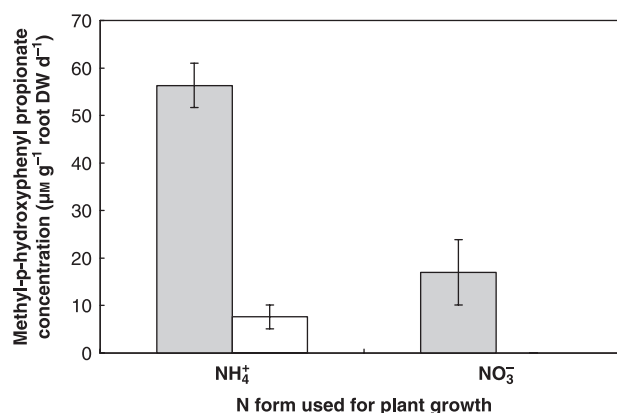
**Fig. 4** Open column chromatography on a reverse-phase C18 column for biological nitrification inhibition (BNI) compounds in the root exudates of sorghum (*Sorghum bicolor*) collected with 1 mM  $\text{NH}_4\text{Cl}$  and dissolved in methanol after evaporation. The column was eluted stepwise with each of the five different concentrations of % methanol (10, 20, 30, 40 and 50%) with 30 fractions. Each fraction was checked for BNI activity and expressed as allylthiourea units (ATU) per fraction.



**Fig. 5** Chemical structure of methyl 3-(4-hydroxyphenyl) propionate.

The physicochemical properties of the compound are as follows – umber gum:  $^1\text{H}$  NMR (800.3 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  2.56 (2H, *t*,  $J = 7.6$  Hz, H-2), 2.81 (2H, *t*,  $J = 7.6$  Hz, H-3), 3.63 (3H, *s*,  $\text{OCH}_3$ ), 6.68 (2H, *m*, H-3' and H-5'), 7.00 (2H, *m*, H-2' and 6');  $^{13}\text{C}$  NMR (201.3 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  31.2 (C-3), 37.1 (C-2), 52.0 ( $\text{OCH}_3$ ), 116.2 (C-3' and C-5'), 130.3 (C-2' and C-6'), 132.7 (C-1'), 156.9 (C-4'), 175.4 (C-1); EIMS  $m/z$  (%) 180 [ $\text{M}$ ] $^+$  (25), 149 (9), 120 (46), 107 (100).

Assignments of the chemical shifts of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were confirmed by HMBC. The NMR spectral data of the isolated compound were identical to those reported for methyl 3-(4-hydroxyphenyl) propionate (Pouchert & Behnke, 1992) and of the authentic preparation. The attachment position of the methyl group was confirmed by HMBC correlation from carbonyl to methyl protons. Thus, the isolated compound was identified as methyl 3-(4-hydroxyphenyl) propionate (Fig. 5). The molecular weight was confirmed by EIMS data. To study the inhibitory properties of the purified compound on *Nitrosomonas* further, we synthesized methyl ester of 3-(4-hydroxyphenyl) propionic acid. These preparations showed inhibitory effect on *Nitrosomonas*. Seventy per cent inhibition ( $\text{IC}_{70}$ ) was used for evaluating the effectiveness



**Fig. 6** The release of methyl 3-(4-hydroxyphenyl) propionate from the roots of sorghum (*Sorghum bicolor*) supplied with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  as the sole nitrogen source. Root exudates were collected with 1 mM  $\text{NH}_4\text{Cl}$  (closed bars) or distilled water (open bars) using a pH-stat system. Error bars indicate SE ( $n = 3$ ). Differences between N form used for growth and exudation collection medium ( $\text{NH}_4\text{Cl}$  and distilled water) were both significant ( $P \leq 0.01$ ).

of the inhibitory effect of this compound. The  $\text{IC}_{70}$  value for methyl 3-(4-hydroxyphenyl) propionate (i.e. the BNI compound isolated from root exudate during this study) is 9.0  $\mu\text{M}$ , and the free-acid form did not have inhibitory activity even at high concentration ( $> 10\,000$   $\mu\text{M}$ ; data not shown).

#### Expt 5: influence of $\text{NH}_4^+$ or $\text{NO}_3^-$ supply on the release of methyl 3-(4-hydroxyphenyl) propionate from roots

The release of methyl 3-(4-hydroxyphenyl) propionate was 3.5-fold higher in  $\text{NH}_4^+$ -grown plants (56.6  $\mu\text{m g}^{-1}$  root DW  $\text{d}^{-1}$ ) than in  $\text{NO}_3^-$ -grown plants (17  $\mu\text{m g}^{-1}$  root DW  $\text{d}^{-1}$ ) when root exudates were collected in 1 mM  $\text{NH}_4\text{Cl}$  solution for 24 h (Fig. 6). When the root exudates were collected in distilled water, the amount of the compound was low (7.6  $\mu\text{m g}^{-1}$  root DW  $\text{d}^{-1}$ ) in comparison to  $\text{NH}_4\text{Cl}$  collection from  $\text{NH}_4^+$ -grown plants, and the compound was not detected in the distilled water-collected root exudates of  $\text{NO}_3^-$ -grown plants (Fig. 6).

