REGULAR ARTICLE

Free fatty acids from the pasture grass *Brachiaria humidicola* and one of their methyl esters as inhibitors of nitrification

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Abstract The tropical pasture grass, *Brachiaria humidicola* (Rendle) Schweick, produces nitrification inhibitory compounds (termed biological nitrification inhibitors or BNIs) in its shoot and root tissues and releases BNIs from its roots. In the present study, two BNI compounds were isolated and identified from the shoot tissue of *B. humidicola* using activity-guided fractionation. The recombinant *Nitrosomonas europaea* containing *lux*AB genes derived from the bioluminescent marine gram-negative bacterium *Vibrio harveyi*, were used to determine BNI activity. The BNI compounds in the shoot tissue were identified as linoleic acid (LA) and linolenic acid (LN) using authentic-chemicals obtained from ©Sigma (ED_{80} 16.0 µg ml^{-1} for both LA and LN) for verification. None of the other tested free fatty acids namely stearic acid, oleic acid, arachidonic acid, and cis-vaccenic acid showed any inhibitory effect on nitrification. Among the fatty acid methyl esters (FAME) evaluated [methyl oleate, methyl linoleate (LA-ME) and methyl linoleneate (LN-ME)], only LA-ME showed an inhibitory effect (ED_{80} 8.0 µg ml^{-1}). The inhibitory effect of LA, LN and LA-ME in the soil was stable for 120 days at 20°C. Soil treated with LA, LN and LA-ME showed a very low accumulation of NO_3^- and the maintenance of soil inorganic N in the NH_4^+ form. The inhibitory effect of LA-ME on soil nitrification was greater than that of LA or LN. In addition to BNI activity, both LA and LA-ME showed a suppressive effect on urea hydrolysis in soil. Both LA and LN blocked the AMO (ammonia monooxygenase) and HAO (hydroxylamino oxidoreductase) enzymatic pathways in *Nitrosomonas*. Since LA and LN can be produced from vegetable oils such as soybean, flax or sunflower, they have the potential for use as nitrification inhibitors in production agriculture.

Keywords Ammonia monooxgynase (AMO) ·
Biological nitrification inhibitors (BNIs) ·
Free fatty acids · Fatty acid methyl esters (FAME) ·
Hydroxylamine oxidoreductase (HAO) ·
Nitrification inhibition · Nitrification ·
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Introduction

The economic and environmental costs of nitrogen (N) losses from farming are large and are of global concern.

A major soil process leading to the loss of N during farming is nitrification, a microbial process that rapidly converts the relatively immobile NH_4^+ to mobile NO_3^-, which is highly susceptible to both leaching and denitrification (Subbarao et al. 2006a). Microbial processes associated with NO_3^- are the principal agricultural sources of N_2O emissions, a greenhouse gas with a global warming potential 300 times greater than CO_2, and is presently the third most important gas contributing to global warming (IPCC 2007). Blocking rapid nitrification will provide more time for the direct uptake and utilization of NH_4^+ by plants leaving less nitrogen to undergo nitrification thus, improving the efficiency of N utilization and at the same time reducing the negative environmental consequences of N fertilization (see review by Subbarao et al. 2006a).

Existence of plant-derived nitrification inhibitors have been known for several decades (Subbarao et al. 2006a). For example, neem (*Azadirachta indica* A. Juss), karanja (*Pongamia glabra* Vent)-products (Parmar et al. 1976; Sahrawat and Mukherjee 1977; Sahrawat et al. 1977) and certain plant oils (Patra et al. 2006) have been reported to show inhibitory effect on soil nitrification. In most cases, the active chemical constituents were either undefined or there were difficulties with consistent delivery of active ingredients in sufficient quantities to maintain inhibition; consequently they have had only limited value in production agriculture (Fillery 2007).

The natural ability of a plant to inhibit soil nitrification by releasing inhibitors from roots is termed, 'biological nitrification inhibition' (BNI) (Subbarao et al. 2006a). Such activity has been reported for the tropical pasture grass, *Brachiaria humidicola* (Ishikawa et al. 2003; Subbarao et al. 2006b, 2007a,b). Synthesis and release of BNIs from plant roots is a highly regulated plant attribute. This is indicated by the requirement for the presence of NH_4^+ in the rhizosphere where it functions as a trigger for initiating the release of BNI (Subbarao et al. 2007a). There are also substantial amounts of BNI in the methanol extracts from the shoot tissue of these plants (200 ATU g^{-1} shoot dry wt; ATU=allylthiourea unit activity) (Subbarao and Nakahara, unpublished results). Given that the production of shoot biomass by *B. humidicola* amounts to about 32 t ha^{-1} $year^{-1}$ (Subbarao and Marco Rondon, unpublished results), the total production of BNIs could be substantial. Recently, two nitrification inhibitors, methyl-*p*-coumarate and methyl ferulate were isolated that are responsible for a

major portion of the BNI activity found in the extracts of the root tissue (Gopalakrishnan et al. 2007). The present investigation was directed towards isolation, and identification of the BNI compounds in the shoot tissue of *B. humidicola* and the characterization of their inhibitory properties.

