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Biological nitrification inhibition (BNI)—is it a widespread phenomenon?

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Abstract Regulating nitrification could be a key strategy in improving nitrogen (N) recovery and agronomic N-use efficiency in situations where the loss of N following nitrification is significant. A highly sensitive bioassay using recombinant luminescent *Nitrosomonas europaea*, has been developed that can detect and quantify the amount of nitrification inhibitors produced by plants (hereafter referred to as BNI activity). A number of species including tropical and temperate pastures, cereals and legumes were tested for BNI in their root exudate. There was a wide range in BNI capacity among the 18 species tested; specific BNI (AT units activity g^{-1} root dry wt) ranged from 0 (i.e. no detectable activity) to 18.3 AT units. Among the tested cereal and legume crops, sorghum [*Sorghum bicolor* (L.)], pearl millet [*Pennisetum glaucum* (L.) R. Br.], and groundnut [*Arachis hypogaea* (L.)] showed detectable BNI in root exudate. Among pasture grasses, *Brachiaria humidicola* (Rendle) Schweick, *B. decumbens* Stapf showed the highest BNI capacity. Several high- and low-BNI genotypes were identified within the *B. humidicola* species. Soil collected from field plots of 10 year-old high-BNI genotypes of *B. humidicola*, showed a near total suppression (>90%) of nitrification; most of the soil inorganic N remained in the NH_4^+ form after 30 days of incubation. In contrast, soils collected from low-BNI genotypes did not show any inhibitory effect; most of the soil inorganic N was converted to NO_3^- after 30 days of incubation. In both the high- and low-BNI genotypes, BNI was detected in root exudate only when plants were grown with NH_4^+, but not when grown with NO_3^- as the sole source of N. BNI compounds when added to the soil inhibited nitrification and the relationship was linear ($r^2 = 0.92^{**}$; $n = 12$). The BNI from high- and low-BNI types when added to *N. europaea* in pure culture, blocked both the ammonia monooxygenase (AMO) and the hydroxylamine oxidoreductase (HAO) pathways. Our results indicated that BNI capacity varies widely among and within species; and that some degree of BNI capacity is likely a widespread phenomenon in tropical pasture grasses. We suggest that the BNI capacity could either be

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managed and/or introduced into pastures/crops with an expression of this phenomenon, via genetic improvement approaches that combine high productivity along with some capacity to regulate soil nitrification process.

Keywords Biological nitrification inhibition (BNI) · Brachiaria · BNI activity · Inter-specific variation · Intra-specific variation · Mode of inhibition · Nitrification · *Nitrosomonas* europaea · Root exudate

Introduction

Nitrification, a microbial process, is a key component and integral part of the soil-nitrogen (N) cycle. Nitrification determines the form of N present and therefore how N is absorbed, utilized or dispersed into the environment. This in turn has large implications as to plant productivity and environmental quality. During nitrification, the relatively immobile NH_4^+ is converted to the highly mobile NO_3^-. This process strongly influences N utilization by plants, because the NO_3^- formed, is highly susceptible to loss from the root zone by leaching and/or denitrification (Raun and Johnson, 1999; Glass, 2003; Giles, 2005; Subbarao et al., 2006a). The loss of N from the root zone has large economic implications, valued at 15 billion US$ annually just in fertilizer loss, plus the unknown cost from the environmental consequences such as nitrate pollution of ground water, eutrophication of surface waters and atmosphere pollution (Raun and Johnson, 1999; Giles, 2005; Subbarao et al., 2006a). Management of nitrification by the application of chemical inhibitors is a proven strategy for improving N recovery, agronomic N-use efficiency (NUE) and at the same time limiting environmental pollution (Jarvis, 1996; Sahrawat and Keeney, 1985; Prasad and Power, 1995; Subbarao et al., 2006a).

Suppression of soil nitrification has been observed to occur naturally in some ecosystems, and is termed biological nitrification inhibition (BNI) indicating that the inhibition originated from plants in the ecosystem (see review by Subbarao et al., 2006a). The conservation of N and the resulting improved N status through BNI is hypothesized as a major driving force for the development of low-NO_3^- ecosystems (Rice and Pancholy, 1972; Lensi et al., 1986; Serca et al., 1998; Lata et al., 2000, 2004; Subbarao et al., 2006a). Some recent studies suggest that certain grass populations are able to influence nitrification in soil (Lata et al., 2004). Several researchers based on empirical studies also indicated that some tropical pasture grasses inhibit nitrification (Robinson, 1963; Lata et al., 2000; also see review by Subbarao et al., 2006a). However, the concept remained largely unsupported for lack of an appropriate methodology to conclusively demonstrate in situ inhibition of nitrification by chemicals released in the plant–soil system (Lata et al., 2004; Subbarao et al., 2006a).

The catabolism of ammonia (by ammonia oxidizing bacteria such as *Nitrosomonas* sp.) takes place in two steps. Ammonia is first oxidized to hydroxylamine by ammonia monooxygenase (AMO), a copper-containing enzyme that is a membrane-bound protein (Basu et al., 2003). Hydroxylamine is then oxidized to nitrite by hydroxylamine oxidoreductase (HAO). This oxidation releases four electrons, two of which are returned to AMO to sustain ammonia oxidation. The remaining two electrons are available for the cell's reductant needs (i.e. generation of NADPH) (see review of Subbarao et al., 2006a for further information), which is the only source of energy for these chemo-lithoautotrophic bacteria (Bock et al., 1991).

Recently, an assay using recombinant luminescent *Nitrosomonas europaea* has been developed that can detect and quantify nitrification inhibitors released in the root zone (Subbarao et al., 2006b). The assay is also a powerful tool for determining the mode of action of BNI compounds released from plant roots (Subbarao et al., 2006b). Most commercially produced nitrification inhibitors such as nitrapyrin and dicyandiamide inhibit soil nitrification by blocking the AMO pathway of *Nitrosomonas*. Monoterpines produced in coastal redwood (*Sequoia sempervirens*) and ponderosa pine trees are widely believed to be responsible for the low nitrification rates and are reported to inhibit *Nitrosomonas* function by blocking the AMO pathway (Ward et al., 1997). Apart from this, very little information is avail-

able as to the mode of action of these plant produced chemicals (i.e. BNI compounds) on the functioning of *Nitrosomonas*.