#### Expt 6: mode of inhibitory action of methyl 3-(4-hydroxyphenyl) propionate

*Nitrosomonas europaea* recovered completely from the DCD inhibitory effect when the AMO product hydroxylamine was added to the assay medium, and the same effect occurred for the BNI compound isolated from the sorghum root exudate, methyl 3-(4-hydroxyphenyl) propionate (Table 1). By contrast, the inhibitory effect of crude root exudates on *N. europaea* did not recover with the addition of hydroxylamine to the assay medium indicating that both the enzymatic pathways are likely to be inhibited (Table 1).

**Table 1** Effect of hydroxylamine on the inhibitory action of biological nitrification inhibition (BNI) compounds in the sorghum (*Sorghum bicolor*) root exudates, methyl 3-(4-hydroxyphenyl)propionate and dicyandiamide (a synthetic nitrification inhibitor) in a culture of *Nitrosomonas europaea*

Treatment	Bioluminescence (RLU ml <sup>-1</sup> )	
	Without hydroxylamine	With hydroxylamine
BNI from sorghum root exudate		
DMSO control	30.2 ± 0.8	232 ± 2.1
BNI from root exudate (% inhibition)	6.80 ± 0.6** (77.5)	33.5 ± 0.5** (86.0)
BNI compound isolated from sorghum root exudate		
DMSO control	18.90 ± 0.80	196.4 ± 14.50
Methyl 3-(4-hydroxyphenyl)propionate (20 µM) (% inhibition)	5.92 ± 0.24** (68.5)	210.4 ± 20.9 (-6.0)
Synthetic nitrification inhibitor		
Water control	38.5 ± 1.2	329 ± 2.2
Dicyandiamide (2200 µM) (% inhibition)	7.50 ± 0.4 (80.1)	308 ± 1.7 ns (6.40)

Values are means ± SE; \*\**P* < 0.001; ns, not significantly different. RLU, relative light units; DMSO, dimethyl sulfoxide.

## Discussion

Sorghum root exudates had previously been found to inhibit nitrification in soils (Alsaadawi, 1988) and, using the bioassay system, the direct inhibitory effect on *Nitrosomonas* bacteria was confirmed (Subbarao *et al.*, 2007a); however, this response had not been characterized in detail. Our results demonstrate for the first time that the production and release of inhibitory compounds from sorghum roots is enhanced by low pH, stimulated by the presence of NH<sub>4</sub><sup>+</sup>, and influenced by plant growth stage. Using an activity-guided fractionation and purification approach, one of the active compounds responsible for inhibitory activity was determined, and release from roots has been shown to be specifically stimulated by the presence of NH<sub>4</sub><sup>+</sup> in the root zone.

### NH<sub>4</sub><sup>+</sup> stimulates the release of BNI compound from sorghum roots

Different forms of N supplied to sorghum roots (NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>) influence the release of BNI compounds, and NH<sub>4</sub><sup>+</sup> is a stimulus for BNI compound release (Fig. 2). Previously, the presence of NH<sub>4</sub><sup>+</sup> has been reported to stimulate BNI compound release from *B. humicola* and *L. racemosus* roots (Subbarao *et al.*, 2007b,c). In both studies, plants were cultivated with N supplied as NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> and then root exudates were collected in 1 mM NH<sub>4</sub>Cl solution or distilled water and pH was not maintained at a steady state. Because NO<sub>3</sub><sup>-</sup> uptake leads to alkalization of the growth media (because of H<sup>+</sup> influx or OH<sup>-</sup> efflux from the plants) and NH<sub>4</sub><sup>+</sup> uptake results in acidification (H<sup>+</sup> efflux; Raven *et al.*, 1992), the final pH after 24 h would be very different between exudate collections using the two forms. Differences in pH in the exudate collection medium, in particular low pH, could result in differences in plasma membrane permeability (Yan *et al.*, 1998). Thus, enhanced plasma membrane permeability

resulting from low rhizosphere pH (because of uptake of NH<sub>4</sub><sup>+</sup>) could be partly responsible for higher BNI release in root exudate solutions containing NH<sub>4</sub><sup>+</sup>. Therefore, earlier studies (Subbarao *et al.*, 2007b,c) indicating the stimulatory role of NH<sub>4</sub><sup>+</sup> in BNI compound release from roots of *B. humicola* and *L. racemosus* were not conclusive.