Materials and methods

Experiment 1a

Extraction of nitrification inhibition activity (i.e. BNI activity) from shoot tissue

Seeds of *B. humidicola* (CIAT 679) were planted on the JIRCAS (Japan International Research Center for Agricultural Sciences, Tsukuba, Japan) experimental farm. Shoot samples were collected 90 days after planting, freeze-dried and finely ground in a cyclone mill (UDY Corporation, Colorado, USA). The ground tissue was extracted with 80% methanol (1:20 *w/w*) for 8 h with stirring, followed by filtration. The methanol was removed under vacuum in a rotary evaporator at 35°C. The aqueous remainder from the previous step was partitioned against diethyl ether (Et_2O) three times. The organic phase (MeOH-Et_2O fraction) was concentrated in vacuo dissolved in methanol and stored at −20°C. Aliquots of these samples (100 μl) were dried in a centrifugal evaporator (model CVE-200D, Eyela, Tokyo, Japan), dissolved in dimethylsulfoxide (DMSO) and the BNI activity determined using the recombinant luminescent *Nitrosomonas* assay (Subbarao et al. 2006b). The BNI activity of the samples is expressed in units defined in terms of 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 AT unit of activity (ATU; Subbarao et al. 2006b).

Isolation and instrumental analysis of BNI activity

The MeOH–Et_2O fraction containing the BNI activity was dissolved in 50% MeOH acidified with 0.5% HCOOH and loaded onto a reversed-phase column (25×2.8 cm, Wakosil 40 C18, Wako) which was then equilibrated with 50% MeOH. The column was eluted with 1 l each of 50%, 75%, 90% and 100% MeOH. The BNI active fractions were further purified by HPLC on a PX-8020 system (Tosoh, Tokyo, Japan)

equipped with a photodiode array detector (Tosoh) with TSKgel Super-ODS (4.6×100 mm or 10× 100 mm) columns (Tosoh). The mobile-phase system was 65% acetonitrile in water and all the peaks were checked for BNI activity. BNI activity was detected in only two fractions. The mass (MS) spectra were recorded on an electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICRMS, Apex II 70e, Bruker Daltonics, Billerica, MA, USA). The ^1H and ^{13}C NMR spectra at 298 K were recorded on DRX 600 and Avance 800 spectrometer (Bruker Biospin, Karsruhe, Germany).

Experiment 1b

Determination of Linolenic acid (LA), Linolenic acid (LN) and total BNI activity in the shoot tissue

Concentrations of LA and LN in the shoot tissue were determined following Sukhija and Palmquist (1998); BNI activity of the shoot tissue was determined following Subbarao et al. (2006b).

Experiment 2

Evaluation of free fatty acids and their methyl esters (FAME) for their inhibitory effect on Nitrosomonas

Fatty acids (namely stearic acid, oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, and *cis*-vaccenic acid) and their fatty acid esters (methyl stearate, methyl oleate, methyl linoleate, ethyl linoleate and methyl α-linolenate) were obtained from Sigma (St. Louis, MO, USA) for the determination of inhibitory activity. All the solvents and other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless otherwise stated. Fatty acids and methyl esters were dissolved in DMSO (except in the case of stearic acid where benzene was used as the solvent) before determining their BNI activity using the bioassay described earlier (Subbarao et al. 2006b).

Experiment 3

Mode of inhibitory action of linoleic acid and linolenic acid on Nitrosomonas

Two purified BNI compounds from the shoot tissue namely linoleic acid (LA) and linolenic acid (LN)

along with the synthetic nitrification inhibitors (allylthiourea, nitrapyrin, and dicyandiamide), were evaluated to determine their inhibitory mode of action on *N. europaea*. Their mode of action was determined by incubating 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). Solutions of (200 μl) of water-soluble inhibitors, AT, DCD and nitrapyrin were added to 250 μl of bacterial culture; and the contents were incubated for 10 min before 200 μl of 1 mM hydroxylamine (to give 307 μM) was added. The total volume of the assay was 650 μl. The mean of the 10 bioluminescence measurements made during the 10 min incubation period was taken as the activity level. Every measurement was repeated three times and they were considered to be replications for the calculation of standard error. The effect of the addition of the AMO enzyme product (i.e. hydroxylamine) to the reaction mixture was evaluated. The inhibitory effect of purified BNI compounds (i.e. LA and LN) and the synthetic inhibitors, allylthiourea, nitrapyrin and DCD on *Nitrosomonas* activity was determined in the presence of hydroxylamine (i.e. inhibition of the HAO enzymatic pathway) and in the absence of hydroxylamine (i.e. inhibition of the AMO enzymatic pathway) as described earlier (Subbarao et al. 2006b). The data were subjected to analysis of variance and the least significant differences at $P< 0.05$ (Fisher LSD) was determined.