The present investigation was aimed at quantifying the inter- and intra-specific variability in BNI in various plant species, including pastures and field crops. This study focused primarily on BNI in tropical forage grasses, in particular *Brachiaria* species, considered to be well adapted to the low-N environments of South American Savannas (Sylvester-Bradley et al., 1988; Ishikawa et al., 2003). A number of field crops were also included in the study to determine the likelihood of the widespread occurrence of BNI, as published information is not available in relation to the distribution of BNI ability. Also, determining the mode of action of BNI compounds (released from *Brachiaria* ssp.) on the functioning of *Nitrosomonas* is another important objective during this investigation.

Materials and methods

Experiment 1: Evaluation of forage grasses and crops for BNI capacity

Several forage grasses [*Brachiaria humidicola* (Rendle) Schweick. (CIAT 679); *B. decumbens* Stapf (CIAT 606); *Melinis minutifolora* Beauv. (CIAT 6374); *Panicum maximum* Jacq. (cv. natsuyutaka); *Lolium perenne* L ssp. Multiflorum (Lam.) Husnot (Italian ryegrass cv. Nioudaichi); *Andropogon gayanus* Kunth. (CIAT 6780); and *B. brizantha* (A. Rich.) Stapf (CIAT 6780)], cereal crops [sorghum (*Sorghum bicolor* (L.) Moench var. hybrid sorgo; pearlmillet (*Pennisetum glaucum* (L.) R. Br. var. CIVT; upland rice (*Oryza sativa* L.) var. Toyohatamochi, var. Sabana 6; maize (*Zea mays* L.) var. Peter No. 610; wheat (*Triticum aestivum* L.) var. Norin 61; barley (*Hordeum vulgare* L.) var. Shunrai], and legumes [peanut (*Arachis hypogaea* L.) var. TMV-2; soybean (*Glycine max* L. Merr.) cv. Orinoquia 3, var. Natsuroyosooi, En 1282 a non-nodulating type; cowpea *Vigna unguiculata* L. Walpers ssp. *unguiculata* var. Caupi) and bean (*Phaseolus vulgaris* L.) accession G 21212] were used in this study. Plants were grown in 2.5-l pots in a sand vermiculite mixture (3:1 v/v). About 10 seeds were sown in each pot, and after germination and seeding establishment (about 10 DAS), the seedlings were thinned to four per pot. The experiment was arranged as a completely randomized block design with four replications in a growth chamber with a day/night temperature regime of 28/22°C, a photosynthetic photon flux density (PPFD) of about 300 μmol m^{-2} s^{-1} and a 14/10 h light/dark photoperiod. For wheat and barley, the growing conditions in the growth chambers were 18/16°C day/night temperature regime and 18/6 h light/dark photoperiod. Plants were watered daily with the nutrient solution of the following composition (mg l^{-1}): KH_2PO_4 38.32, K_2SO_4 31.02, $CaCl_2$ $2H_2O$ 10.5, $MgSO_4$ $7H_2O$ 36.93, Fe–EDTA 15.1, H_3BO_3 0.57, $CuSO_4$ $5\ H_2O$ 0.078, $MnSO_4$ $6H_2O$ 2.35, Na_2MoO_4 $2H_2O$ 0.126, $ZnSO_4$ $7H_2O$ 0.220. The pH of the nutrient solution was adjusted to 6.5 before use. N was supplied as $(NH_4)_2SO_4$ (1 mM) in the nutrient solution. Plants were grown for 60 days after germination before being used for the collection of root exudate as described earlier (Subbarao et al., 2006b).

Experiment 1a: Collection of root exudate, and determination of BNI

The intact plants were removed from pots, washed with running tap water, and deionized water, and were then placed in a 1-l black bottle with 800 ml of aerated ultra-purified distilled water; and the root exudate were allowed to accumulate for 24 h. After collection, the root exudate was stored at 5°C until the quantification of BNI. Following the collection of root exudate, the roots and shoots were separated and dried at 70°C for 48 h in a forced air-circulating oven before determining dry weights. Root exudate was evaporated to dryness using a rotary-evaporator under vacuum at 40°C; this was followed by extraction of the residue with 20 ml of methanol. The methanol extract was then evaporated to dryness using a rotary-evaporator at 35°C, and the resulting residue was extracted with 200 μl of dimethyl sulfoxide (DMSO); the DMSO extract was then used for the determination of BNI using a modified bioassay that utilizes recombinant

luminescent *Nitrosomonas* (Iizumi and Nakamura, 1977; Iizumi et al., 1998; Subbarao et al., 2006b). The detailed methodology of the bioassay for the detection, quantitative determination and expression of BNI has been described previously (Subbarao et al., 2006b). The inhibitory effect of the root exudate is expressed as the equivalent of the effect of a given amount of a standard inhibitor allylthiourea (AT) (Subbarao et al., 2006b). One AT unit of BNI activity is defined as equal to the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ (Subbarao et al., 2006b). The data were subjected to ANOVA using SigmaStat-3.1 and the least significant difference at $P < 0.05$ (Fisher LSD) were calculated.

