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Potential for biological nitrification inhibition to reduce nitrification and N_2O emissions in pasture crop–livestock systems

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Agriculture and livestock production systems are two major emitters of greenhouse gases. Methane with a GWP (global warming potential) of 21, and nitrous oxide (N_2O) with a GWP of 300, are largely emitted from animal production agriculture, where livestock production is based on pasture and feed grains. The principal biological processes involved in N_2O emissions are nitrification and denitrification. Biological nitrification inhibition (BNI) is the natural ability of certain plant species to release nitrification inhibitors from their roots that suppress nitrifier activity, thus reducing soil nitrification and N_2O emission. Recent methodological developments (e.g. bioluminescence assay to detect BNIs in plant root systems) have led to significant advances in our ability to quantify and characterize the BNI function. Synthesis and release of BNIs from plants is a highly regulated process triggered by the presence of NH_4^+ in the rhizosphere, which results in the inhibitor being released precisely where the majority of the soil-nitrifier population resides. Among the tropical pasture grasses, the BNI function is strongest (i.e. BNI capacity) in Brachiaria *sp. Some feed-grain crops such as sorghum also have significant BNI capacity present in their root systems. The chemical identity of some of these BNIs has now been established, and their mode of inhibitory action on* Nitrosomonas *has been characterized. The ability of the BNI function in* Brachiaria *pastures to suppress N_2O emissions and soil nitrification potential has been demonstrated; however, its potential role in controlling N_2O emissions in agro-pastoral systems is under investigation. Here we present the current status of our understanding on how the BNI functions in* Brachiaria *pastures and feed-grain crops such as sorghum can be exploited both genetically and, from a production system's perspective, to develop low-nitrifying and low N_2O-emitting production systems that would be economically profitable and ecologically sustainable.*

Keywords: biological nitrification inhibition, climate change, global warming, greenhouse gases, nitrous oxide emissions

Implications

Nitrous oxide (N_2O), the most powerful greenhouse gas, is emitted largely from agricultural systems primarily through soil biological processes – nitrification and denitrification. Modern agricultural systems have become high-nitrifying, N-inefficient and leak large amounts of reactive nitrogen (N) to the environment. Biological nitrification inhibition (BNI) is the natural ability of certain plant species to release nitrification inhibitors from roots to suppress nitrification and N_2O emission. The BNI function in *Brachiaria* pastures and feed-grain crops (e.g. sorghum) can be exploited both genetically and, from a cropping system's perspective, to develop low-nitrifying and low N_2O-emitting production systems that would benefit both agriculture and the environment.

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Introduction

Nitrification and denitrification are the biological drivers for N_2O production

Nitrification and subsequent denitrification are the primary drivers for the generation of nitrous oxide (N_2O), the most powerful greenhouse gas with a global warming potential (GWP) of 300 times greater than that of CO_2 (Hahn and Crutzen, 1982; Kroeze, 1994; Intergovernmental Panel on Climate Change (IPCC), 2012). N_2O is emitted during two enzymatic pathways ((ammonia mono-oxygenase (AMO) and hydroxylamine oxidoreductase (HAO)) involved in the oxidation of ammonia (NH_3^+) to nitrite (NO_2^-) and nitrate (NO_3^-) (Prosser, 1989; Supplementary Figure S1). In addition, during denitrification (i.e. reduction of NO_3^- into N_2), N_2O is emitted (Supplementary Figure S1; Prosser, 1989). Nearly 70% of the global N_2O emissions come from agricultural systems, and nitrification–denitrification is the only

known soil biological process responsible for the generation of N_2O (Bremner and Blackmer, 1978; Smith *et al.*, 1997; Hofstra and Bouwman, 2005; Tubiello *et al.*, 2013). As denitrification cannot take place without substrate NO_3^- (produced by nitrification), controlling nitrification thus is the most effective strategy to reduce N_2O emissions from agricultural systems (Subbarao *et al.*, 2012 and 2013b).

Two groups of soil bacteria – ammonia-oxidizing bacteria (AOB; mainly *Nitrosomonas* spp. and *Nitrosospira* spp.) and ammonia-oxidizing archaea (AOA) – are largely responsible for the biological oxidation of NH_3^+ to NO_3^- (Leninger *et al.*, 2006; Taylor *et al.*, 2010). As a cation, NH_4^+ is electrostatically held by the negatively charged clay surfaces and functional groups of soil organic matter (SOM) that reduce the loss of NH_4^+-N by leaching (Sahrawat, 1989). In contrast, NO_3^-, with negative charge, does not readily bond to the soil, and is more labile to be leached out of the root zone. In addition, several heterotrophic soil bacteria denitrify NO_3^- under anaerobic or partially anaerobic conditions (which can often coincide with temporary water-logging of a soil after a heavy rainfall or irrigation in fields that have improper drainage; Bremner and Blackmer, 1978; Mosier *et al.*, 1996). The loss of nitrogen (N) during and following nitrification reduces the effectiveness of N fertilization, causing environmental degradation, loss of biodiversity, loss of ecosystem services, emergence of pathogens and threatening the long-term sustainability of agricultural production systems (Clark, 1962; Jarvis, 1996; Vitousek *et al.*, 1997a and 1997b; Dalgaard *et al.*, 2012).

