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ORIGINAL ARTICLE

Biological nitrification inhibition by *Brachiaria humidicola* roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms

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Abstract

The tropical pasture grass *Brachiaria humidiola* (Rendle) Schweick releases nitrification inhibitory compounds from its roots, a phenomenon termed 'biological nitrification inhibition' (BNI). We investigated the influence of root exudates of *B. humidicola* on nitrification, major soil microorganisms and plant growth promoting microorganisms using two contrasting soil types, Andosol and Cambisol. The addition of root exudates (containing BNI activity that is expressed in Allylthiourea unit (ATU) was standardized in a bioassay against a synthetic inhibitor of nitrification, allylthiourea, and their function in soil was compared to inhibition caused by the synthetic nitrification inhibitor dicyandiamide. At 30 and 40 ATU g^{-1}soil, root exudates inhibited nitrification by 95% in fresh Cambisol after 60 days. Nitrification was also similarly inhibited in rhizosphere soils of Cambisol where *B. humidicola* was grown for 6 months. Root exudates did not inhibit other soil microorganisms, including gram-negative bacteria, total cultivable bacteria and fluorescent pseudomonads. Root exudates, when added to pure cultures of *Nitrosomonas europaea*, inhibited their growth, but did not inhibit the growth of several plant growth promoting microorganisms, *Azospirillum lipoferum*, *Rhizobium leguminosarum* and *Azotobacter chroococcum*. Our results indicate that the nitrification inhibitors released by *B. humidicola* roots inhibited nitrifying bacteria, but did not negatively affect other major soil microorganisms and the effectiveness of the inhibitory effect varied with soil type.

Key words: *Brachiaria humidicola*, inhibitors, nitrification inhibition, root exudates, soil microorganisms.

INTRODUCTION

Nitrification is an important biological process in the nitrogen cycling of both natural and managed agricultural ecosystems. Nitrification products (i.e. nitrite and nitrate) are vulnerable to leaching and denitrification, resulting in the loss of 45–60% of applied nitrogenous fertilizer (Jarvis 1996). Field evaluations using synthetic nitrification inhibitors have determined that if nitrification is reduced in agricultural systems, plants have more time to take up available N, thereby improving N recovery and uptake and reducing NO_3^- leaching and associated off-farm environmental impacts (Subbarao *et al.* 2006b). However, widespread adoption of synthetic nitrification inhibitors has not occurred, primarily because they are not economical for use in farming systems. Synthetic inhibitors also tend to be inconsistent in performance or are not persistent under field conditions (Fillery 2007; Slangen and Kerkhoff 1984; Subbarao *et al.* 2006b).

For many years it has been hypothesized that plants release compounds from their root systems that are capable of inhibiting the nitrification process (Lata *et al.* 2004; Moore and Waid 1971; Russell 1914). This hypothesis has not been supported because of ambiguity resulting from the complexity of real soil systems. A limitation to past research supporting this hypothesis has been the lack of a direct way to examine the effect. Recently, a bioassay

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using a genetically recombinant *Nitrosomonas europaea* was developed that determined that compounds present in root exudates could directly inhibit *N. europaea* bacteria. This new method allowed the inhibition effect to be standardized against a known synthetic nitrification inhibitor, allylthiourea (AT) (Iizumi and Nakamura 1997; Iizumi *et al.* 1998; Subbarao *et al.* 2006a). This phenomenon was termed biological nitrification inhibition (BNI) to distinguish it from chemical nitrification inhibition caused by synthetic nitrification inhibitors (Subbarao *et al.* 2006a).

Creeping signal grass or false creeping paspalum, *Brachiaria humidicola* [Rendle] Schweick, is a native pasture grass of Africa and is grown widely in the humid-tropical countries of South America, the Pacific Islands, South-East Asia and in coastal northern Australia (Schultze-Kraft and Teitzel 1992). Lower levels of NO_3^- have been found in fields of *B. humidicola* than in other forage grasses, including *Melinis minutiflora* Beauv., *Andropogon gayanus* Kunth. and *Brachiaria decumbens* Stapf. in the acid soils of Colombia (International Center for Tropical Agriculture 1985; Sylvester-Bradley *et al.* 1988). *Brachiaria humidicola* is also less responsive to the application of inorganic N fertilizer than other forage grasses (Sylvester-Bradley *et al.* 1988). Populations of ammonia-oxidizing bacteria (AOB) are reported to be suppressed by *B. humidicola* (Ishikawa *et al.* 2003; Sylvester-Bradley *et al.* 1988).