Experiment 2: Evaluation of BNI capacity of tropical pasture grasses grown on acid soil

A sandy loam Oxisol (4 kg of soil pot^{-1}) from the Llanos (Matazul) of Colombia was used to grow the plants. A basal level of nutrients was applied to the soil before planting (kg ha^{-1}): 40 N, 50 P, 100 K, 66 Ca, 28.5 Mg, 20 S and micronutrients at 2 Zn, 2 Cu, 0.1 B and 0.1 Mo (Rao et al., 1995). Six tropical grasses [(*B. humidicola* (Rendle) Schweick. CIAT 679, *B. decumbens* Stapf CIAT 606; *B. dictyoneura* (Fig. & De Not.) Stapf CIAT 6133; *B. hybrid* cv. Mulato; *B. brizantha* (A. Rich.) Stapf CIAT 6780; *P. maximum* CIAT 16028)] were grown at two levels of $(NH_4)_2SO_4$ application (0 and 100 kg N ha^{-1}). The experiment was arranged as a completely randomized block design with four replications in a greenhouse where the mean day/night temperatures were 28/22°C during the growth period. At 11 and 24 weeks after planting, N was applied (at a rate equivalent to 100 kg N ha^{-1}) as a solution of $(NH_4)_2SO_4$ and plants were grown for 180 days. Intact plants were removed from the soil; the soil attached to the roots was gently washed away in flowing tap water followed by deionized water. The root exudate was then collected in distilled water. The BNI activity of the root exudate was quantified as described earlier. The data were subjected to ANOVA using SigmaStat-3.1 and the least significant difference at $P < 0.05$ (Fisher LSD) were calculated.

Experiment 3: Evaluation of *Brachiaria humidicola* germplasm accessions for BNI activity in root exudate

Six accessions of *B. humidicola* (CIAT 26159, CIAT 26427, CIAT 26430, CIAT 26438, CIAT 26149 and CIAT 682) along with the standard cultivars CIAT 679, and *P. maximum* (CIAT 16028) were grown (4 plants pot^{-1}) in sandy loam Oxisol from the Llanos (Matazul) of Colombia (4 kg of soil pot^{-1}). Plants were propagated from the vegetative cuttings of the germplasm lines maintained at the CIAT-Popayan experimental station and used for planting in pots. The experiment was arranged as a completely randomized block design with four replications. A basal level of nutrients was applied as described in Experiment 2. Nitrogen was added to the pots @100 kg N as $(NH_4)_2SO_4$ ha^{-1}. Plants were grown for 180 days. For collecting root exudate, plants were removed from the soil, washed with running tap water and root exudate was collected using deionized water for 24 h. The BNI activity of the root exudate was quantified as described earlier. Plant roots and shoots were oven dried and their dry weights determined. The data were subjected to ANOVA using SigmaStat-3.1 and the least significant difference at $P < 0.05$ (Fisher LSD) were calculated.

Experiment 4: Influence of N forms (i.e. NH_4^+ versus NO_3^-) on plant growth and release of BNI from roots of *Brachiaria humidicola* and *P. maximum* that have contrasting BNI capacity

Experiment 4a: Plant culture

Seeds of *B. humidicola* (two genotypes, the standard cultivar CIAT 679, the high-BNI genetic stock CIAT 26159) and *P. maximum* (cv natsuyutake) were sown in a sand vermiculite mixture (3:1 v/v) and the plants were grown for 2 weeks. Only distilled water was added during this period. The plants were grown in a growth chamber with a day/night temperature regime of 30/28°C, a PPFD at plant height averaging 300 μmol m^{-2} s^{-1} and a 14/10 h light/dark photoperiod. The seedlings when 14 days old were transferred to a

hydroponic culture system where the plants were grown in 50-l tanks on styrofoam blocks having eight holes with four plants per hole. The plants were kept in place with the support of sponges. The nutrient solutions were constantly aerated. The composition of the full-strength nutrient solution (mg l^{-1}) was: KH_2PO_4 38.31, K_2SO_4 31.02, $CaCl_2$ $2H_2O$ 10.5, $MgSO_4$ $7H_2O$ 36.93, Fe-EDTA 15.1, H_3BO_3 0.57, $CuSO_4$ $5H_2O$ 0.078, $MnSO_4$ $6H_2O$ 2.35, Na_2MoO_4 $2H_2O$ 0.126, $ZnSO_4$ $7H_2O$ 0.220. The pH of the nutrient solution was adjusted to 5.0 with 1 M NaOH or 1 M HCl on a daily basis and replaced at weekly intervals. There were two N treatments 1 mM N added as $(NH_4)_2SO_4$ or KNO_3. The experiment had three replications.

Experiment 4b: Collection of root exudate

Root exudate was collected from intact plants at 40, 50 and 60 DAT (days after transplantation to the hydroponic system). One mM of N was added either as $(NH_4)_2SO_4$ or as KNO_3 depending on the treatment, to the nutrient solution tanks 2 h prior to the collection of the root exudate. Collecting root exudate non-destructively involved removing 12 intact plants from the nutrient solution, washing them in tap water followed by immersing them in aerated double-distilled water for a collection period of 24 h. The solutions with the root exudate were then immediately stored at 5°C until the determination of BNI. After the collection of root exudate, roots and shoots were separated, dried at 70°C for 48 h in a forced air-circulating oven before determining dry weights.

Experiment 4c: Preparation of root exudate for the soil application

For the incubation experiments with soil, the methanol extracts of root exudate were evaporated and finally extracted with double-distilled water; the pH of the resulting extract was around 6.8. Only the root exudate from NH_4^+ grown plants of high BNI genotype (CIAT 26159) was used for the soil incubation experiments.

Experiment 5: Determine the effectiveness of BNI activity in inhibiting nitrification in soil

The soil used for the incubation studies was a volcanic ash soil, Typic Hapludands [(pH H_2O) 6.0, clay 54.8%, silt 26.3%, sand 18.9%, total carbon = 29.2 mg C g^{-1} soil; total N = 2.5 mg N g^{-1} soil; and C/N ratio 11.7], collected from the JIRCAS (Japan International Research for Agricultural Sciences) experimental field site in Tsukuba, Japan. The soil was air-dried, and passed through a 2-mm sieve before use. Two grams of soil was weighed into a 10-ml glass bottle with 0.72 ml of $(NH_4)_2SO_4$ solution providing 182 mg N kg^{-1} soil; and this was used as the control treatment. The soil water status was maintained at a level where 60% of the pore space was water filled which is considered optimum for nitrification (WFPS) (0.36 ml was required g^{-1} to give 60% WFPS for this soil) (Mosier et al., 1996).