In the present study, a pH-stat system was used to keep the pH in the growth and exudate collection medium constant, removing the secondary effects of rhizosphere pH associated with the uptake from N forms on BNI compound release from roots. Our results show that NH<sub>4</sub><sup>+</sup> is the major contributing factor in the release of BNI compounds and that rhizosphere pH is also important but influences BNI compound release from roots to a lesser extent (Fig. 2). The effect of different concentrations of NH<sub>4</sub><sup>+</sup> in the exudate collection medium on the release of BNI compounds from NH<sub>4</sub><sup>+</sup>- and NO<sub>3</sub><sup>-</sup>-grown plants was also studied (Fig. 3). Continuous exposure of NH<sub>4</sub><sup>+</sup> during plant growth was also found to be an important factor resulting in greater release of BNI compounds. The results also show that high concentrations of NH<sub>4</sub><sup>+</sup> can stimulate greater release of BNI compounds under pH-stat conditions. This demonstrates conclusively that NH<sub>4</sub><sup>+</sup> is the key contributing factor to the production and release of BNI compounds by sorghum. Presently, it is unknown why this link could be ecologically significant, or what benefit the exudation of these compounds could have to whole-plant nutrition.

The supply of nitrogen as NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> is known to have many other influences on root systems. The morphological effect of increased root branching with supply of NH<sub>4</sub><sup>+</sup> compared with NO<sub>3</sub><sup>-</sup> has been thoroughly characterized in many species (Martins-Lucaio *et al.*, 2000; Mahmood *et al.*, 2002). Supply of NH<sub>4</sub><sup>+</sup> has also previously been associated with increased root respiration and sugar exudation (Martins-Lucaio *et al.*, 2000). The genetic response of roots to differing forms of N supply has also been extensively studied. Supplying NH<sub>4</sub><sup>+</sup> has been associated with various aspects of plant growth and

metabolism, for example, carbon and nitrogen partitioning (Cramer & Lewis, 1993), solute composition and energy status of roots (Lang & Kaiser, 1994), and leaf morphogenesis (Walch-Liu *et al.*, 2000). Given the many different morphological, chemical and genetic responses that have previously been associated with differing forms of N supply, a link between  $\text{NH}_4^+$  acquisition and the production and/or release of compounds for the purpose of nitrification inhibition, thereby retaining more N as  $\text{NH}_4^+$  in soil, is not altogether surprising, but at this stage it is poorly understood. Isolation and identification of BNI compounds from root exudates may help to further our understanding of the stimulatory role of  $\text{NH}_4^+$  on the production and/or release of BNI compounds from sorghum roots. Whether the stimulatory role of  $\text{NH}_4^+$  in the root zone for the release of BNI compounds is the result of its uptake or assimilation remains unclear from this study, and will be the subject of further investigations.

### The properties of methyl 3-(4-hydroxyphenyl) propionate with respect to BNI

The active BNI compound in the sorghum root exudate, methyl 3-(4-hydroxyphenyl) propionate, showed a strong inhibitory effect on *Nitrosomonas* activity in the assay, as indicated by the  $\text{IC}_{70}$  value of  $9.0 \mu\text{M}$ . This is the first time that a nitrification inhibitor has been isolated from the root exudate of any plant species. There have been previous reports of nitrification inhibitors extracted from different plant parts. Gopalakrishnan *et al.* (2007) isolated ferulic acid and *p*-coumaric acid from the root tissue of *B. humidicola*; however, these compounds were not present in root exudates. Other examples of plant tissue-extracted substances that can inhibit nitrification include neem oil from the neem tree (*Azadirachta indica*) (Kumar *et al.*, 2007), and the extracts of karanj (*Pongamia glabra*) seed, bark and leaves of neem (Sahrawat *et al.*, 1974). Crude extracts from the roots of *Astragalus mongholicus* have also been reported to inhibit soil nitrification (Mao *et al.*, 2006).

The inhibitory properties of 3-(4-hydroxyphenyl) propionic acid and chemically synthesized methyl 3-(4-hydroxyphenyl) propionate were further confirmed by *in vitro* assay. The nitrification enzymes and related electron carrier system of *N. europaea* are located in the periplasm or inner membrane (Iizumi & Nakamura, 1997). The results suggest that methyl 3-(4-hydroxyphenyl) propionate may have such polarity that can reach the active site of a bacterial cell. By contrast, the presence of a polar  $-\text{COOH}$  group of the free acids might make their permeability too low to approach the action point. This implies that ester forms of the compound are preferable to prevent nitrification by the bacterial cell. Similarly, it has also been reported that the esters of phenolic acids are more toxic to fungi than their corresponding free acids (Daayf *et al.*, 2000). Methyl 3-(4-hydroxyphenyl) propionate can be formed from the free-acid form in the presence of methanol under