For LA, LN, LA (dissolved in DMSO) calculation for the inhibition were:

1. Inhibition (%) on AMO pathway=[100−((Bioluminescenece in LA or LN-water treatment/ Bioluminescence in DMSO-water control)×100)]
2. Inhibition (%) on HAO pathway=[100−((Bioluminescenece in LA or LN-HA treatment/Bioluminescence in DMSO-water control)×100)]

For water soluble synthetic inhibitors (AT, DCD, nitrapyrin) the calculations for inhibition were:

3. Inhibition (%) on AMO pathway=[100− ((Bioluminescenece in inhibitor-water treatment/ Bioluminescence in water control)×100)]
4. Inhibition (%) on HAO pathway=[100−((Bioluminescenece in inhibitor-HA treatment/Bioluminescence in water control)×100)]

Experiment 4

Ameliorative effects of the fatty acid binding protein, BSA (bovine serum albumin) on the inhibitory effect of Linolenic acid (LN) on Nitrosomonas

To determine the nature of the inhibitory function of LN, a fatty acid binding protein, BSA was introduced into the assay during the incubation with LN. The bacterial culture (250 µl) was incubated with LN (50 µM in the assay medium) for 10 minutes before 200 µl of BSA solution (to give BSA concentration of 1.53 mg/ml in assay) was added and incubated for an additional 20 min at 20°C before measuring biolumi-nescence. The total volume of the assay was 650 µl. Similarly, the root exudate containing BNI activity from *B. humidicola* was included as a treatment to determine whether their inhibitory effect would also be influenced by the BSA protein (details on collection and extraction of BNIs from root exudate were described earlier, Subbarao et al. 2006b). Data were subjected to analysis of variance and the least significant differences at $P<0.05$ (Fisher LSD) was determined.

Experiment 5

Inhibitory effect of linoleic acid (LA), linolenic acid (LN) and linoleic acid methyl ester (LA-ME) on soil nitrification

To characterize the inhibitory function of LA, LN and LA-ME in soil, several soil incubation experiments were conducted. Pure compounds of LA, LN and LA-ME (obtained from Sigma) were added to soil (and mixed thoroughly) to prepare a stock soil with a concentration of 10,000 µg g^{-1} soil for each inhibitor. This stock soil was used to create treatment soils with varying concentrations of the test compound. For the control and the nitrapyrin treatment, a similar amount of the ground soil was added. Nitrapyrin was dissolved in a small amount of ethanol and then diluted to several thousand times before adding it to the soil (4.5 µg g^{-1} soil) as described earlier (Subbarao et al. 2006b). 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 g^{-1} soil; total N=2.5 mg g^{-1} soil; C/N ratio of 11.7 and CEC=

119.1 me/100 g], collected from the JIRCAS (Japan International Research Center for Agricultural Sciences) experimental farm in Tsukuba, Japan. The soil was passed through a 2-mm sieve before use. The soil water status during the experiment was maintained at a level where 60% of the soil pore space was water filled, which is considered -optimum for nitrification (WFPS) (0.36 ml of water was required per gram to give 60% WFPS for this soil; Mosier et al. 1996). To confirm the effectiveness of nitrification inhibition by LA, LN and LA-ME in the soil, two incubation experiments were carried out. The first incubation experiment was aimed at evaluating the nitrification inhibitory activity of LA and LN at various concentrations (0 to 1,000 µg g^{-1} soil) in the soil. For each treatment, 400 µg of N as $(NH_4)_2SO_4$ was added to bottles containing 2 g soil. The remaining details of the soil incubation study were as described earlier (Subbarao et al. 2006b). The experiment was replicated three times. The second incubation study was aimed at evaluating the stability of these nitrification inhibitors, LA, LN and LA-ME (1,000 µg g^{-1} soil) in soil over a 120 days incubation period. The experiment consisted of four sets of bottles with treatment soils (i.e. control, LA, LN, LA-ME and nitrapyrin), incubated at 20°C and 85% humidity in a temperature humidity controlled incubator (Bench-top type temperature and humidity chamber, ESPEC Corp., Osaka, Japan). Sequential sampling of the incubated soils was done at 30 d intervals for up to 120 days. One set of the bottles were used for each sampling. After the incubation period, the soil samples were extracted by shaking with 20 ml of 2 M KCl for 30 min, and then they were filtered through Wattmann no. 1 filter paper. The filtrate was then analyzed colorimetrically for NH_4^+ (indophenol method) using an auto ion analyzer (model AA II, Brant + Luebbe, Germany; Litchfield 1967; Varley 1966). Data were subjected to analysis of variance and the least significant differences at $P<0.05$ (Fisher LSD) was determined.