When tested in the bioassay, exudates released from *B. humidicola* roots have a strong inhibitory effect on *N. europaea* function and were shown to inhibit nitrification in air-dried and then rewetted soil (Subbarao *et al.* 2006a). The bioassay has also been used to isolate compounds from root (methyl-*p*-coumarate and methyl ferulate) and shoot tissues (linoleic and linolenic acid) of *B. humidicola* (Gopalakrishnan *et al.* 2007; Subbarao *et al.* 2008). These compounds may be important for nitrification inhibition resulting from root or shoot degradation and organic matter turnover in natural ecosystems (Gopalakrishnan *et al.* 2007). The compounds responsible for the inhibition of *N. europaea* have not yet been successfully isolated from root exudates.

The presence of NH_4^+ has been found to stimulate the release of BNI compounds from *B. humidicola* (Subbarao *et al.* 2007a). Root exudates from *B. humidicola* can block both the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) pathways. This contrasts with the function of the synthetic inhibitor AT, which only blocks the AMO pathway (McCarty 1999; Subbarao *et al.* 2007a). At present, very little information is available on the function of these root exudates on soil microbes. We hypothesize that BNIs released from the roots of *B. humidicola* inhibit nitrifying bacteria, but do not inhibit other soil microorganisms, and that the effectiveness of nitrification inhibition may vary with soil type. Testing this hypothesis should provide critical insights into the specificity of the inhibitors released from *B. humidicola* roots in inhibiting the nitrification process and how this can differ with soil type, thereby providing evidence on the physiological relevance of BNIs to this species. Therefore, the main objectives of the present study were to: (1) evaluate the effectiveness of BNI activity (of root exudates of *B. humidicola*) on soil nitrification using contrasting soil types, Andosol and Cambisol, (2) investigate the impact of BNI activity on major soil microorganisms and plant growth promoting soil microorganisms in the rhizosphere and in pure culture, allowing for conclusions to be drawn on whether the BNIs released from the roots have a targeted function (e.g. suppressing nitrifier activity) or are more allelopathic in function.

MATERIALS AND METHODS

Soil type and characteristics

Two types of fresh soil contrasting in physical and chemical characteristics, Andosol (volcanic ash soil collected from the Japan International Research Center for Agricultural Sciences [JIRCAS] experimental site, Tsukuba, Japan; 36°4′N. 140°7′E) and Cambisol (collected from the JIRCAS subtropical station, Ishigaki Island, Okinawa Prefecture, Japan; 24°2′N, 124°9′E) were used. The physical and chemical characteristics of the Andosol and Cambisol soils are shown in Table 1.

Plant material and growing conditions

Seeds of *B. humidicola* (Rendle) Schweick (CIAT 679) were germinated at 25°C in a sand–vermiculite mixture (3:1) and watered with distilled water to maintain 75% Relative Humidity (RH). Two-week-old plants were transferred into an aerated nutrient solution. A modified Arnon and Hoagland nutrient solution was used to grow the plants (Subbarao *et al.* 2006a). The composition of the nutrient solution (mg L^{-1}) was: KH_2PO_4 38.31, K_2SO_4 31.02, $CaCl_2{\cdot}2H_2O$ 10.5, $MgSO_4{\cdot}7H_2O$ 36.93, Fe-ethylenediaminetetraacetic acid15.1, H_3BO_3 0.57, $CuSO_4{\cdot}5H_2O$ 0.078, $MnSO_4{\cdot}6H_2O$ 2.35, $Na_2MoO_4{\cdot}2H_2O$ 0.126 and $ZnSO_4{\cdot}7H_2O$ 0.220. Nitrogen at 1 mmol L^{-1} was added as $(NH_4)_2SO_4$ to the nutrient solution and the solution was constantly aerated. The pH of the nutrient solution was adjusted to 5.0 (twice per week) and the solution was replaced with fresh solution at weekly intervals. Plants were grown in 50 L tanks on Styrofoam blocks with 10 holes and four plants per hole, supported with sponge.

Collection and concentration of root exudates

Root exudates were collected twice per week for 4 months. At the time of collection, four plants were

Table 1 Physical and chemical characteristics of the Andosol and Cambisol soils

Soil	pH (H_2O)	Clay (%)	Silt (%)	Sand (%)	Total carbon (mg g^{-1} soil)	Total nitrogen (mg g^{-1} soil)	C/N ratio
Andosol	5.9	54.8	26.3	18.9	29.2	2.5	11.7
Cambisol	6.9	17	12	71	5.8	0.62	9.35

removed from the culture tanks, rinsed thoroughly in tap water followed by ion-exchange water, and then placed in 1 L of aerated double distilled water for 24 h for the release of exudates before being returned to the culture tanks. Following collection, the root exudates were passed through Whatman No. 1 filter paper to remove pieces of roots and debris and evaporated to dryness using a rotary evaporator at 35°C. The residues were dissolved in a minimal volume of methanol.