Low N recovery is the major cause of N pollution and N_2O emissions from agricultural systems

Industrially fixed N (i.e. N fertilizers) is the primary driver of agricultural productivity since the 1960s. Massive amounts of N fertilizer transformed agricultural production to feed the growing population during the last five decades (i.e. from 1960 to 2010) (Broadbent and Rauschkolb, 1977; Matson *et al.*, 1999; Tilman *et al.*, 2001 and 2002; Hungate *et al.*, 2003; Sutton *et al.*, 2011). Global cereal production has tripled in the last 50 years, largely driven by an eightfold increase in N-fertilizer consumption, coupled with the use of N-responsive high-yielding crop cultivars, a combination often referred as 'Green Revolution' (Smil, 2001; Tilman *et al.*, 2001 and 2002; Steinfeld and Wassenaar, 2007; Food and Agriculture Organization (FAO), 2009; Pelletier and Tyedmers, 2010; Sutton *et al.*, 2011). By 2050, global population is projected to be 50% larger than at present; global grain demand and N-fertilizer consumption are projected to double during this period (Cassman and Pingali, 1995; Alexandratos, 1999; Cassman, 1999; Cohen and Federoff, 1999; Tilman *et al.*, 2001). Doubling food production again (i.e. by 2050) and to sustain food production at that level without compromising on environmental integrity and public health are the greatest challenges to humankind (Alexandratos, 1999; Ruttan, 1999; Tilman *et al.*, 2002).

N efficiency (mega tons of cereal grain produced per mega ton of N fertilizer applied) in cereal production has declined from about 80 in 1960s to 20 at present (Tilman *et al.*, 2002; Figure 1), suggesting a diminishing returns to N-fertilizer applications; this is largely associated with the accelerated soil-nitrifier activity led to diminished ability to retain soil-N. In addition, this implies that further applications may not be effective in increasing yields in the future (Cassman and Pingali, 1995; Tilman *et al.*, 2002; Cassman *et al.*, 2003; Zhang *et al.*, 2008). Several changes in agricultural management practices during the twentieth century led to the development of high-nitrifying soil environments, and they are largely responsible for N loss (through NO_3^- leaching and gaseous N emissions (N_2O, NO)) and N pollution of the environment (NO_3^- pollution of water bodies and global warming) (Vitousek *et al.*, 1997a and 1997b; Matson *et al.*, 1998; Tilman *et al.*, 2001 and 2002; Dinnes *et al.*, 2002; Wagner-Riddle *et al.*, 2007; Turner *et al.*, 2008).

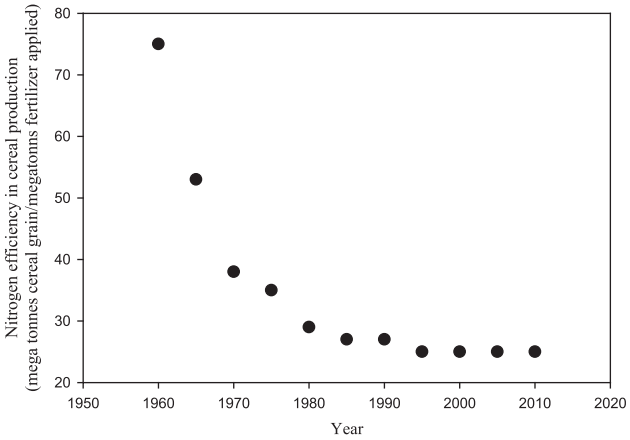

Figure 1 Trends in N-fertilization efficiency in cereal production (annual global cereal production divided by annual global application of N fertilizer) systems – global food production has tripled during this period (1960 to 2010), but the N fertilizer applied has increased eightfold (Adapted from Tilman *et al.*, 2002 and FAO, 2012).

N recovery in various components of agro-ecosystems that include agriculture (i.e. pasture/crop production), livestock production and human systems indicate a diminishing flow of N from agriculture (through N fertilization) to human nutrition (either vegetable or animal protein) (Supplementary Figure S2, Figure 2). Only 30% of the applied N fertilizer is taken by crops to produce plant protein (Raun and Johnson, 1999; Smil, 1999; Cassman *et al.*, 2002). Nearly 70% of the 150 Tg N as N fertilizer applied to the agricultural systems is lost either through NO_3^- leaching or gaseous N emissions; moreover, a large proportion of the leached NO_3^- is eventually denitrified, generating N_2O and NO (Peterjohn and Schlesinger, 1990; Vitousek and Howarath, 1991; Vitousek *et al.*, 1997a and 1997b; Matson *et al.*, 1998 and 1999; Smil, 1999; Tilman *et al.*, 2001; Wagner-Riddle *et al.*, 2007; Jahangir *et al.*, 2012). The N-recovery efficiency by the livestock sector is only about 10% at best (ranging from 5% for beef cattle, 13% for dairy cows, about 20% for pigs and 34% for poultry, i.e. for converting plant protein to animal protein) (van der Hoek, 1998), losing 90% of the fertilizer N to the environment through NO_3^- leaching or gaseous

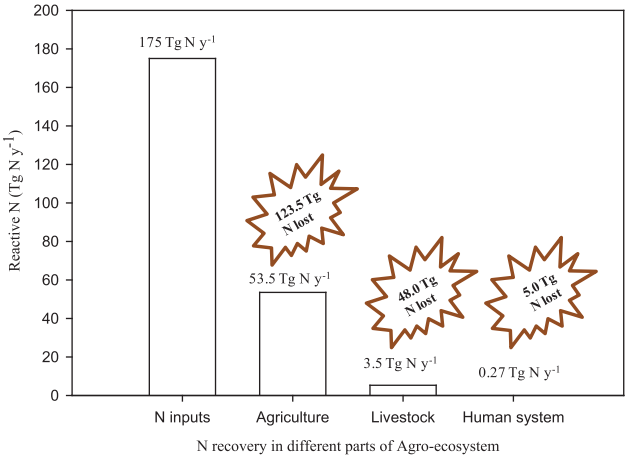

Figure 2 Nitrogen recovered in different components of an agro-ecosystem [calculated on the basis of the assumption that total N input into agricultural systems is 175 Tg N/year (25 Tg N/year is from biological nitrogen fixation from legumes and from N fertilizer is at 150 Tg N/year (Smil, 1999); 30% of this N recovered by crops to produce plant protein (Raun and Johnson, 1999; Cassman *et al.*, 2002); the N-recovery efficiency by the livestock sector is about 10% at best (van der Hoek, 1998); the N-retention capacity in human systems is about 5% of the protein-N intake (van der Hoek, 1998)).