Experiment 6

Influence of LA and LA-ME on urea hydrolysis in soil

Soil was mixed with LA, and LA-ME (similar to the soil incubation study mentioned above) to give a concentration of 500 or 1,000 µg g^{-1} soil. A known urease inhibitor, hydroquinone at 50 µg g^{-1} soil was
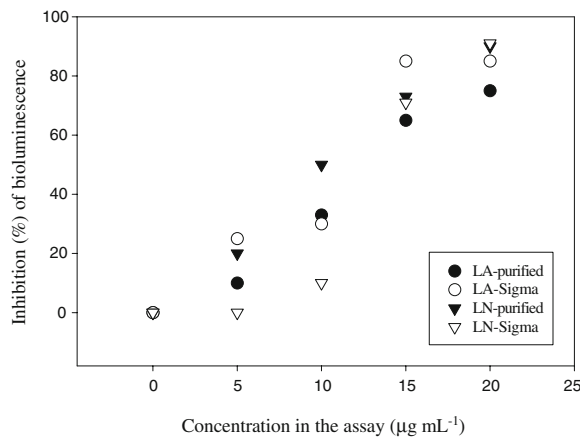
Fig. 1 Inhibitory effect on *Nitrosomonas* activity (*in vitro* assay) from linoleic acid (*LA*) linolenic acid (*LN*) isolated from shoot tissue of *B. humidicola* and authentic samples obtained from Sigma

included as an additional treatment along with a water control. Soil urease activity was determined according to Douglas and Bremner (1971). The detailed protocol is as follows: Urea (1,700 $\mu g\ g^{-1}$ soil, equivalent to 800 $\mu g\ N\ g^{-1}$ soil) was added to the soil and 0.72 ml of distilled water was added to each bottle containing the 2 g of soil to give a water status of 60% WFPS. The experiment consisted of four sets of bottles with treatment soils (i.e. control, LA, LA-ME, hydroquinone), incubated at 20°C and 85% humidity in a temperature and humidity controlled incubator (Bench-top type temperature and humidity chamber, ESPEC Corp, Osaka, Japan). Sequential sampling was done on day 3, 6, 8 and 10 after incubation. The experiment was replicated three times. The mouth of each bottle was sealed with parafilm in which a pinhole was made to provide adequate aeration. The bottles containing the soil were incubated at 20°C in a temperature humidity controlled incubator. After incubation, the soil sample was shaken for 2 h with 20 ml of 2 M KCl containing 5 ppm phenyl mercuric acetate to extract the NH_4-N. The sample was then filtered using Whattman no. 1 filter paper and the filtrate was analyzed colorimetrically for NH_4^+ (indophenol method) using an auto ion analyzer as described earlier.

Percentage inhibition of urea hydrolysis in the soil sample was calculated as suggested by Douglas and Bremner (1971).

Inhibition (%) of soil urease activity = $[(100 - (NH_4-N$ concentration in treatment soil/NH_4-N concentration in control soil) × 100)]

Results

BNI activity isolated from *B. humidicola* shoot tissue

The activity-guided fractionation of the methanol extract from shoot tissue of *B. humidicola* led to the isolation of two BNI active compounds. Mass spectrometry and ^1H, ^{13}C NMR determined them to be α-linolenic acid (LN), and linoleic acid (LA) by direct comparison with authentic chemicals. Their inhibitory activity was also confirmed by comparison with authentic chemicals obtained from ©Sigma (Fig. 1). In the shoot tissue, the concentration of LA is 1.35 mg g^{-1} DW and for LN it is 2.88 mg g^{-1} DW; BNI activity is 215 ATU g^{-1} DW (Table 1).