For testing the effectiveness of BNI compounds in soils, aliquots of BNI from the root exudate from CIAT 26159 (i.e. 0, 3.5, 7.0, 10.5 and 16.8 AT units of BNI g^{-1} of dry soil) were added to the soil along with NH_4^+ as the source of nitrogen. The entire experiment was laid out as two complete sets, one set of bottles was incubated for 26 days and the second set was incubated for 56 days. The experiment was replicated three times. The mouth of the bottles were sealed with laboratory parafilm and a needle with 0.35 mm in diameter was used to make a hole for aeration. Each sample bottle along with the soil and treatment solution were weighed and weights recorded. The samples were placed in a temperature- and humidity-controlled incubator (Bench-top type temperature and humidity chamber, ESPEC Corporation, Japan), at 20°C and 95% humidity. Sample bottles were weighed at 15-day intervals, and the water status adjusted by adding distilled water to the initial weight of the sample. After the incubation period, the soil samples were extracted by shaking with 20 ml of 2 M KCl for 30 min, and then filtered through Wattman No. 1 filter paper. The filtrate was then analyzed colorimetrically for NH_4^+ (indophenol method) and NO_3^- (sulfanilamide—α—naphthlamine method) using an Auto Ion analyzer

(model AAII, Brant+Luebbe, Germany) (Anon, 1974; Litchfield, 1967; Varley, 1966).

Experiment 6: Determination of the nitrification potential of soils collected from 10-year old field plots of *Brachiaria humidicola* accessions with contrasting BNI release characteristics

Six *B. humidicola* germplasm accessions (CIAT 26159, CIAT 26427, CIAT 26430, CIAT 26438, CIAT 26149 and CIAT 682) along with the standard cultivar CIAT 679 were used for this study. Plant cuttings from these plots were also used for Experiment 3. These field plots have been maintained at the CIAT-Popayan experimental station (225′N 76 40′W at 1730 m altitude with a mean temperature of 20.1°C, annual precipitation of 2124 mm, annual potential evapo-transpiration of 1530 mm, and a relative humidity of 74%) for about 10 years. The soil is an Andisol with soil organic matter levels of about 15%, and is considered highly fertile. Soils were sampled from a depth of 0–15 cm, using a 2-cm soil auger. For each germplasm accession plot, about 10 core samples were sampled and were pooled to make one composite sample that was air-dried. For the control, soil was sampled near the experimental plot in a plant-free area with similar soil physical and chemical characteristics. For the seven germplasm accessions, six sets of soil samples were collected as control soils. The roots were removed to the extent possible before the soil was crushed and passed through a 2-mm sieve after air drying for 48 h. Air-dried soil of 2 gm was weighed into a bottle, and 200 mg kg^{-1} N as $(NH_4)_2SO_4$ was added along with sufficient distilled water to fill 60% of the pore space. The experiment was replicated two times. The mouth of the bottle was sealed with parafilm with a pinhole opening at the center and incubated at 25°C for 30 days. After incubation, the soil was extracted with 2 M KCl and the NO_3^- and NH_4^+ contents were determined using an autoanalyzer. The data were subjected to ANOVA using SigmaStat-3.1 and the least significant difference at $P < 0.05$ (Fisher LSD) were calculated.

Experiment 7: Mode of action of BNI compounds from two genotypes of *Brachiaria humidicola*, CIAT 679 and CIAT 26159

The BNI compounds from the root exudate of two genotypes of *B. humidicola* (CIAT 679 and CIAT 26159) that were genetically contrasting for BNI capacity (medium- and high-BNI types) were evaluated further for their mode of inhibitory action on *N. europaea*. Plants were grown with NH_4^+ as their N source and the remaining details of the plant culture and root exudate collection and BNI determination were same as in Experiment 4. The mode of inhibitory action of the BNI compounds from the two contrasting germplasm accessions of *B. humidicola* was determined by incubating the bacteria in the presence and absence of hydroxylamine in the bioassay medium. After the bacterial cells were incubated with either BNI or AT for 10 min, 200 µl of 1 mM hydroxylamine was added to give 307 µM in the assay and the bioluminescence was measured at 1 min intervals by injecting 100 µl of the reaction mixture into the luminometer. For the hydroxylamine control, 200 µl of distilled water was added to the assay that had 250 µl culture, 10 µl BNI dissolved in DMSO and 190 µl distilled water. Making the total volume of the control assay 650 µl. The mean of the 10 measurements made during the 10 min incubation period was taken as the active level. The effect of the addition of the AMO enzyme product (i.e. hydroxylamine) to the reaction mixture was evaluated. The known AMO inhibitor, AT was included in these experiments as a standard. The inhibitory effect of BNI was determined in the presence and the absence of hydroxylamine. The data were subjected to'*t*-tests' to determine the effect of BNI on bioluminescence in *N. europaea*.

Results

BNI capacity of selected pastures and field crops

Root exudate collected from sand-vermiculite grown plants of several tropical, temperate pas-

ture grasses, cereals and legume crops showed a wide range in BNI capacity (i.e. the ability to release BNI compounds from roots) (Table 1). Plant species differed significantly ($P < 0.05$) in BNI capacity (Table 1). Among the species tested, *Brachiaria* species in general, *B. humidicola*, and *B. decumbens* in particular showed the highest specific BNI (13.4 to 18.3 AT units activity gm^{-1} root dry wt). Several of the other pasture grasses tested showed a specific BNI that ranged from 2.0 (*B. brizantha*) to 7.7 AT units activity (*A. gayanus*) (Table 1). Among field crops, only sorghum, pearlmillet and groundnut showed detectable BNI in their root exudate where specific BNI ranged from 1.8 to 5.2 AT units of activity (Table 1). There was no detectable BNI in root exudate of rice, maize, barley, wheat, soybean, mungbean and bean (Table 1). A further evaluation of several *Brachiaria* pasture grasses grown on a sandy loam Oxisol from the Llanos (Matazul) of Colombia (where *Brachiaria* grasses are the predominant pastures dominating the Savannas), indicated that BNI capacity is widespread among tropical pasture grasses, with significant ($P < 0.05$) inter-specific differences (Table 2). There was no detectable BNI in the root exudate of *P. maximum* (Table 2). The BNI (both total and specific BNI) was significantly ($P < 0.001$) high in plants from N-fertilized (NH_4^+) treatment compared to non-N fertilized treatment (Table 2).