Removal of inorganic salts from the root exudates

To remove inorganic salts, concentrated root exudates were diluted with water (to make a final concentration of 10% methanol) and passed through a solid phase extraction cartridge (Sep-Pak Plus C18 cartridges; Waters Corporation, Massachusetts, USA). After washing with water the cartridge was eluted with a minimal volume of methanol and the eluate was stored in a freezer at −20°C. Fractions of the above samples (1 mL) were analyzed for carbon and nitrogen levels using a CN analyzer (Sumigraph NC-900; Sumika Chemical Analysis Services, Tokyo, Japan; Shimadzu C-R6A Chromatopac, Kyoto, Japan (carbon content were determined as 60 μg ATU^{-1}). Aliquots of these samples (100 μL) were further concentrated on a centrifugal evaporator (model CVE-200D; Eyela, Tokyo, Japan) and the residues were dissolved in 25 μL dimethyl sulfoxide for the determination of BNI activity.

Nitrification inhibition bioassay

Biological nitrification inhibition activity of the samples was determined by a bioassay that uses recombinant luminescent *Nitrosomonas* as earlier described (Iizumi *et al.* 1998; Subbarao *et al.* 2006a). The BNI activity is expressed in ATU, which is defined as the inhibitory effect of 0.22 μmol L^{-1} AT in an assay containing 18.9 mmol L^{-1} of NH_4^+ (Subbarao *et al.* 2006a).

Effect of root exudates on nitrification

Four levels of BNI activity (extracted from root exudates; 10, 20, 30 and 40 ATU g^{-1} soil) and a synthetic nitrification inhibitor, dicyandiamide (DCD) (50 mg kg^{-1} soil), were tested for their effects on soil nitrification using Andosol and Cambisol soils (5 g by dry weight); the experiment was replicated three times. The soils were collected fresh from the field and passed through a 2-mm mesh before being placed in a 20 mL soil incubation vial. Nitrogen in the form of $(NH_4)_2SO_4$ solution (at 182 mg N kg^{-1} soil) was added to all treatments. Water status in the soil during incubation was maintained at 50% water-filled pore space (WFPS) for Cambisol and 60% WFPS for Andosol, which is considered optimum for nitrification (Mosier *et al.* 1996). Root exudates were prepared by dissolving the dried root exudates in a trace amount of acetonitrile, which was then diluted with milli-Q water (15 mL). Root exudates or DCD and $(NH_4)_2SO_4$ solution were mixed with the soil samples and incubated at 20°C with 90% humidity for 60 days using a temperature and humidity controlled incubator (Biotron LPH 200; Nippon Medical and Chemical Instruments, Osaka, Japan) as described earlier (Subbarao *et al.* 2006a). Sterilized distilled water was added to the soils at the end of 30 days to maintain the WFPS. Samples (5 g) taken at 15-days intervals were extracted with 50 mL of 2 mol L^{-1} KCl for 2 h and filtered through Whatman No. 1 filter paper and the filtrates were analyzed for NH_4^+ and NO^-_3 using an Auto Ion analyzer (model AAII; Brant+Luebbe, Norderstedt, Germany) as described previously (Anon 1974; Varley 1966). An additional set of the above experiments was conducted with air-dried Andosol soil samples, instead of fresh Andosol soil; all the other experimental conditions were the same.

Effect of root exudates on major soil microorganisms

Root exudates (30 ATU g^{-1} soil) were added to fresh Andosol and Cambisol soils (5 g by dry weight) and incubated for 60 days at 20°C and 90% humidity. Controls were water and DCD (at 50 mg kg^{-1} soil). Nitrogen was added as $(NH_4)_2SO_4$ solution (at 182 mg N kg^{-1} soil). Microbial counts (total cultivable bacteria, aerobic spore forming bacteria, fluorescent pseudomonads, Gram-negative bacteria, AOB and nitrite oxidizing bacteria [NOB]) were monitored every 15 days for 60 days.

Gram-negative bacterial counts were determined using the plate-dilution frequency technique (Harris and Sommers 1968). Soil samples were serially diluted and spread plated onto Petri plates containing MacConkey agar (Nihon Seiyaku, Tokyo, Japan). Plates were incubated at 30°C for 72 h in the dark before enumeration of the colonies.