emissions (Supplementary Figure S2, Figure 2). Some of the reactive N excreted from intensive livestock systems (i.e. urine and feces) is recycled through agricultural systems in a limited way, results in N-saturation hotspots (farm soil NO_3^- levels often reaching 300 to 400 kg N/ha per year), causing NO_3^- contamination of groundwater (Dalgaard *et al.*, 2012; Hansen *et al.*, 2012). A major portion of the reactive N excreted from livestock systems is not recycled effectively through modern production systems as N source (Wagner-Riddle *et al.*, 2007; Centner and Newton, 2008; Schlesinger, 2009). The situation is same in the case of N excreted by humans (i.e. domestic sewage), as most of this reactive N (human urine and fecal matter) is lost directly to the environment. However, the greatest loss of reactive N occurs from agricultural (crop/pasture) systems (Supplementary Figure S2, Figure 2) (Peterjohn and Schlesinger, 1990; Vitousek and Howarath, 1991; Smil, 1999).

Greenhouse gas emissions associated with N-fertilizer production

Substantial amounts of GHGs (e.g. CO_2, N_2O and CH_4) are emitted during the production of N fertilizers, which is expressed as CO_2 equivalents per unit mass of fertilizer (g CO_2-e/kg N fertilizer) on the basis of their GWP (IPCC, 2012). Synthesis of NH_3^+ (the basic ingredient of all N fertilizers) is an energy-intensive process, and requires about 25 to 35 GJ/t of NH_3^+ (Kongshaug, 1998). Nearly 5% of the natural gas produced in the world is used for the manufacture of N fertilizers (Smil, 2001). The emission factor is about 4 kg CO_2-e/kg urea N, about 10 kg CO_2-e/kg N in complex fertilizers (i.e. NPK; Kuesters and Jenssen, 1998; Kramer *et al.*, 1999). With the current levels of annual N-fertilizer applications in agriculture (i.e. 150 Tg N/year), this amounts to annual emissions of 458 Tg of CO_2 associated with the manufacture of N fertilizer alone (excluding GHG emissions during transportation of N fertilizers from factory to farm; Smil, 2001). These GHG emissions are similar in magnitude to annual CO_2-e emissions from running motor vehicles, which are at 900 Tg CO_2-e (Schafer and Victor, 1999). By 2050, GHG emissions from global N-fertilizer production will reach 1200 to 3000 Tg CO_2-e (on the basis of the current estimates that N-fertilizer usage will reach 300 Tg by 2050; Galloway *et al.*, 2008; Schlesinger, 2009). Currently, global CO_2 emissions are at 34 000 Tg CO_2-e (IPCC, 2012) and GHG emissions from N-fertilizer production accounts for about 2% to 4% of the global CO_2 emissions.

High-nitrifying modern agricultural systems are inherently N-inefficient and affect global environment

Unlike most climax ecosystems that have tightly closed N cycling to protect N from leaking, the modern agricultural systems have open N cycling, extremely leaky and inherently N-inefficient (Rice and Pancholy, 1972 and 1974; White, 1991; Nasholm *et al.*, 1998; Paavolainen *et al.*, 1998; Cassman *et al.*, 2002). Large amounts of fertilizer N are added from industrial processes; moreover, N is being continuously removed from the system (through harvested food/feed grains) to support intensive livestock feed and human food systems, often located away from the primary production sites. This results in not returning the reactive N (i.e. N excreted from livestock and humans) to the agricultural systems for nutrient cycling (Dinnes *et al.*, 2002). The intensification of agricultural practices coupled with the separation of crop production from livestock production has disrupted natural nutrient cycling, deplete SOM levels, changes in soil, physical and chemical properties, brought major shifts in soil microbial activity and diversity, resulted in the development of high-nitrifying soil environments, where NO_3^- accounts for $>$95% of the crop N uptake in modern agricultural systems (Supplementary Figure S3) (Elliot, 1986; Ross, 1993; Tiessen *et al.*, 1994; Matson *et al.*, 1998; Poudel *et al.*, 2002; Celik, 2005; Khan *et al.*, 2007; Mulvaney *et al.*, 2009; Russell *et al.*, 2009; van Wesemael *et al.*, 2010). These high-nitrifying soil environments are largely responsible for the loss of 70% N fertilizer applied to the production systems (Peterjohn and Schlesinger, 1990; Vitousek and Howarath, 1991; Raun and Johnson, 1999). With the worldwide N-fertilizer application reaching 150 Tg/year (Smil, 1999; Galloway *et al.*, 2008) and the cost of urea N ranging from US$ 0.80 to 0.54/kg N, the direct annual economic loss is estimated at nearly US$ 90 billion (Fertilizer Market Bulletin, 2008; Mulvaney *et al.*, 2009; Subbarao *et al.*, 2013b). Fertilizer-N use is projected to double by 2050 to reach close to 300 Tg/year, (Tilman *et al.*, 2001; Turner *et al.*, 2008; Schlesinger, 2009), and N lost from NO_3^- leaching from agricultural systems can be at 61.5 Tg N/year (Schlesinger, 2009). Nearly 17 Tg N is emitted as N_2O, which is expected to quadruple by 2100 largely because of an increase in the use of N fertilizers (Galloway *et al.*, 2008; Schlesinger, 2009; Burney *et al.*, 2010; Kahrl *et al.*, 2010).