Genotypic variability of BNI capacity in the germplasm lines of *Brachiaria humidicola*

Significant ($P < 0.05$) genotypic variability in BNI capacity was found in *B. humidicola* germplasm accessions (Table 3). Based on a preliminary evaluation of 32 germplasm accessions for BNI activity (data not reported here), seven accessions were chosen (based on contrasting abilities for BNI capacity) for a more detailed study, the

Table 1 The BNI released from intact plant roots of vdarious plant species grown in sand-vermiculite (3:1 v/v) for 60 days (Based on Experiment 1)

Serial no.	Plant species	Total BNI released from four plants (AT units)	Specific BNI (AT units g^{-1} root dry wt)
	Pasture grasses		
1.	*Brachiaria humidicola* (Rendle) Schweick.	51.1	13.4
2.	*B. decumbens* Stapf	37.3	18.3
3.	*Melinis minutiflora* Beauv.	21.4	3.8
4.	*Panicum maximum* Jacq.	12.5	3.3
5.	*Lolium perenne* L ssp. *Multiflorum* (Lam.) Husnot	13.5	2.6
6.	*Andropogon gayanus* Kunth	11.7	7.7
7.	*B. brizantha* (A. rich.) Stapf	6.8	2.0
	Cereal crops		
8.	*Sorghum bicolor* (L.) Moench var. hybrid sorgo	26.1	5.2
9.	*Pennisetum glaucum* (L.) R. Br. var. CIVT	7.0	1.8
11.	*Oryza sativa* L. var Sabana 6	0	0
	Oryza sativa L. var. Toyo	0	0
12.	*Zea mays L.* var. Peter no. 610	0	0
13.	*Hordeum vulgare* L. var. Shunrai	0	0
14.	*Triticum aestivum* L. var. Norin-61	0	0
	Legume crops		
15.	*Arachis hypogaea* L. var. TMV 2	9.4	2.5
16.	*Glycine max* (L.) Merr. var. Orinoquia 3	0	0
	Glycine max. (L.) Merr var. Natsuroyosooi	0	0
	Glycine max(L.) Merr non-nodulating type—EN 1282	0	0
17.	*Vigna unguiculata* (L. Walpers ssp. unguiculata var. Caupi	0	0
18.	*Phaseolus vulgaris* (L.) (Accession G 21212)	0	0
	LSD (0.05)	7.1	2.8

Note: '0' activity refers that the inhibitory effect is possibly below the detection limit of our assay system

Table 2 The BNI released from roots of tropical pasture grasses grown in an acid soil from the Llanos of Colombia

Serial No.	Plant species	N fertilizer application	Total BNI released from four plants (AT units)	Specific BNI (AT units g^{-1} root dry wt)
1	*B. humidicola*	YES	82.0	17.3
2	*B. decumbens*	YES	77.4	12.3
3	*B. dictyonara*	YES	70.5	5.9
4	*B. hybrid*	YES	84.5	10.2
5	*B. brizantha*	YES	49.6	8.4
6	*P. maximum*	YES	0	0
1	*B. humidicola*	NO	13.1	2.9
2	*B. decumbens*	NO	21.4	7.2
3	*B. dictyonara*	NO	19.2	2.9
4	*B. hybrid*	NO	23.2	6.5
5	*B. brizantha*	NO	23.9	6.8
6	*P. maximum*	NO	1.8	0.7
	LSD (0.05)		6.5	1.6

Four plants per pot were grown for 180 days before collecting root exudate using distilled water (Based on Experiment 2)
Note: '0' activity refers that the inhibitory effect is possibly below the detection limit of our assay system

results of which are reported here. The root exudate from *P. maximum* did not show BNI, which is similar to the result of Experiment 2. The specific BNI in root exudate ranged from 6.5 to 46.3 AT units of activity among the seven germplasm accessions (Table 3). The high-BNI accessions had 3 to 5 times higher specific BNI in their root exudate than the low-BNI types (Table 3). The incubation study with soils collected from high-BNI and low-BNI field plots, showed that NO_3^- formation was significantly ($P < 0.05$) higher in soils from high-BNI plot compared to low-BNI field plots (Table 4; Fig. 1). A major portion (70 to 90%) of the inorganic N remained in NH_4^+ form at the end of 30 days incubation period in soils from high-BNI plots (Table 4). In contrast, >70% of the total inorganic N was in the NO_3^- form in the soils from low-BNI field plots (Table 4). Only 200 ppm N as NH_4^+ was added to the soils at the beginning of the 30 days incubation study, but total inorganic N in treatment soils exceeded 4,000 ppm in some cases on 30th day; this could be due to mineralization of organic N. Organic matter levels in these soils are very high and normally exceed 15%. Thus a major portion of the inorganic nitrogen in the soil at the end of a 30 days incubation period had originated from the mineralization of organic N.

Influence of N forms (NH_4^+ versus NO_3^-) on plant growth and release of BNI from roots of *Brachiaria humidicola* and *Panicum maximum* that have contrasting BNI capacity

There was little if any influence of the source of N (i.e. NH_4^+ or NO_3^-) in the nutrient solutions on the

Table 3 Genotypic variation in BNI released from roots of *B. humidicola* accessions. Four plants per pot were grown for 180 days before collecting root exudate using distilled water (Based on Experiment 3)

Serial No.	Accession No.	Total BNI released from four plants (AT units activity)	Specific BNI (AT units g^{-1} root dry wt)
1	CIAT 26159	126.2	46.3
2	CIAT 26427	118.5	31.6
3	CIAT 26430	151.0	24.1
4	CIAT 679	68.8	17.5
5	CIAT 26438	93.5	6.5
6	CIAT 26149	22.3	7.1
7	CIAT 682	53.4	7.5
8	*P. maximum*	0.6	0.1
	LSD (0.05)	21.7	6.0

Table 4 Differential nitrification in soils (0–15 cm) collected from field plots planted to pasture grasses *B. humidicola* for the last 10 years (Based on Experiment 6)