Microbial counts were done using the most probable number (MPN) method (Alexander 1965; Ishikawa *et al.* 2003). Immunoplates (Nunc A/S, Kamstrupvej 90,

Roskilde, Denmark; containing 96 wells) were inoculated with the serially diluted soil samples and incubated at 30°C for 72 h in the dark before enumeration. Total cultivable bacteria and aerobic spore forming bacteria were determined on Tryptic Soy Broth (Becton and Dickinson, Sparks, MD, USA). S1 medium consisting of (g L^{-1}) saccharose (10.0), K_2HPO_4 (2.3), casein (5.0), $NaHCO_3$ (1.0), $MgSO_4 \cdot 7H_2O$ (1.0) and glycerol (10.0 mL) was used for fluorescent pseudomonads, which were observed under ultraviolet light after incubation for 72 h. For aerobic spore forming bacterial counts, diluted soil samples were kept in a boiling water bath for 1 h to kill the vegetative cells before inoculation.

The MPN method was also used for the enumeration of AOB and NOB. The medium composition for the AOB counts was (all ingredients in g L^{-1} of distilled water; Tiedje 1982) $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.3), NaCl (0.3), $FeSO_4 \cdot 7H_2O$ (0.03) and $CaCO_3$ (7.5); and for the NOB counts the composition was $NaNO_2$ (0.049), K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.1), NaCl (0.3), $FeSO_4 \cdot 7H_2O$ (0.03) and $CaCO_3$ (1.0). Serially diluted soil samples were inoculated into these solutions and the immunoplates were incubated in the dark for 21 days at 30°C. Nitrite and nitrate concentrations were determined using the Griess–Ilosvay assay method (Hewitt and Nicholas 1964). After incubation for 3 weeks, Griess–Ilosvay reagent was added to each sample well. If AOB or NOB were present, they would convert the ammonia into nitrite or nitrate, respectively. The Griess–Ilosvay reagent reacts with nitrite as indicated by the formation of a red color, but not with nitrate. Therefore, in AOB medium, if a red color is formed, AOB are present, but if no color is formed AOB are either absent or not present in sufficient numbers to convert ammonia to nitrite (Note: we did not test NO_3^- accumulation in the AOB medium), whereas it is the reverse for NOB medium, that is, if a red color is formed either NOB are absent or not present in sufficient strength to convert nitrite to nitrate, and if no color is formed NOB are present. The immunoplates were visually scored for the presence or absence of red color in each sample well and AOB and NOB populations were estimated using MPN tables (Alexander 1965).

Effect of root exudates on plant growth promoting microorganisms

Pure cultures of plant growth promoting microorganisms viz. *Azotobacter chroococcum* (American Type Culture Collection [ATCC] 4412), *Nitrosomonas europaea* (ATCC 25978), *Rhizobium leguminosarum* (ATCC 11444), *Azospirillum lipoferum* (ATCC 29707) and *Escherichia coli* (non-pathogenic control; ATCC 33767) were acquired (American Type Culture Collection, Manassas, VA, USA) and grown in their respective broths viz. *Azotobacter* basal medium (no. 12), *Nitrosomonas* medium (no. 2265), *Rhizobium* X medium (no. 111), *Spirillum* nitrogen-fixing medium (no. 838) and LB medium (Luria-Bertani medium) (no. 1065) as per the product information sheet for ATCC cultures. Pure cultures of the above were serially diluted and spread plated onto media containing root exudates (15 ATU mL^{-1} medium; prepared by dissolving the dried root exudates in a trace amount of acetonitrile first, followed by diluting with milli-Q water, followed by filter sterilization before applying to the bacterial medium (0.2 μm). The plates were incubated at 26 or 30°C in the dark, depending on the requirement of the individual cultures. Control plates contained no root exudates. Counting was done as per the standard protocol of the plate-dilution frequency technique (Harris and Sommers 1968).

Nitrification and bacterial populations in rhizosphere soils

Seeds of *B. humidicola* (CIAT 679) were germinated as described earlier. Two-week-old seedlings were transplanted into Wagner pots (0.5 L), filled with fresh Andosol and Cambisol soils (250 g by dry weight), and grown in a green house for 9 months (maintained at 33°C in the summer and 26°C in the winter). Control pots contained no plants and were maintained throughout the 9 months as barren soil. Each pot (including the control pots) was supplied once per month with NH_4^+-N, as $(NH_4)_2SO_4$ at 50 kg ha^{-1} soil, and the pots were watered regularly. At the end of every month, shoots were removed from three pots each of both Andosol and Cambisol soils (pots with and without plants giving a total of 12 pots). The soil from each pot was thoroughly mixed (after the roots and root debris were carefully removed) and a subsample (10 g pot^{-1}) of the homogenized soil was used for the determination of total cultivable bacteria, fluorescent pseudomonads and AOB using the MPN method.

Rhizosphere soils of Andosol and Cambisol (obtained from pots where *B. humidicola* were grown for 6 months) along with barren soils (four treatments in total) were analyzed for their ability to inhibit nitrification. Nitrogen in the form of $(NH_4)_2SO_4$ solution (providing 182 mg N kg^{-1} soil) was incorporated into all treatments and incubation was as described previously.