A case for moving toward low-nitrifying agricultural systems

Nitrification is one of the several pathways (e.g. N fixation, organic matter mineralization, ammonification, nitrification and denitrification) in the soil-N cycle. Most climax ecosystems tightly control nitrification by suppressing nitrifier activity, and N flow is facilitated through multiple paths of the N cycle; a variety of organic and inorganic N forms are used as N source for uptake and assimilation to conserve N and to have a closed N cycling (Vitousek and Matson, 1984; Northup *et al*., 1995; Smolander *et al*., 2012). In contrast, nitrification became a dominant pathway for N flow; NO_3^- is the primary N form for uptake and assimilation (>95% of the N uptake is in NO_3^- form) in the intensively managed high production systems, making N cycling extremely inefficient and leaky to the environment (Supplementary Figure S3) (Galloway *et al*., 2008; Schlesinger, 2009; Subbarao *et al*., 2012 and 2013b).

High-nitrifying soil environments rapidly convert NH_4^+ to NO_3^-, which results in inefficient use of both soil N (i.e. N mineralized from SOM) and applied N (N fertilizer) as NO_3^- is lost to the environment either through leaching or denitrification (Poudel *et al*., 2002). In addition, the assimilation of NO_3^-, but not of NH_4^+, results in the direct emission of N_2O from crop canopies, further reducing nitrogen use efficiency (NUE; Smart and Bloom, 2001). Thus, maintaining soil N in NH_4^+ form is advantageous even after taking into consideration the potential negative effects of rhizosphere acidification from its uptake and assimilation (caused by H^+ excretion). By slowing the soil nitrification rates, NH_4^+ can move into the microbial pool (i.e. microbial immobilization) where it is converted to slow-release N source (Vitousek and Matson, 1984; Hodge *et al*., 2000). Most plants have the ability to use either NH_4^+ or NO_3^- as their N source (Haynes and Goh, 1978; Salsac *et al*., 1987; Boudsocq *et al*., 2012). Reducing nitrification rates in agricultural systems thus do not alter the intrinsic ability of plants to absorb N, but increases N-retention time in the root zone as NH_4^+, which is less mobile than NO_3^-, provides additional time for plants to absorb N. This in turn reduces the amount of N lost through leaching and denitrification, and thus leads to improved N recovery and NUE in agricultural systems (Hodge *et al*., 2000; Subbarao *et al*., 2012). Restricting the nitrification pathway by suppressing soil nitrifier activity thus could be a key strategy to shift the current NO_3^- dominated crop N nutrition toward NH_4^+ as the primary N form for uptake and assimilation (Subbarao *et al*., 2012 and 2013b). Such a paradigm shift in the N nutrition of field crops and pastures is necessary for developing next-generation N-efficient production systems that leak less N, thus contributing to the ecological and economic intensification of agriculture and livestock production. Many of these advantages associated with inhibiting nitrification in improving crop yield, grain quality, livestock production and environmental quality have been demonstrated using chemical nitrification inhibitors (Slangen and Kerkhoff, 1984; Prasad and Power, 1995; Subbarao *et al*., 2006a; Giltrap *et al*., 2010; Dennis *et al*., 2012).

Biological nitrification inhibition (BNI)

The BNI concept

The natural ability of some plants to produce and release nitrification inhibitors from roots to suppress nitrifier activity in soils is termed 'biological nitrification inhibition (BNI)' (for details see Figure 3) (Subbarao *et al*., 2006a, 2006b, 2007a, 2007b, 2007c, 2008, 2009a, 2009b, 2012 and 2013b). As nitrification is the most important process determining the N-cycling efficiency (i.e. proportion of N that stays in the ecosystem during a complete recycling loop), restricting nitrification will minimize the N leakage and facilitate N flow through the NH_4^+ assimilation pathways (Subbarao *et al*., 2012 and 2013b). Agronomic NUE ($NUE_{agronomic}$ = grain yield per unit of applied N) is a function of both intrinsic NUE ($NUE_{intrinsic}$ = dry matter produced per unit N absorbed), HI (harvest index optimized for most high yielding cultivars) and N uptake (Raun and Johnson, 1999). $NUE_{intrinsic}$ is physiologically conserved (Glass, 2003), and thus improvements in $NUE_{agronomic}$ can only come from improvements in crop-N uptake (Finzi *et al*., 2007), which is largely a function of recovering the applied N fertilizer. The BNI function in plants thus can exert a positive influence on $NUE_{agronomic}$ by reducing N loss associated with nitrification–denitrification (Subbarao *et al*., 2012 and 2013b). Recent modeling studies suggest that tropical grasses that inhibit nitrification exhibit a twofold greater productivity than those that lack such ability (Lata *et al*., 1999; Boudsocq *et al*., 2009 and 2012).

Recent methodological developments have facilitated the detection and quantification of nitrification inhibitors from plant roots using a recombinant luminescent *Nitrosomonas* construct (Iizumi *et al*., 1998; Subbarao *et al*., 2006b); the inhibitory activity released from roots is termed 'BNI activity' (expressed in ATU (allylthiourea unit) the inhibition caused by 0.22 μM AT in the assay is defined as one ATU); and the ability to release BNI activity is termed BNI capacity of the plant root system (Subbarao *et al*., 2006b). These recently developed research tools facilitated the characterization of BNI function in plants (Subbarao *et al*., 2006b). Soil-based assays to determine the changes in nitrification potential of rhizosphere soil complement this characterization of BNI capacity in plant root systems. The changes in potential soil nitrification by BNI function can be determined by monitoring NH_3^+-oxidizing activity (Subbarao *et al*., 2009a; Smits *et al*., 2010).