Serial No.	Accession No.	NO_3^- (mg kg^{-1})	NH_4^* (mg kg^{-1})	Total N (mg kg^{-1})	NO_3^- (% of total inorganic N)
1	CIAT 26159	135	2338	2473	5.4
2	CIAT 26427	94	987	1080	8.7
3	CIAT 26430	77	828	905	8.5
4	CIAT 679	67	808	875	7.6
5	CIAT 26438	1540	657	2197	70.1
6	CIAT 26149	1771	305	2076	85.3
7	CIAT 682	4586	223	4809	95.4
8	Control soil	577	158	735	78.5
	LSD (0.05)	330.1	82.1	124.5	

biomass production in medium-BNI (CIAT 679), or high-BNI (CIAT 26159) *B. humidicola* over a 60 days period (data presented only for root dry weight; Fig. 2). However, in *P. maximum*, NH_4^+ grown plants produced significantly higher biomass compared to NO_3^- grown plants (Fig. 2). Only NH_4^+ grown plants released BNI from roots (Fig. 3). No detectable BNI was found in the root exudate from NO_3^- grown plants (Fig. 3). The high-BNI genotype (CIAT 26159) showed consistently higher BNI in their root exudate than the medium-BNI type (i.e. CIAT 679) at 40, 50 and 60 days samplings (Fig. 4). Root exudate from *P. maximum* showed very low or no detectable BNI most of the time (Fig. 4).

BNI compounds from root exudate of high BNI genetic stock inhibits soil nitrification

The stability and effectiveness of BNI compounds (from root exudate) of *B. humidicola* CIAT 26159 (a high BNI-type) in inhibiting soil nitrification was evaluated during this study. BNI compounds ranging from 4 AT units g^{-1} to 17 AT units g^{-1} soil was added, and NO_3^- formation in the soil was determined after 26 and 51 days of incubation at 20°C. In the control soil (i.e. no BNI added) all the added NH_4^+ (i.e. 182 mg kg^{-1} soil) was nitrified by 51 days. Additions of BNI to the soil inhibited nitrification (i.e. NO_3^- formation) in the soil. The degree of inhibition on nitrification in the soil was a function of the amount of BNI added to the soil. The relationship between the amount of BNI added and inhibitory effect on nitrification was linear and highly significant ($P < 0.001$) after both 26 and 51 days of incubation (Figs. 5, 6). Nitrate levels were nearly 70% lower than the control when 17 AT units of BNI were added to g^{-1} soil (Figs. 5, 6).

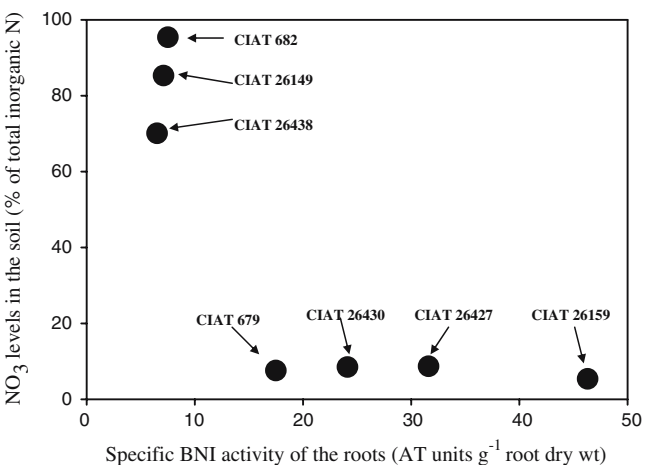

Fig. 1 Relationship between specific BNI in root exudate (Experiment 3) and NO_3^- levels observed in soils (after 30 days incubation; Experiment 6) collected from 10-year old pastures of *B. humidicola* genotypes

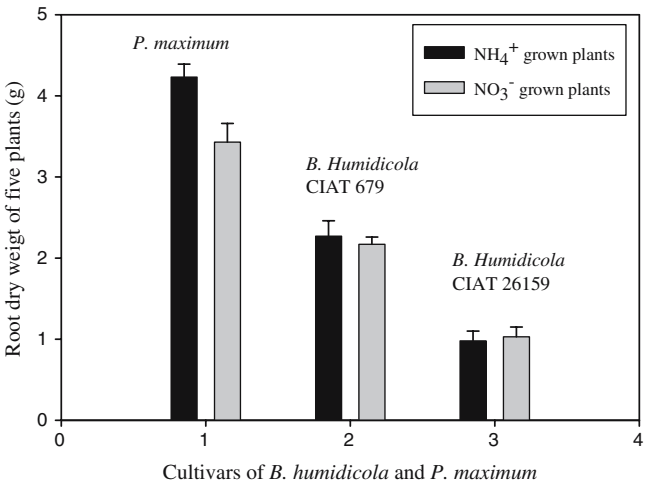

Fig. 2 Root dry weight of three tropical pasture grasses (*B. humidicola* CIAT 679, CIAT 26159 and *Panicum maximum* CIAT 16028) at 60 days of growth with NH_4^+ or NO_3^- as the sole source of N in nutrient solution (Based on Experiment 4)

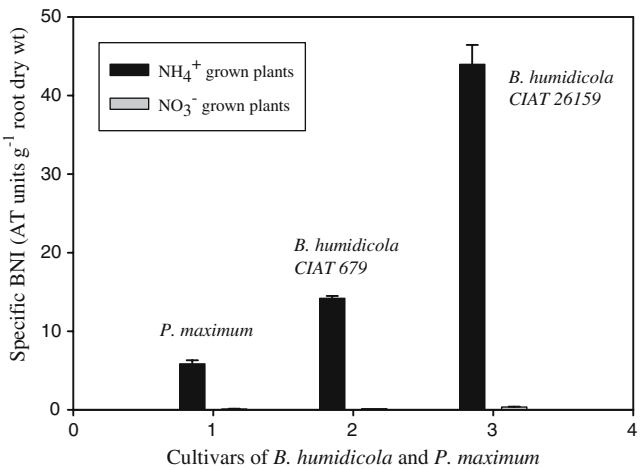

Fig. 3 Specific BNI (AT units g^{-1} root dry wt) from roots of three tropical pastures (two genotypes of *B. humidicola* CIAT 679; CIAT 26159; and *P. maximum*) at 60 days of growth in hydroponic culture (Based on Experiment 4)