Statistical analyses

The experimental data were subjected to one-way ANOVA using the Genstat 7.1 statistical package (Lawes Agricultural Trust 2004). Means are presented with standard errors. Differences among means were tested at a significance of $P < 0.05$.

RESULTS

Effect of root exudates on nitrification

After 60 days, root exudates of *B. humidicola* inhibited nitrification at 10 ATU g^{-1} soil (29%) in Cambisol soil and inhibition was greater at 20 ATU g^{-1} soil (70%). At 30 and 40 ATU g^{-1} soil inhibition was complete (87 and 89%, respectively) when compared with DCD-amended soils (92%; Fig. 1). In contrast, in fresh Andosol soils, 20–30% of the NH_4^+ form of nitrogen was not accounted for in treatments where root exudates were incorporated and hence it was not possible to estimate the percentage that underwent nitrification (Fig. 2). When Andosol samples were air-dried, however, inhibition of soil nitrification was evident (67 and 76% for 30 and 40 ATU g^{-1} soil, respectively) when compared with DCD-amended soil (85%) following the addition of root exudates (Fig. 3).

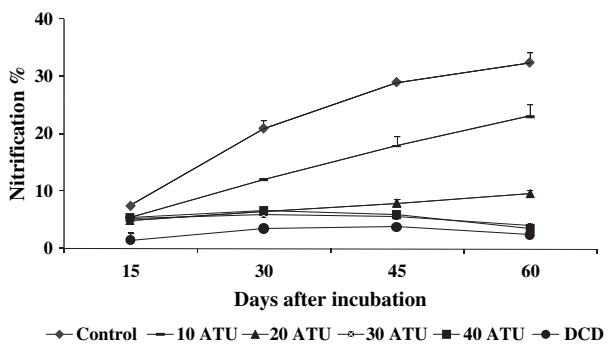

Figure 1 Effect of root exudates of *Brachiaria humidicola* (10, 20, 30 and 40 ATU g^{-1} soil) and dicyandiamide (DCD; 50 mg kg^{-1} soil) on nitrification in fresh Cambisol. Nitrification (%) = (NO_3^--N/(NO_3^--N + NH_4^+-N)) ×100; Error bars indicate the standard error (n = 3).

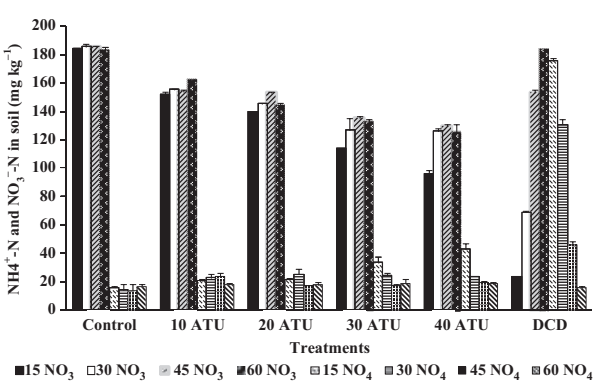

Figure 2 Effect of root exudates of *Brachiaria humidicola* (10, 20, 30 and 40 ATU g^{-1} soil) and dicyandiamide (DCD; 50 mg kg^{-1} soil) on NH_4^+-N and NO_3^--N levels during the incubation period of 15, 30, 45 and 60 days in fresh Andosol soil. Error bars indicate standard error (n = 3).

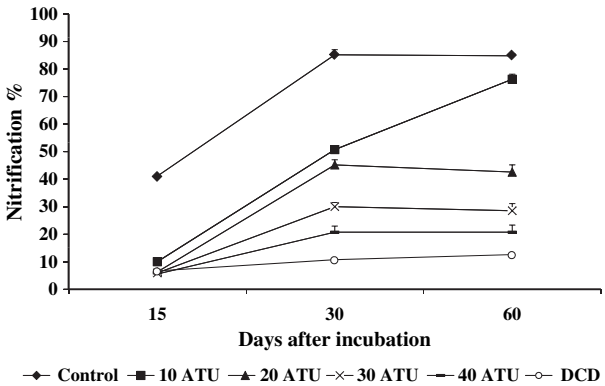

Figure 3 Effect of root exudates of *Brachiaria humidicola* (10, 20, 30 and 40 ATU g^{-1} soil) and dicyandiamide (DCD; 50 mg kg^{-1} soil) on nitrification in air-dried Andosol soils. Note: Nitrification (%) = (NO_3^--N/[NO_3^--N + NH_4^+-N]) ×100. Error bars indicate standard error (n = 3).