BNI activity of free fatty acids and fatty acid esters (FAME)

None of the other fatty acids evaluated showed measurable BNI activity at a concentration of 20 μg ml^{-1} (or about 71 μM) in the assay, except LA and LN (Table 2). With the exception of methyl linoleate (LA-ME), none of the fatty acid esters tested showed BNI activity (Table 2). It is interesting to note that LA lost its nitrification inhibitory ability when converted into the ethyl ester (LA-EE), but showed a larger inhibitory effect when converted into methyl ester (i.e. LA-ME) (Table 3); this is further illustrated in the dose-response relationships shown in Fig. 2. Linolenic acid (LN) lost its inhibitory ability when converted into methyl linolenate (LN-ME) (Tables 2 and 3). The ED$_{80}$ (effective dose for 80% inhibition) for LA, LN, their esters and standard nitrification inhibitors (nitrapyrin and dicyandiamide) indicated

Table 1 Linoleic acid (LA), Linolenic acid (LN) levels and BNI activity of the shoot tissue of *B. humidicola*

BNI compound	Concentration in shoot tissue (mg g^{-1} dry weight)	SE
Linoleic acid (LA)	1.35	0.05
Linolenic acid (LN)	2.88	0.09
BNI activity	ATU g^{-1} dry weight[a]	
Freeze-dried shoot tissue extracted with 70% methanol	215	5.8

[a] *ATU* Allylthiourea unit activity – The inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one AT unit of activity (ATU).
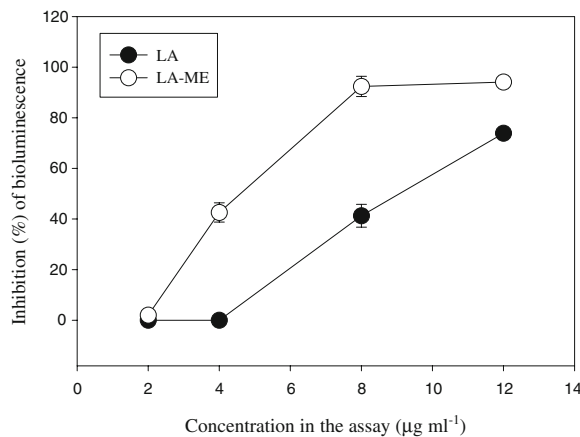
Fig. 2 Relative effectiveness of linoleic acid (*LA*) and methyl linoleate (*LA-ME*) (authentic chemicals from ©Sigma) in inhibiting *Nitrosomonas* activity in an in vitro assay; *vertical bars* represent standard error of means (*n*=3)

that nitrapyrin and LA-ME had the most potent nitrification inhibitory activity (Table 3). The standard nitrification inhibitor, dicyandiamide required substantially higher concentrations for the same degree of nitrification inhibition (Table 3).

Mode of inhibitory action on *Nitrosomonas*

The inhibitory effect of synthetic nitrification inhibitors, nitrapyrin and dicyandiadiamide on *N. europaea* was eliminated in the presence of hydroxylamine, suggesting that only the ammonia monooxygenase

(AMO) enzymatic pathway was blocked (Table 4). However, the nitrification inhibitory effect by LA and LN was not alleviated in the presence of hydroxylamine, suggesting that both AMO and hydroxylamine oxidoreductase (HAO) enzymatic pathways of *N. europaea* were blocked (Table 4). The inhibitory effect of LN was partially alleviated when a fatty acid binding protein such as bovine serum albumin (BSA) was introduced into the assay, suggesting that the inhibitory effect could be reversible (Fig. 3). Inhibitory effect from the BNI activity of root exudate however, was not relieved in the presence of BSA, indicating that the BNI compounds in the root exudate had a different mode of action than the BNIs obtained from the shoot tissue of *B. humidicola* (Fig. 3).

Inhibitory effects of LA, LN and LA-ME on nitrification

The inhibitory effects of LA, and LN on soil nitrification were further characterized with a 30 d soil incubation study (Table 5). The conversion of NH_4^+ to NO_3^- in soil decreased as the concentration of LA and LN increased and reached a near total suppression of nitrate formation at ≥ 600 μg g^{-1} soil (Table 5). Net immobilization was not enhanced as there was likely mineralization and immobilization turnover in soil but not net immobilization of inorganic N; most inorganic N remained as NH_4^+ due to the inhibitory effect from LA or LN (Table 5).

Table 2 Influence of free fatty acids (FA) and fatty acid methyl esters (FAME) on *Nitrosomonas* function (i.e. bioluminescence) in in vitro assay

Compound	No. of double bonds	Concentration in in vitro assay (μg ml^{-1})	Inhibition (%) on bioluminescence
Free fatty acids			
Stearic acid	0	20	−35.9[a]
Oleic acid	1	20	−4.5
Linoleic acid (LA)	2	20	95.0
Linolenic acid (LN)	3	20	87.9
Arachidonic acid	4	20	−135.3
cis-Vaccenic acid			−17.2
Fatty acid esters			
Methyl stearate	0	20	−120.0
Methyl oleate	1	20	−110.0
Methyl linoleate (LA-ME)	2	20	95.10
Ethyl linoleate (LA-EE)	2	20	−68.7
Methyl linolenate (LN-ME)	3	20	−26.2

[a] Negative inhibitory activity indicates that the compound has a stimulatory effect on *Nitrosomonas* function in in vitro assay.