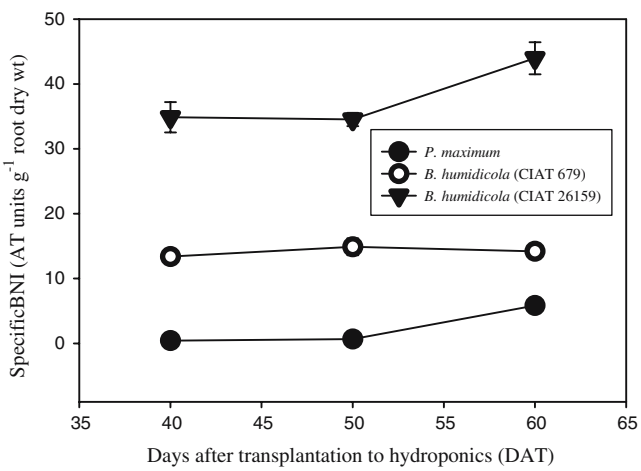

Fig. 4 Specific BNI of roots (AT units activity g^{-1} root dry weight) of three tropical pasture grasses (two genotypes of *B. humidicola* CIAT 679, CIAT 26159 and *Panicum maximum* CIAT 16028) at 40, 50 and 60 days of growth in solution culture (Based on Experiment 4)

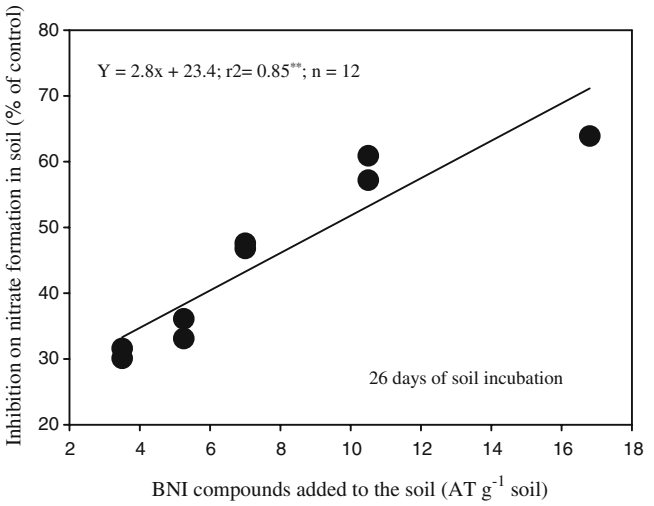

Fig. 5 Relationship between BNI compounds (from root exudate of *B. humidicola* CIAT 26159) added to the soil and inhibition on nitrate formation during a 26-day incubation period (Note: for the control treatment, i.e. no BNI activity is added to the soil) (Based on Experiment 5)

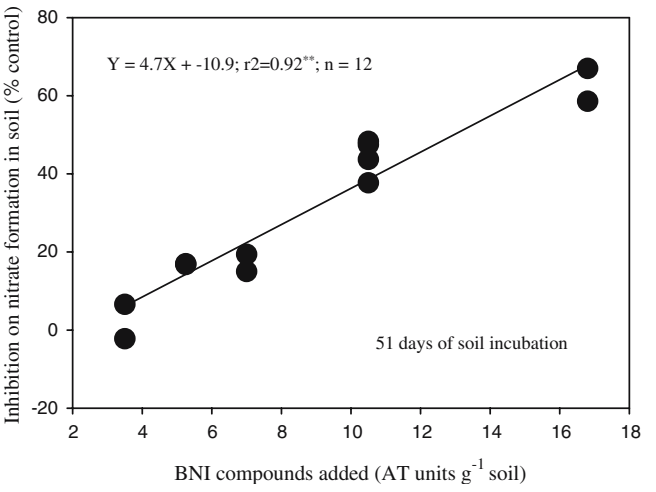

Fig. 6 Relationship between BNI compounds (from root exudate of *B. humidicola* CIAT 26159) added to the soil and inhibition on nitrate formation during a 51 days incubation period (Note: for the control treatment, i.e. no BNI is added to the soil, all the N added 182 mg kg^{-1} of NH_4^+ was nitrified by 51st day) (Based on Experiment 5)

Mode of action of BNI for both the low-BNI and high-BNI type of *B. humidicola*

The inhibitory effect of the synthetic nitrification inhibitor, AT was nearly absent when hydroxylamine was added to the *N. europaea* cultures (Table 5). The BNI compounds from CIAT 679, a medium-BNI type had a similar inhibitory effect on both the AMO and HAO pathways, as additions of hydroxylamine to the bacterial cultures did not alleviate the inhibitory effect (Table 5). The BNI compounds from CIAT 26159, a high-BNI type, had a greater inhibitory effect on HAO than the AMO enzymatic pathway of *Nitrosomonas* (Table 5).

Discussion

BNI is the release of biologically active compounds from roots that suppress or regulate nitrifiers function and growth which limits nitrification in soils. Although earlier researchers had suspected the existence of BNI based on empirical studies and field surveys (Theron, 1963; Lata et al., 2004; also see Subbarao et al., 2006a for review), there was little progress in establishing the existence of BNI phenomenon due to the lack of suitable methodology to detect and quantify nitrification inhibitors released from plant–soil systems. The development of a bioassay to detect inhibitors released from plant roots (i.e. BNI) has

Table 5 The effect of added hydroxylamine on the inhibitory action of BNI compounds (from root exudate of medium- and high-BNI genotypes of *B. humidicola*) added to a pure culture of *N. europaea* (Based on Experiment 7)

Treatment	Bioluminescence (RLU ml^{-1})	
	Without hydroxylamine addition in the assay	With hydroxylamine in the assay
DMSO control	21	210
BNI compounds from root exudate of CIAT 679	7**	64**
% inhibition	64	70
DMSO control	20	208
BNI compounds from root exudate of CIAT 26159	13**	49**
% inhibition	35	76
Water control	49	261
AT (100 µl)	9**	234ns
% inhibition	82	10