Effect of root exudates on major soil microorganisms

In Cambisol soils root exudates (30 ATU g^{-1} soil) significantly enhanced the populations (by at least 1 log units) of total cultivable bacteria and fluorescent pseudomonas when compared with water or the DCD controls. Enhancement was evident in all incubations up to 60 days of observation (Fig. 4). Conversely, AOB and NOB

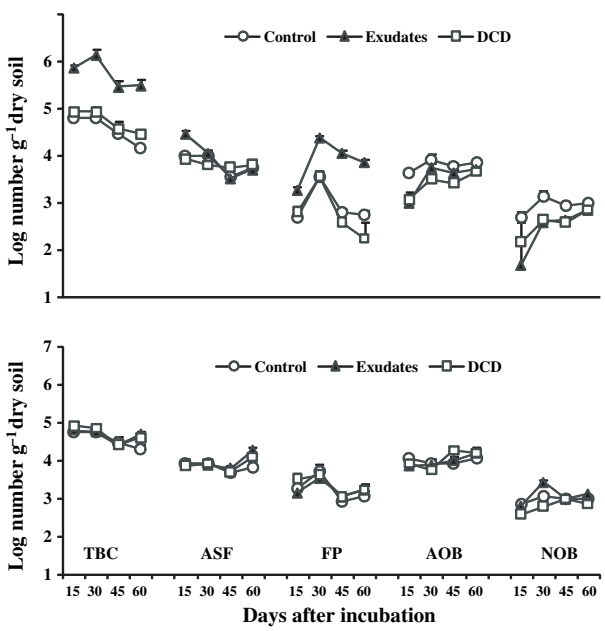

Figure 4 Effect of root exudates of *Brachiaria humidicola* (30 ATU g^{-1} soil) and dicyandiamide (DCD; 50 mg kg^{-1} soil) on major soil microorganisms in fresh Cambisol and Andosol soils. AOB, ammonia-oxidizing bacteria; ASF, aerobic spore formers; FP, fluorescent pseudomonads; NOB, nitrite-oxidizing bacteria; TBC, total cultivable bacteria. Error bars indicate standard error (n = 3).

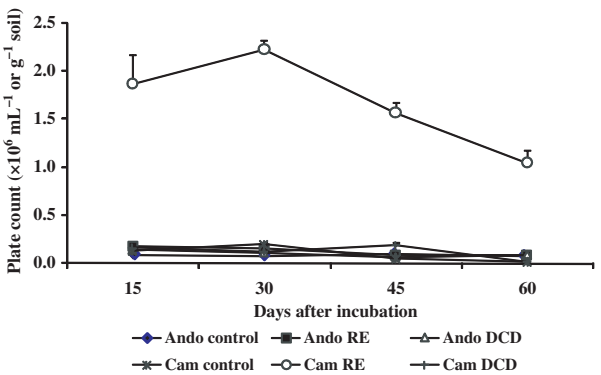

Figure 5 Effect of root exudates (RE) of *Brachiaria humidicola* (30 ATU g^{-1} soil) and dicyandiamide (DCD; 50 mg kg^{-1} soil) on the Gram-negative bacterial count in fresh Cambisol (Cam) and Andosol (Ando) soils. Error bars indicate standard error (n = 3).

populations were reduced (by at least 0.5 log units) in root exudates or DCD-amended soils; however, aerobic spore former counts were not affected (Fig. 4). No significant effects of root exudates were found in any of the microbial populations in Andosol soils (Fig. 4). In addition, root exudates did not negatively influence Gram-negative bacterial populations in Andosol soils, whereas in the Cambisol soils root exudates stimulated the Gram-negative bacterial population nearly 10-fold (Fig. 5).

Effect of root exudates on plant growth promoting microorganisms

When supplemented into the respective media for cultivating plant growth promoting microorganisms, root exudates (at 15 ATU mL^{-1} medium) did not inhibit *A. lipoferum*, *R. leguminosarum* or *A. chroococcum*, but did inhibit (a 10-fold reduction) the AOB *N. europaea* (Table 2).

Table 2 Effect of root exudates of *Brachiaria humidicola* (15 ATU mL^{-1} medium) on plant growth promoting microorganisms

Pure cultures of	ATCC no.	With RE† (SE)	With out RE† (SE)
Azospirillum lipoferum	29707	3.7 (0.10)	5.4 (0.10)
Rhizobium leguminosarum	11444	56.0 (3.00)	52.0 (1.00)
Azotobacter chroococcum	4412	49.0 (1.60)	32.5 (1.50)
Nitrosomonas europaea	25978	2.6 (0.30)	25.6 (0.80)
Escherichia coli	33767	239.0 (1.20)	230.5 (1.00)

†Plate count (×10^6 cells mL^{-1} broth). Figures are plate counts in millions with standard errors (SE) in parentheses (n = 3). ATCC, American Type Culture Collection; RE, root exudates.