Table 3 Relative effectiveness of free fatty acids, fatty acid esters, and standard chemical nitrification inhibitors on *Nitrosomonas* in in vitro bioassay

Compound	ED$_{80}$ [effective dose (μg ml^{-1}) for 80% inhibition on *Nitrosomonas* function (i.e. bioluminescence) in in vitro bioassay system]
Synthetic nitrification inhibitors	
©Nitrapyrin	4.0
©Dicyandiamide	185.0
Free fatty acids	
Linoleic acid (LA)	16.0
Linolenic acid (LN)	16.0
Fatty acid esters	
Methyl linoleate (LA-ME)	8.0
Ethyl linoleate (LA-EE)	400.0
Methyl linolenate (LN-ME)	>2,000.0

The nitrapyrin treatment lost a major part of its nitrification inhibitory effect by 120 days, while LA lost nearly 50% of its nitrification inhibitory effect during the same period. The inhibitory effect of LN and LA-ME on nitrate formation remained stable during the entire 120 days incubation period (Fig. 4).

Inhibitory effects of LA, and LA-ME on soil urease activity

Soil urease inhibitory activity from LA, and LA-ME lasted only 3 to 6 days (Table 6). The inhibitory effect of LA-ME was stronger than that of LA. In comparison, the inhibitory effect on soil urease activity from hydroquinone (a well known soil urease inhibitor) was higher and lasted longer than 10 days (Table 6).

Discussion

The BNI activity is attributed to LA and LN, which accounts for nearly 70% of the total fatty acid profile of the shoot tissue (data not presented), with concentrations of LA + LN reaching 4 mg g^{-1} DW. Given that the potential biomass production of these plants is about 32 t ha^{-1} year^{-1}; (Subbarao and Marco Rondon, unpublished results), the potential production of BNIs (i.e. LA + LN) from the shoot biomass of *B. humidicola* could be substantial. In systems where the biomass

is recycled back into the soil the release of BNIs from shoot tissue during decomposition could contribute significantly to the nitrification inhibition from *B. humidicola*. However, in pasture systems where the above ground biomass is either removed or grazed, nitrification inhibitors in the shoot tissue may have only limited significance. Nitrification inhibitors exuded from roots and those released from root tissue during root decomposition (methyl-*p*-coumarate and methyl ferulate) play a major role in reducing soil nitrification rates in *B. humidicola* ecosystems (Subbarao et al. 2006b; Gopalakrishnan et al. 2007).

The inhibitory effect of LA increased when converted into methyl ester (LA-ME), but was lost when converted into ethyl ester (LA-EE). In contrast, the inhibitory effect of LN was lost when converted to the methyl ester (LN-ME), indicating that there may be a high degree of specificity in the chemical structure needed to inhibit *Nitrosomonas* function. The inhibitory effect of LA, LN and LA-ME on nitrification is also shown from the soil incubation studies. Further, the nitrification inhibitory effect of LN and LA-ME was more persistent than that from nitrapyrin, lasting for the entire 120 days incubation period. The concentrations of LA and LN ($\geq$600 μg g^{-1} soil) required for an inhibitory effect on soil nitrification is substantially higher than that from nitrapyrin (4.5 μg g^{-1} soil) or DCD (20 to 30 μg g^{-1} soil; Subbarao et al. 2007c). The inhibitory effect of LA, LN or LA-ME on soil nitrification is more stable

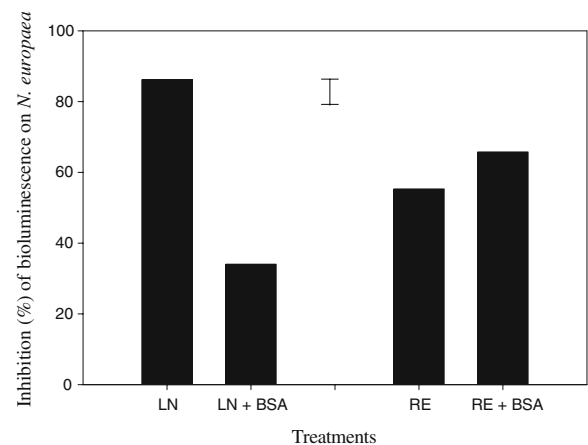**Fig. 3** Influence of bovine serum albumin (*BSA*; 1.53 mg ml^{-1}) on the inhibitory function of LN (linolenic acid, 16 μg ml^{-1} in the assay medium) and root exudate (*RE*) from *B. humidicola*, in the in vitro *N. europaea* assay; *Vertical bar* represents Fisher LSD (*P*<0.001) for the interaction term (inhibitor × BSA)