DMSO = dimethyl sulfoxide

** $P < 0.001$(based on the *t*-test between bioluminescence at control and inhibitor treatment)

ns = not significantly different from control

allowed the revisiting of these earlier hypotheses of nitrification inhibitors being released from plant systems especially tropical pasture grasses. Our results provide the first direct evidence for the existence of BNI capacity in tropical pastures, in particular *Brachiaria* ssp (Subbarao et al., 2006b). The degree of BNI capacity varied widely, and there were substantial inter-specific differences among the tropical pasture grasses. Pastures of *B. humidicola* and *B. decumbens* that are highly adapted to low-N production environments of the South American Savannas (Rao et al., 1996) showed the highest BNI capacity. In contrast, *P. maximum*, which is adapted to high input and intensive production systems (Rao et al., 1996) showed the least BNI capacity. Among the field crops evaluated, only sorghum, pearl millet and peanut showed detectable BNI compounds in their root exudate.

Inhibition of nitrification (i.e. BNI capacity) is likely part of the adaptation mechanisms to conserve and use N efficiently in some of the natural systems where N is the principal limiting factor for growth and reproduction (see review by Subbarao et al., 2006a). Thus N stress could be the dominant force driving the evolution of BNI as an adaptive mechanism (Rice and Pancholy, 1972; Lata et al., 2004). For legumes, it is likely that the BNI attribute will have little or no adaptive value due to their ability to fix N symbiotically. Conserving N from mineralization thus would not offer them any additional competitive advantage. Several of our preliminary studies indicate that soybean root exudate stimulated nitrification and N_2O emissions in soils (G.V. Subbarao and M. Rondon, unpublished results). Several forest systems that are dominated by leguminous trees (*Acacia mangium*, and *A. auriculaeformis*) have either no influence or stimulated soil nitrification; this contrasts with the observation that forest soils dominated by non-legume tree systems (*Eucalyptus citriodora*, *Pinus elliotii*, and *Schima superba*) usually have low nitrification rates (Li et al., 2001).

We demonstrated significant and substantial genotypic variability in BNI capacity in *B. humidicola*. Several high- and low-BNI genotypes were identified in *B. humidicola*, further suggesting that BNI is a genetically controlled attribute. The high-BNI genotypes released three to five times more BNI than the low-BNI genotypes. Furthermore, nitrification and NO_3^- formation was low in soils collected from plots planted to high-BNI genotypes, and most of the mineralized N was in NH_4^+ form. This is in contrast to lack of inhibition on nitrification was observed in soils collected from low-BNI genotype planted field plots, where most of the inorganic N was in NO_3^-form after a

30-day incubation period. This provides further evidence that high-BNI genotypes can have a strong influence on soil nitrification through the release of BNI compounds. Earlier, we showed that a threshold level of BNI, which is about 5 AT units g^{-1} soil needs to be reached before the inhibitory effect becomes effective; above that level, BNI shows an inhibitory effect on nitrification following a typical dose response curve (Subbarao et al., 2006b). The high-BNI genotypes likely reach this threshold level much earlier than the low-BNI genotypes, thus they can influence nitrification rates longer than the low-BNI genotypes. Our study is the first to demonstrate genetic differences (i.e. intra-specific variability) in influencing soil nitrification through the release of BNI compounds from roots and the ecological value of such a plant attribute under field conditions in controlling soil nitrification. Several high- and low-BNI genotypes identified during this study could become valuable genetic stocks/tools for understanding the physiological, agronomic and genetic characteristics of the BNI attribute.

Both genotypes of *B. humidicola* (CIAT 679 and CIAT 26159) released BNI from roots only when grown with NH_4^+, but not with NO_3^- as the N source. This is in agreement with our recent findings on the regulatory role of NH_4^+ (in the root environment) in the release of BNI compounds from *B. humidicola* roots (Subbarao et al., 2006c). Further, BNI (both total BNI and specific BNI) released from N-fertilized *Brachiaria* ssp. were substantially higher than that of from non-N fertilized plants (based on Table 2). This raises the possibility that the presence of NH_4^+ in the root environment is necessary for the release of BNI from roots, thus likely to be part of an adaptive mechanism in *Brachiaria* pastures to protect NH_4^+ being nitrified by *Nitrosomonas* sp.

The known AMO inhibitor, AT blocks only the AMO pathway, as the inhibitory effect of AT was completely overcome in the presence of hydroxylamine in the assay. This is in confirmation of our earlier report on the potential value of the assay to analyze the mode of action from inhibitory compounds that include root exudate (Subbarao et al., 2006b). The BNI compounds from CIAT 679 (a medium-BNI genotype) have inhibitory effect on both AMO and HAO pathways that agrees with earlier reports (Subbarao et al., 2006b). However, the BNI compounds from a high-BNI type (CIAT 26159) suppressed HAO pathway more effectively than the AMO pathway in *N. europaea.* This suggests that BNI from CIAT 679 is likely to be different from that of CIAT 26159. It could be that the BNI is composed of more than one active substance and composition of these inhibitors may differ between CIAT 679 and CIAT 26159. The BNI activity blocked both AMO and HAO enzymatic pathways of *Nitrosomonas*. This is in contrast to most synthetic nitrification inhibitors such as AT, nitrapyrin, and DCD that suppress *Nitrosomonas* function mostly by blocking the AMO pathway (Hauck, 1980; McCarty, 1999; see Subbarao et al., 2006a). Thus, the inhibitory effect from BNI is likely to be ecologically more effective than that of synthetic nitrification inhibitors.

Conclusions

The capacity to inhibit nitrification through the release of BNI compounds from roots appears to be widespread among *Brachiaria* ssp. with significant inter- and intra-specific variability. Several high- and low-BNI genotypes of *B. humidicola* were identified. BNI compounds from root exudate when added to the soil showed effective and stable inhibitory effect on NO_3^- formation for more than 50 days. The BNI compounds appear to block both AMO and HAO pathways of *Nitrosomonas*.

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