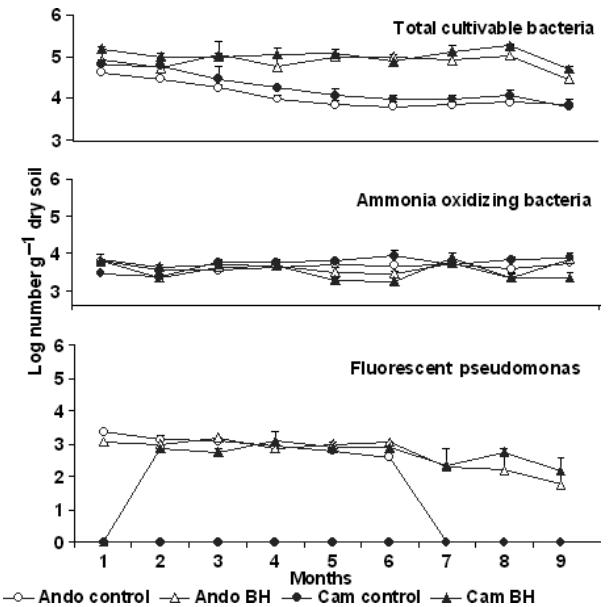

Figure 6 Evaluation of total cultivable bacteria, fluorescent pseudomonads and ammonia oxidizing bacteria in rhizosphere (*Brachiaria humidicola* grown) and control soils. Ando control, barren Andosol soil; Ando BH, rhizosphere Andosol soil; Cam control, barren Cambisol soil; Cam BH, rhizosphere Cambisol soil. Error bars indicate standard error (n = 3).

Nitrification and bacterial populations in rhizosphere soils

Brachiaria humidicola rhizosphere soils, in both Andosol and Cambisol, significantly enhanced (by at least 1 log unit) the population of total cultivable bacteria when compared with barren soils (Fig. 6). This trend was consistent over the 9-month observation period. Fluorescent pseudomonads were not found in barren Cambisol on any sampling occasion, whereas they were found in all Cambisol rhizosphere soil samples except in the first month. In the barren Andosol soil, fluorescent pseudomonads decreased with time and were not detected after 6 months, but were found in all rhizosphere samples. The AOB populations were not significantly different in barren and rhizosphere samples of both Andosol and Cambisol soils at all sampling dates (Fig. 6). Nitrification was inhibited in the *B. humidicola* rhizosphere samples from Cambisol soils after 6 months of growth compared with barren soil (Fig. 7). Immobilization of the NH_4^+ ions did not allow estimation of the percentage reduction in nitrification in the Andosol rhizosphere samples.

DISCUSSION

Influence of root exudates on nitrification

To compare the effectiveness of nitrification inhibition by *B. humidicola* root exudates, fresh Andosol and Cambisol

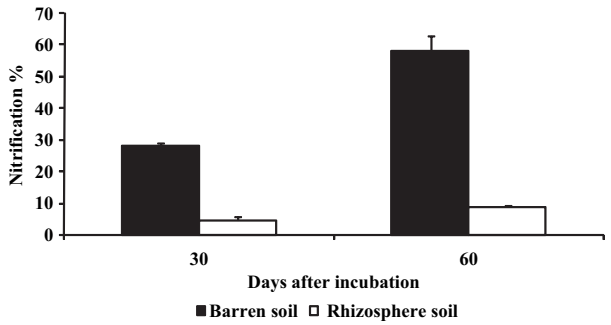

Figure 7 Inhibition of nitrification by Cambisol soil in which *Brachiaria humidicola* had been grown (rhizosphere soil) and in control (barren) soil. Nitrification (%) = (NO_3^--N/(NO_3^--N + NH_4^+-N)) ×100. Error bars indicate standard error (n = 3).

soils contrasting in pH, clay, silt, sand, total carbon and nitrogen contents and C/N ratio were used in our study (Table 1). The root exudates of *B. humidicola* at 30 and 40 ATU g^{-1} soil completely inhibited nitrification in fresh Cambisol soil over a period of 60 days (Fig. 1). This is the first time that nitrification inhibition by root exudates of *B. humidicola* has been demonstrated in fresh Cambisol soil. In the fresh Andosol soil, significant amounts of the NH_4^+ form of nitrogen (20–30%) were not accounted for (Fig. 2), thus preventing us from interpreting the results; one possibility is microbial immobilization of nitrogen in these carbon-rich Andosols, but why this would happen in fresh Andosols and not in air-dried Andosols is puzzling and requires further investigation.