BNI capacity in selected field crops and pasture grasses

Tropical pasture grasses and selected field crops showed a wide range in the BNI capacity in their root systems (Figure 4; Subbarao *et al*., 2007b). Forage grasses of *Brachiaria humidicola*, which are highly adapted to low-N production environments of South American savannas (Miles *et al*., 2004) showed the greatest BNI capacity (Subbarao *et al*., 2007b). By contrast, *Panicum maximum*, which is adapted to high-N availability environments, showed the least BNI capacity among tropical pasture grasses (Subbarao *et al*., 2007b). Among the cereal crops evaluated, only sorghum

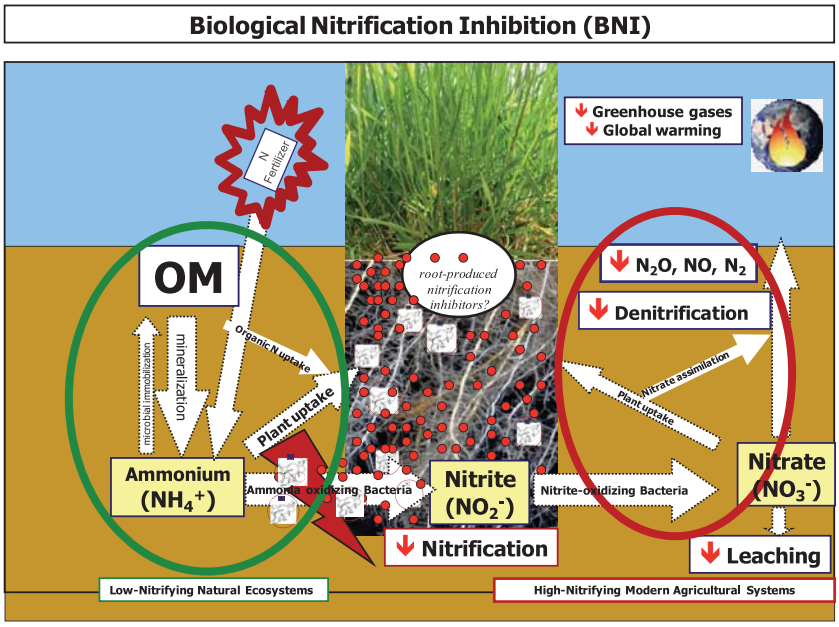

Figure 3 Schematic representation of the biological nitrification inhibition (BNI) interfaces in the N cycle. The BNI exuded by the plant root systems inhibits the first step of nitrification. In ecosystems with high BNI (e.g. brachialactone), such as with *Brachiaria* grasses, the flow of nitrogen from NH_4^+ to NO_3^- is restricted, and NH_4^+ and microbial N rather than NO_3^- accumulate in the soil and root system. In systems with little or no BNI, such as modern agricultural systems, nitrification occurs at a rapid rate, converting NH_4^+ to NO_3^-, which is highly susceptible to loss from the system by denitrification and or leaching (adapted from Subbarao *et al.*, 2012).

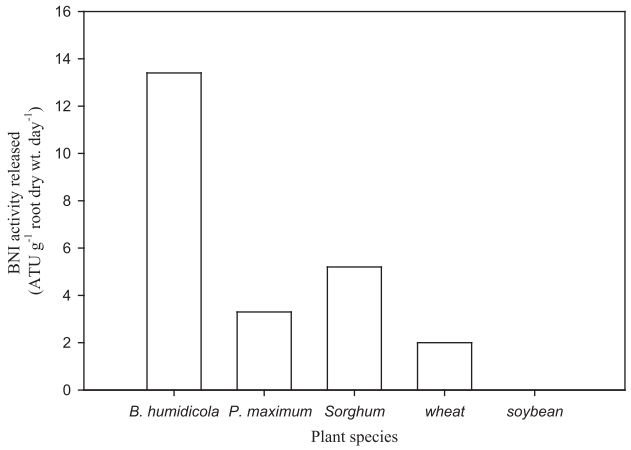

Figure 4 The biological nitrification inhibition (BNI) activity released from intact roots of various plant species grown in sand-vermiculite (3 : 1 v/v) culture for 60 days (*Source*: Subbarao *et al.*, 2007b).

(*Sorghum bicolor*), which is adapted to low N-input conditions showed significant BNI capacity (Subbarao *et al.*, 2007b and 2013a). Other cereal crops including rice, maize, wheat and barley lacked detectable BNI capacity in their root systems during the initial screening studies (Subbarao *et al.*, 2007b and 2012; Zakir *et al.*, 2008). Most legumes evaluated showed stimulation of nitrification and showed no BNI capacity in their root systems (Subbarao *et al.*, 2007b). Inhibition of nitrification is likely to be part of an adaptation mechanism to conserve and use N efficiently in natural systems where N is the most limiting nutrient determining the ecosystem productivity (Lata *et al.*, 2004; Subbarao *et al.*, 2007a), and in driving the evolution of BNI function (Rice and Pancholy, 1972; Lata *et al.*, 2004). The lack of BNI capacity in legumes is not surprising as the BNI attribute may have no adaptive value owing to their ability to fix N symbiotically. Conserving N thus may not offer much of an advantage for legumes as it may attract non-legumes as competitors (Subbarao *et al.*, 2009b and 2013b).

Characterization of BNI function in sorghum and B. humidicola

Two categories of biological nitrification inhibitors (BNIs) released from roots of sorghum (Supplementary Figure S4):

(a) Hydrophilic BNIs
(b) Hydrophobic BNIs

These two BNI fractions differ in their mobility in the soil and their solubility in water. The hydrophobic BNIs may remain close to the root as they could be strongly sorbed on the soil particles, increasing their persistence; their movement in soil is likely to be via diffusion across the concentration gradient and is likely to be confined to the rhizosphere (Dayan *et al.*, 2010; Subbarao *et al.*, 2012). In contrast, the hydrophilic BNIs may move further from the point of release owing to their solubility in water, and this may improve their capacity to control nitrification beyond the rhizosphere (Subbarao *et al.*, 2012 and 2013a). However, the distribution of hydrophobic and hydrophilic BNIs in the rhizosphere likely differs and may have complementary functional roles such as differential inhibitory effects on AOB *v.* AOA (Subbarao *et al.*, 2013a). In sorghum, the production