acidic conditions. To rule out the possibility that the methyl ester is an artefact formed during the sample preparation process, after rotary evaporation the root exudate residue was extracted with chloroform instead of methanol. Subsequent HPLC analysis determined a concentration of  $60 \mu\text{M g}^{-1}$  root DW  $\text{d}^{-1}$  of methyl 3-(4-hydroxyphenyl) propionate for  $\text{NH}_4^+$ -supplied plants when root exudates were collected in  $1 \text{ mM NH}_4\text{Cl}$  solution for 24 h. This result confirmed that methyl 3-(4-hydroxyphenyl) propionate is not an artefact. Our results thus indicate that methyl ester (i.e. methyl 3-(4-hydroxyphenyl) propionate) is naturally synthesized and released from sorghum roots. However, the isolated compound, methyl 3-(4-hydroxyphenyl) propionate, has not previously been reported to be released from plants or for its inhibitory effects on nitrification.

### Modes of action of the compound for nitrification inhibition

Some progress has been made towards understanding the modes of inhibition of *Nitrosomonas* spp. by synthetic chemical inhibitors. Two enzymatic pathways are involved in *N. europaea* for nitrite formation. Ammonia is first converted to hydroxylamine by AMO, and hydroxylamine is then converted into nitrite by hydroxylamine oxidoreductase (HAO). Ammonia monooxygenase has a broad substrate range for catalytic oxidation, and the inhibitory effect of synthetic nitrification inhibitors is the result of competition for the active site of AMO, but they do not inhibit HAO (Murakami *et al.*, 1995; Arp & Stein, 2003). In our study, a known synthetic nitrification inhibitor, DCD, was found to inhibit the AMO pathway by more than 80%, and it does not have any inhibition function on HAO (Table 1). This confirms that it is specific to the AMO enzymatic pathway, as previously reported by McCarty (1999). In contrast to synthetic inhibitors such as DCD, root exudates inhibited the function of both the enzymes (Table 1). Root exudates of *B. humidicola* and *L. racemosus* have also been reported to inhibit the function of both the enzymes (Subbarao *et al.*, 2007b,c); however, the chemical identity of these BNI compounds has not yet been resolved. Methyl 3-(4-hydroxyphenyl) propionate, however, did not inhibit the function of both enzymes. The isolated compound, methyl 3-(4-hydroxyphenyl) propionate, has a similar mode of inhibitory action to the synthetic inhibitor DCD, suggesting that other compounds that have not yet been purified must be responsible for inhibition of the HAO enzyme.

### How might $\text{NH}_4^+$ be a stimulus for BNI compound synthesis and/or release?

The presence of  $\text{NH}_4^+$  has a direct influence on the release of the methyl 3-(4-hydroxyphenyl) propionate from roots (Fig. 6), and on the release of other unidentified compounds (Fig. 2). The isolated BNI compound is phenolic in nature



and might be synthesized through a modified phenyl propanoid metabolism pathway. This can be speculated because the structure of the compound has similarity with methyl *p*-coumarate, only it does not have double bond in the side-chain of position 2. These types of compound are derived mainly from L-phenylalanine in the initial step of phenyl propanoid synthesis mediated by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) (Jozef *et al.*, 2007). Ammonium is eliminated along with the pro-S hydrogen atom from L-phenylalanine to form secondary phenylpropanoid products in the reaction catalysed by PAL. Ammonium ion released by the PAL enzyme can be assimilated via the GS/GOGAT system (Razal *et al.*, 1996). It has been reported that low PAL activity accumulates high amounts of endogenous ammonia, and that PAL activity and endogenous concentrations of ammonia are known to affect each other in a plant cell (Maldonado *et al.*, 2002). There is a possibility that endogenous  $\text{NH}_4^+$  may be a regulator in adjusting PAL activity or other related enzymes, which could lead on to other metabolic changes, including the production of BNI compounds, as well as giving a signal to the membrane for BNI compound release. We now know that assimilation of  $\text{NH}_4^+$  and associated metabolic processes have a definite contribution to the release of BNI compounds from roots; however, little is known about the in-depth induction mechanisms of  $\text{NH}_4^+$  on the release of BNI compounds from sorghum roots. Future studies will be required to understand this process.

## Conclusion

The release of nitrification inhibitors from the roots of crop and pasture species may be a useful tool for decreasing the emission of nitrogenous greenhouse gasses from soil and reducing off-farm impacts associated with nitrate leaching. The isolation of a compound from the root exudates of sorghum that is responsible for nitrification inhibition has many implications for understanding the BNI phenomenon. In particular, it will allow for more detailed physiological characterization of BNI compound release mechanisms with regard to the effects of  $\text{NH}_4^+$  assimilation. Our ongoing research is aimed at further characterization of the BNI phenomenon in sorghum.

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