Immobilization is the process whereby microorganisms take up nutrients from soil and root exudates (following mineralization) that gets incorporated into proteins, nucleic acids and other constituents of microbial cells (Robertson and Groffman 2007). The BNI activity of the root exudates contained 60 μg C ATU^{-1}; the fresh Andosol had higher carbon and nutrient levels than the fresh Cambisol soil (Table 1). In addition to BNIs, several carbon compounds that include simple and complex sugars, growth regulators, amino acids, organic acids, phenolic acids, flavonoids, enzymes, fatty acids, nucleotides, tannins, steroids, terpenoids, alkaloids and vitamins are released from the roots (Subbarao *et al.* 2006a; Uren 2000). Many of these compounds can be utilized by soil microbes (Inderjit and Weston 2003; Juma and Paul 1983), and could stimulate microbial immobilization of inorganic nitrogen (Burger and Jackson 2003; Wickramasinghe *et al.* 1985).

Root exudates effectively inhibited nitrification in air-dried Andosol soil (Fig. 3). Effective nitrification inhibition by *B. humidicola* and *Leymus racemosus* (a wild-wheat) root exudates in air-dried Andosol has previously been reported (Subbarao *et al.* 2006a, 2007b). Root exudates inhibited nitrification in the Cambisol over a 60-day period. Given the contrasting results observed between the Andosol and Cambisol soils in the present study, it seems that the effectiveness of BNI function can vary with soil type.

Dicyandiamide at 10–15% of the amount of applied nitrogen has previously been reported to inhibit soil nitrification over a 60 day period (Slangen and Kerkhoff 1984). The DCD inhibited nitrification in the fresh Cambisol and air-dried Andosol soils (Figs 1 and 3); however, it's inhibitory effect was evident for only 15 days in the fresh Andosol (Fig. 2). The stability of DCD can be affected by temperature, soil moisture conditions, carbon availability, pH and microbial activity, affecting its effectiveness in inhibiting soil nitrification (Slangen and Kerkhoff 1984). Dicyandiamide is also known to have a bacteriostatic effect on *Nitrosomonas* bacteria (Rodgers 1986; Sturm *et al.* 1994; Zacherl and Amberger 1990a,b).

Influence of root exudates on major soil microorganisms, AOB and NOB populations *in vitro*

Root exudates at 30 ATU g^{-1} soil inhibited nitrification over a 60-day period during the incubation study in the Cambisol. Root exudates were tested at 30 ATU g^{-1} soil for their influence on major soil microorganisms; for plant growth promoting microorganisms, root exudates at 15 ATU mL^{-1} medium were tested on pure cultures of plant growth promoting microorganisms. The root exudates and rhizosphere soil of *B. humidicola* significantly increased the populations of major soil microorganisms, with the exception of AOB, and did not influence plant growth promoting microorganisms (Figs 4–6; Table 2). Root exudates suppressed populations of AOB and NOB in the Cambisol soil (Fig. 4; Table 2).

Root exudates can influence the rhizosphere and its microbial composition through their effects on soil microflora and microfauna. These influences can be either positive, to produce chemical signals to attract bacteria or induce chemotaxins with plant growth promoting rhizobacteria, or negative, such as the secretion of antimicrobials, phytotoxins, nematicidal, and insecticidal compounds (Bais *et al.* 2006). The BNI hypothesis proposes that a specific negative plant–microbe interaction can exist whereby the root exudates of some species inhibit nitrifying bacteria as a specific function to control nitrification in soil (Subbarao *et al.* 2007a). The finding that root exudates from *B. humidicola* increase the population of major soil microorganisms, with the exception of AOB, and do not inhibit plant growth promoting micro organisms provides additional support for this hypothesis.

Effect of root exudates *in vivo*

When *B. humidicola* was grown in Andosol or Cambisol there was no effect on AOB counts over nine consecutive months of observation (Fig. 6). This finding conflicts with

a previous report by Ishikawa *et al.* (2003) where AOB populations in bulk Andosol were found to be significantly lower than a control when *B. humidicola* had been grown for only 60 days. Under *in vitro* conditions AOB populations were inhibited, suggesting that BNIs inhibit AOB population growth. Rhizosphere samples showed lower nitrification potential in Cambisol soil during laboratory incubation studies, providing additional evidence for BNI function in *B. humidicola*.

Conclusions

Our results demonstrate that BNIs released from the roots of the tropical pasture grass *B. humidicola* are effective in suppressing soil nitrification, but did not negatively affect soil microbial populations or plant growth promoting microorganisms. However, the effectiveness of the suppressing effect from BNIs varied with soil type. These results provide additional evidence for the hypothesis that certain plants can suppress soil nitrification by releasing inhibitors from their roots, a plant function termed BNI.

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