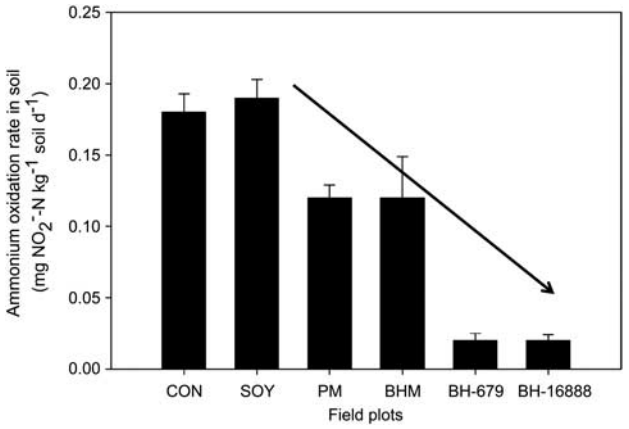

Figure 5 Soil ammonium oxidation rates (mg of NO_2^--N/kg soil per day) in field plots planted to tropical pasture grasses (differing in BNI capacity) and soybean (lacking BNI capacity in roots) (covering 3 years from establishment of pastures (September 2004 to November 2007); for soybean, two planting seasons every year and after six seasons of cultivation). CON, control (plant-free) plots; SOY, soybean; PM, *P. maximum*; BHM, *Brachiaria* hybrid cv. Mulato; BH-679, *B. humidicola* CIAT 679 (commercial cultivar); BH-16888, *B. humidicola* accession CIAT 16888 (a germplasm accession). Values are means ± s.e. of three replications (adapted from a study by Subbarao *et al.*, 2009a).

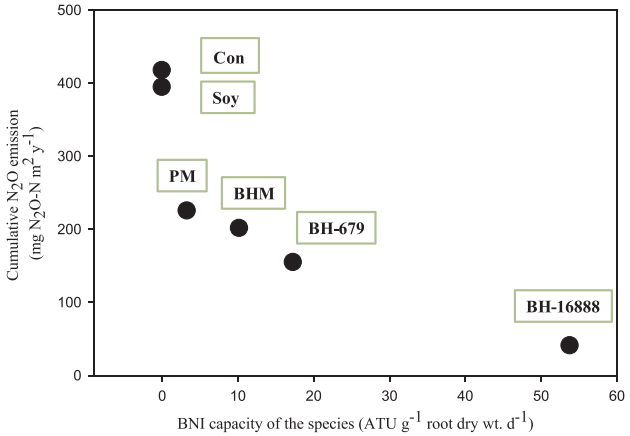

Figure 6 Relationships of the biological nitrification inhibition (BNI) capacity of plant species with N_2O emitted from field plots. The N_2O emission was monitored over a period of 3 years (adapted from a study by Subbarao *et al.*, 2012; see Figure 5 for abbreviations and treatment details).

and release of hydrophilic and hydrophobic BNIs appear to be of similar magnitude during crop development (Subbarao *et al.*, 2013a). On the basis of the BNI activity release observed from a number of studies, we estimated that the amounts of BNIs (hydrophilic plus hydrophobic) released from sorghum during a 130-day growing period (i.e. nearly up to physiological maturity) can reduce nitrification in about 500 g soil per plant (Subbarao *et al.*, 2013a).

For *Brachiaria* sp. (*B. humidicola*), assuming the average live root biomass from a long-term grass pasture at 1.5 Mg/ha (Fisher *et al.*, 1994) with a BNI capacity of 17 to 70 ATU/g root dry wt per day (Subbarao *et al.*, 2007a), it was estimated that BNI activity of 2.6×10^6–7.5×10^6 ATU/ha per day can potentially be released (Subbarao *et al.*, 2007a and 2009a). This estimate amounts to an inhibitory potential equivalent to that by the application of 6.2 to 18 kg of nitrapyrin/ha per year (based on 1 ATU being equal to 0.6 μg of nitrapyrin), which is large enough to have a significant influence on the function of soil nitrifier population and nitrification rates (Subbarao *et al.*, 2009a). Field studies indicate a 90% decline in soil ammonium oxidation rates owing to extremely small populations of nitrifiers ((AOB and AOA); determined as *amoA* genes) within 3 years of establishment of *B. humidicola* (Subbarao *et al.*, 2009a; Figure 5). N_2O emission was also suppressed by >90% in field plots of *B. humidicola* (CIAT 16888) compared with soybean (*Glycine max* (L.) Merr.), which lacks BNI capacity in its roots or control plots (plots without plants). Two other pasture grasses *P. maximum* and *Brachiaria* spp. hybrid cv. Mulato that have a low to moderate level of BNI capacity (3 to 10 ATU/g root dry wt. per day) showed only an intermediate level of inhibitory effect on soil ammonium oxidation rate. A negative relationship was observed between the BNI capacity of roots of a species and N_2O emissions, on the basis of field monitoring of N_2O emissions over a 3-year period in tropical pasture grasses having a wide range of BNI capacity in roots (Figure 6).

BNIs and their mode of action

Several BNIs that belong to different chemical functional groups have been isolated and identified (Subbarao *et al.*, 2006b, 2008 and 2009a; Zakir *et al.*, 2008). A phenyl propanoid isolated from root exudates of hydroponically grown sorghum, methyl 3-(4-hydroxyphenyl) propionate (MHPP), has been identified as the hydrophilic BNI component of the inhibitory activity released from sorghum roots (Zakir *et al.*, 2008). The IC_{50} (concentration required for 50% inhibition) value for MHPP is 9×10^{-6} M (Zakir *et al.*, 2008). The mode of inhibitory action for MHPP is based on the disruption of the AMO enzymatic pathway, and it does not affect the HAO enzymatic pathway as has been observed in the case of synthetic nitrification inhibitors (Zakir *et al.*, 2008). Sorgoleone, a *p*-benzoquinone, exuded from sorghum roots has a strong inhibitory effect on *Nitrosomonas* sp., and it contributes significantly to the hydrophobic BNI capacity in sorghum (Subbarao *et al.*, 2012; Supplementary Figure S5a).