Table 4 Inhibitory strength of BNIs (LA and LN) isolated from shoot tissue of *B. humidicola* and synthetic chemical inhibitors (allylthiourea, nitrapyrin and dicyandiamide) on AMO or HAO enzymatic pathways of *N. europaea*

Compound	Concentration (μM) in in vitro assay	Inhibition (%)	
		AMO pathway	HAO pathway
BNIs from B. humidicola			
Linoleic acid (LA)	86.9	96.1	99.4
Linoleneic acid (LN)	86.9	94.0	98.4
Synthetic nitrification inhibitors			
Allylthiourea	0.2	81.4	8.2
Nitrapyrin	2.6	76.5	10.9
Dicyandiamide	2200.0	88.0	7.9
Dicyandiamide	4,400.0	89.4	7.3
LSD (0.05)		3.2	2.3

and effective than that from nitrapyrin due to the greater volatility of nitrapyrin. Because of its volatility, nitrapyrin is not persistent at soil temperatures >10°C, thus not effective under tropical conditions (Slangen and Kerkhoff 1984). In addition, LA and LN are hydrophobic, making them relatively immobile which results in them remaining close to the site of application. In contrast, DCD is highly mobile and is often leached away from the application zone, making its affect inconsistent under field conditions (McCarty and Bremner 1989). The effectiveness of LA, and LA-ME could be enhanced due to their suppressive effect on soil urease activity. Currently, urea is the dominant form of nitrogen fertilizer used worldwide (Kroschwitz and Howe-Grant 1995). Available urease inhibitors such as hydroquinone have neither consis-

Fig. 4 Relative stability of the inhibitory effects on soil nitrification from linoleic acid (*LA*; 1,000 μg g^{-1} soil), linolenic acid (*LN*; 1,000 μg g^{-1} soil), methyl linoleate (LA-ME; 1,000 μg g^{-1} soil) and nitrapyrin (4.5 μg g^{-1} soil) during 120-day incubation period at 20°C. V*ertical bar* represents Fisher LSD (*P*<0.001) for the interaction term (inhibitor × soil incubation period)

tency in performance nor are they cost-effective (Mulvaney and Bremner 1978).

In these studies there did not appear to be any direct relationship between the number of double bonds in the unsaturated fatty acids and their inhibitory effect on *Nitrosomonas* function. Saturated fatty acids (i.e. no double bonds) such as stearic acid and unsaturated fatty acids with four double bonds such as arachidonic acid, both showed no inhibitory effect on *Nitrosomonas*. This is in contrast to previous work that suggested that the biological activity of free fatty acids may be influenced by the number and position of their double bonds as they could determine stereo-structure, permeability and affinity to lipophilic proteins (Richieri and Kleinfeld 1989; Gottlicher et al. 1992; Kray et al. 1997).

Two key enzymes, ammonia monooxygenase (AMO, a membrane-bound enzyme) and hydroxylamine oxidoreductase (HAO, cytosolic enzyme) play a critical role in the oxidation of NH_4^+ to NO_2^- in *Nitrosomonas* spp. (Bock et al. 1991). The synthetic nitrification inhibitors, nitrapyrin and dicyandiamide (DCD) both blocked only the AMO pathway of *Nitrosomonas*, and have no effect on the HAO enzymatic pathway. For DCD, doubling the concentration in assay from 2,200 to 4,400 μM did not alter its specificity to AMO, agreeing with the earlier reports (McCarty 1999; Subbarao et al. 2007c). In contrast, LA and LN blocked both the AMO and the HAO enzymatic pathways, similar to the BNIs in the root exudate released from *B. humidicola* (Subbarao et al. 2006b). Also, palmitoleic acid, palmitic acid and oleic acid are reported to block only the AMO pathway in *Nitrosomonas* at concentrations >240 μM, and this inhibitory effect is reversible (Rottenberg and Hashimoto 1986; Skulachev 1991). Fatty acids, such

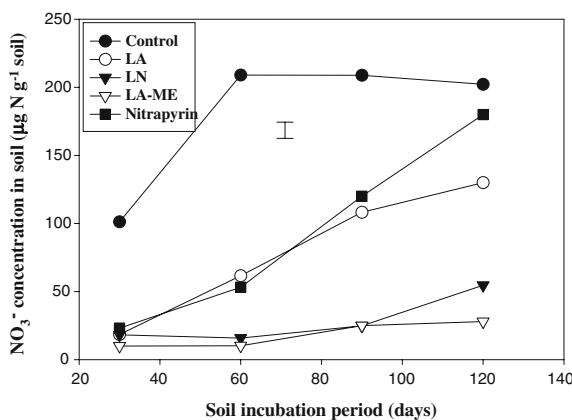**Table 5** Effectiveness of linoleic acid (LA) and linolenic acid (LN) in inhibiting nitrification in soil after 30 days of incubation at 20°C

Treatment	Concentration in soil ($\mu g\ g^{-1}$ soil)	NH_4^+–N concentration in soil ($\mu g\ N\ g^{-1}$ soil)	NO_3^-–N concentration in soil ($\mu g\ N\ g^{-1}$ soil)	NO_3^--N as % of total inorganic N	Inhibition (%) of NO_3^- formation
Linoleic	0	120.1	87.2	42.1	
acid (LA)	50	119.5	72.7	37.8	16.6
	100	119.8	71.3	37.3	18.2
	200	129.7	55.3	29.9	36.6
	400	149.1	35.8	19.4	58.9
	600	151.4	25.8	14.6	70.4
	800	158.1	15.3	8.8	82.5
	1,000	169.5	10.9	6.1	87.5
LSD (0.05)		14.0	3.7		
Linolenic	50	122.9	72.7	37.2	16.6
acid (LN)	100	132.1	61.6	31.8	29.4
	200	146.1	44.8	23.5	48.6
	400	167.6	20.1	10.7	77.0
	600	174.1	13.4	7.2	84.6
	800	175.6	9.8	5.3	88.8
	1,000	173.1	7.9	4.4	90.9
LSD (0.05)		12.1	6.8		

Table 6 Influence of linoleic acid (LA), methyl linoleate (LA-ME), and hydroquinone on soil urease activity during a 10 day incubation period at 20°C

Soil treatment	Incubation period (days)	Soil urease activity NH_4^+ -N formed ($\mu g\ g^{-1}$ soil)	SE	Inhibition of Soil urease activity (%)
Control	3	234.3	3.4	
	6	470.7	3.2	
	8	577.4	9.8	
	10	790.0	4.5	
Linoleic acid (LA) – 500 $\mu g\ g^{-1}$ soil	3	135.6	6.1	42.0
	6	465.0	7.4	1.2
	8	612.3	6.1	0
	10	800.0	10.1	0
Linoleic acid (LA) – 1,000 $\mu g\ g^{-1}$ soil	3	107.4	2.4	54.2
	6	250.7	1.4	46.7
	8	518.6	5.8	10.2
	10	805.3	20.7	0
Methyl linoleate (LA-ME) – 500 $\mu g\ g^{-1}$ soil	3	121.8	1.31	48.0
	6	461.4	33.2	1.9
	8	570.7	8.3	1.2
	10	790.0	6.6	0
Methyl linoleate (LA-ME) – 1,000 $\mu g\ g^{-1}$ soil	3	103.1	6.7	56.0
	6	213.2	3.5	54.7
	8	405.2	12.7	29.8
	10	639.4	6.9	19.1
Hydroquinone – 50 $\mu g\ g^{-1}$ soil	3	59.4	2.2	74.6
	6	196.8	10.3	58.2
	8	258.9	2.2	55.2
	10	356.4	7.7	54.9

as undecynoic acid are reported to bind to the active AMO site in a similar fashion to acetylene, a suicidal inhibitor where the inhibitory effect is irreversible (Hyman et al. 1988; McCarty 1999). The reversible nature of the LN's inhibitory effect in the presence of BSA (a fatty acid binding protein) indicates that the inhibitory effect of LN and possibly LA may not be suicidal.

The finding that LA and LN are the predominant nitrification inhibitors in the shoot tissue of *B. humidicola* is interesting as they are essential constituents of the fatty acid profile in many forage grasses and vegetable oils (Fan and Chapkin 1998; Clapham et al. 2005). The possibility of using vegetable oils as the raw material for the synthesis of these inhibitors enhances the chances of the successful application of these findings.

Conclusions

Nitrification inhibitors were isolated from the shoot tissue of *B. humidicola*, identified and characterized. The study presented here demonstrates the potent and durable nitrification inhibitory activity of LA, LN and LA-ME. Given the desirability of developing the next generation nitrification inhibitors that are cost-effective, environmentally friendly and functionally effective in providing reliable control of soil nitrification, these findings may be a significant step in that direction.

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