Several isothiocyanate-based compounds such as 2-propenyl-glucosinolate, methyl-isothiocyanate, 2-propenyl isothiocyanate, butyl-isothiocyanate, phenyl-isothiocyanate, benzyl-isothiocyanate and phenethyl-isothiocyanate are formed during the degradation of cruciferous tissues and they have been reported to have varying degree of inhibitory effects on nitrification (Bending and Lincoln, 2000). Preliminary evaluation of these isothiocyanates showed inhibitory activity in the bioassay, indicating the possibility of incorporating cruciferous crop residues as a means to control soil nitrification in agricultural systems (G.V. Subbarao, JIRCAS, unpublished results).

The compounds with BNI activity in the aerial parts of *B. humidicola* are unsaturated free fatty acids, linoleic acid (LA) and α-lenolenic acid (LN; Subbarao *et al.*, 2008), which are relatively weak inhibitors of nitrification with IC_{50} values

of 3×10^{-5} M, whereas the IC_{50} value of the synthetic nitrification inhibitor AT is 1×10^{-7} M. Both LA and LN inhibit *Nitrosomonas* by blocking of both the AMO and HAO enzymatic pathways (Subbarao *et al*., 2008). In addition, BNIs could also disrupt the electron transfer pathway via HAO to ubiquinone and cytochrome (which need to be maintained to generate reducing power, i.e. NADPH), which is crucial to the metabolic functions of *Nitrosomonas* (Subbarao *et al*., 2009a). Most synthetic nitrification inhibitors (e.g. nitrapyrin, dicyandiamide (DCD) and 3,4-dimethylpyrazolephosphate) suppress *Nitrosomonas* activity by suppressing the AMO enzymatic pathway (McCarty, 1999; Subbarao *et al*., 2006a). Two phenyl propanoids, methyl-*p*-coumarate and methyl ferulate were identified and accounted for the BNI activity in the root tissues of *B. humidicola* (Gopalakrishnan *et al*., 2007).

The major nitrification inhibitor released from the roots of *B. humidicola*, a cyclic diterpene, has been discovered and termed 'brachialactone' (Supplementary Figure S5b; Subbarao *et al*., 2009a). This compound has a dicyclopenta [a,d] cyclooctane skeleton (5–8–5 ring system) with a γ-lactone ring bridging one of the five-membered rings and the eight-membered rings (Subbarao *et al*., 2009a). Brachialactone, with an ED_{80} (effective dose for 80% inhibition) of 10.6 μM, is considered as one of the most potent nitrification inhibitors compared with nitrapyrin or DCD, two of the synthetic nitrification inhibitors most commonly used in practical agriculture (ED_{80} of 5.8 μM for ©nitrapyrin and 2200 μM for ©dicyandiamide). Brachialactone inhibits *Nitrosomonas* sp. by blocking both the AMO and HAO enzymatic functions, but appears to have a relatively stronger effect on the AMO than on the HAO enzymatic pathway (Subbarao *et al*., 2009a). About 60% to 90% of the inhibitory activity released from the roots of *B. humidicola* is brachialactone, and its release is triggered by NH_4^+ in the rhizosphere. In addition, brachialactone release is confined to the root regions where NH_4^+ is present, and is mostly localized in the nature (Subbarao *et al*., 2009a).

Genetic improvement of BNI capacity in pasture grasses and cereal crops

Availability of genetic variability is a prerequisite for the genetic improvement of any plant trait through conventional and/or molecular breeding approaches. Significant genetic variability exists for the BNI capacity in *B. humidicola* (Subbarao *et al*., 2007b). Specific BNI activity (ATU/g root dry wt. per day) ranged from 7.1 to 46.3, indicating a significant potential for genetic improvement of the BNI capacity by selection and recombination (Subbarao *et al*., 2007b and 2009). Recent findings suggest substantial genetic variability for brachialactone release in the germplasm accessions of *B. humidicola*, and several genetic stocks with contrasting ability (nearly 10-fold differences) for brachialactone release capacity have been identified (G.V. Subbarao and K. Nakahara, JIRCAS, unpublished results), suggesting the potential of breeding for high-brachialactone release genetic stocks to improve the BNI capacity in *B. humidicola*. The discovery of sorgoleone's BNI function adds a new dimension to the functional significance of its release from sorghum roots (Subbarao *et al*., 2013a). A substantial genetic variability for sorgoleone (a major component of hydrophobic BNI activity released from sorghum roots) release has been found in sorghum germplasm (G.V. Subbarao and C.T. Hash, JIRCAS & ICRISAT, unpublished results). Several mapping populations of recombinant inbred lines (RIL) based on crosses of sorghum parental lines differ in their capacity to exude sorgoleone, and these results are being currently used to map additional sorghum genomic regions contributing to genetic variation in sorgoleone exudation. As these populations are generally based on elite germplasm, this approach has the advantage of facilitating deployment of BNI traits in relevant high-yielding cultivars of sorghum. Preliminary evaluation suggested a lack of significant BNI capacity in the cultivated wheat. Subsequent evaluation of wild wheats indicated that roots of *Leymus racemosus*, a wild relative of wheat, possess high-BNI capacity (Subbarao *et al*., 2007c). The rate of suppression by *L. racemosus* was effective in reducing soil nitrification (Subbarao *et al*., 2007c). Using chromosome addition lines derived from the hybridization of *L. racemosus* with cultivated wheat, it was shown that genes conferring high-BNI capacity were located on chromosomes *Lr#n*, *Lr#l* and *Lr#J*, and they could successfully be introduced into and expressed in cultivated wheat (Subbarao *et al*., 2007c). These results indicate that there is a potential for developing future wheat cultivars with BNI capacity to suppress soil nitrification in wheat production systems (Subbarao *et al*., 2007c; Zahn, 2007).

Strategies for deployment of BNI function in production agriculture

In the case of annual crops with duration usually $<$120 days may not be adequate with the current BNI-activity release rates observed for sorghum and other major food crops (Subbarao *et al*., 2007b, 2007c, 2009a and 2013a; Zakir *et al*., 2008; Zhu *et al*., 2012) to reach the critical threshold levels (i.e. $>$6 ATU/g soil) needed to substantially reduce nitrification in the bulk soil (Subbarao *et al*., 2013a). It is thus likely that the impact of BNI from annual crops may be confined to the rhizosphere soil environment, where NH_4^+ oxidation can be substantially reduced to make NH_4^+ available for crop uptake and assimilation, thus helping to shift more toward NH_4^+ form of N in annual field crops with high-BNI capacity in the root systems (Subbarao *et al*., 2013a). But for tropical pastures (e.g. *Brachiaria* spp.) with high-BNI capacity in root systems and extensive root systems coupled with perennial habits can significantly reduce the soil nitrification potential and nitrifier populations (i.e. low-nitrifying production environments; Subbarao *et al*., 2009a and 2012a). This could be exploited for the benefit of annual crops, such as maize and wheat that receive most of the N fertilization, but at present have little inherent BNI capacity in their root systems, by integrating pastures with high-BNI capacity with crop production using agro-pastoral systems or mixed crop–livestock systems (Subbarao *et al*., 2013a). The pasture component could provide the required BNI activity to

suppress soil nitrifier activity to improve N economy of the annual crops (a weak contributor of BNIs) that follow the pasture phase. The stability of the residual BNI effects (determined as NH_4^+ oxidation rates) from *Brachiaria* pastures, where an annual crop such as maize or soybean is grown after pasture phase is not yet known, but this is needed to determine the cropping duration between pasture phases in such agro-pastoral systems (Subbarao *et al*., 2012 and 2013b). For crops that produce BNIs in their plant tissues, but do not release them from their root systems, for example, crucifers (Bending and Lincoln, 2000), the incorporation of plant residues into the soil could be an alternative way to control soil nitrification (Subbarao *et al*., 2013b). In addition, *Brachiaria* grasses could also be used as short-term cover crops for using the BNIs produced in their biomass as mulch after 3 to 4 months of growth, followed by direct sowing of maize or soybean into the mulch. Appropriate agronomic practices need to be developed to supplement the addition of BNIs by *Brachiaria*'s shoot tissues (Subbarao *et al*., 2008), in addition to that added from the root systems. Increased reliance on soil microbial root and microbial rhizosphere processes through 'ecological intensification' in agroecosystems generate environmental benefits and decrease reliance on fossil fuel-based fertilizers (Jackson *et al*., 2012). Thus, multi-disciplinary efforts are needed for crop and forage genetic improvement in the BNI capacity coupled with agronomic practices in suitable cropping systems that could be used to utilize BNI function to promote low-nitrifying production systems in agriculture.

The way forward

Global food systems have a profound impact on disrupting the N cycle with introducing massive amounts of reactive N through industrial fertilizer production (Socolow, 1999; Tubiello *et al*., 2013). Half of the synthetic N fertilizer ever used on Earth has been applied in just the last 15 to 20 years (Pelletier and Tyedmers, 2010); most of this reactive N is routed through just 11% of the Earth's surface leading to degradation of soil quality, reduction of ecosystems ability to provide goods and services, resulting in serious environmental problems (Newbould, 1989; Tilman *et al*., 2001). Despite efforts over the last 40 years involving genetic and cultural improvements, a 66% decline was observed in global agronomic N efficiency (Tilman *et al*., 2002). Global food demand is expected to double by 2050 (Alexandratos, 1999; Cassman, 1999; Cohen and Federoff, 1999), and the world's N-fertilizer consumption will double from the present levels by 2050 reaching 300 Tg N/year, unless there is a substantial improvement in NUE of our production systems (Cassman and Pingali, 1995; Tilman *et al*., 2001; Galloway *et al*., 2008; Schlesinger, 2009). There is serious concern that reactive N levels in the environment have already reached a critical planetary boundary limit and that further increase will threaten the future habitability of our planet Earth (Rockstrom *et al*., 2009). In the worst-case scenario, we would move toward a N-saturated planet – not a pleasant situation (e.g. algae-infested green lakes with reduced aquatic life and NO_3^--contaminated drinking water supplies unfit for human consumption without treatment; Galloway *et al*., 2008).

The problem is that at present we waste most of the Haber's N fertilizer, manufactured using vast amounts of energy and by emitting enormous amounts of CO_2. Of the 150 Tg N fertilizers we presently apply to agricultural fields annually, 70% is lost, largely because of the high-nitrifying nature of our production systems. The NUE of the world's cereals has fallen from 80% in 1960s to just below 30% at present (Raun and Johnson, 1999; Tilman *et al*., 2002). In our quest for enhancing food production, we rather failed to consider the flow of industrially produced reactive N through the multiple pathways of soil N cycling. The consequence is the emergence of nitrification as the major N-flow pathway, acting as a powerful driving force, largely responsible for the inefficient use of N and for the resulting N pollution (Vitousek *et al*., 1997a and 1997b; Matson *et al*., 1998; Tilman *et al*., 2001; Mulvaney *et al*., 2009; Subbarao *et al*., 2013b). It is neither necessary nor prudent for most N to be cycled through the nitrification pathway to achieve higher productivity.

Nature has shown that by routing reactive N through multiple pathways and restricting the flow through nitrification path, N can be cycled more effectively with limited leakage into the environment (Vitousek and Matson, 1984; Cooper, 1986; White, 1991; Northup *et al*., 1995; Stelzer and Bowman, 1998; Harrison *et al*., 2007; Ashton *et al*., 2010; Smolander *et al*., 2012). As nitrification and denitrification are the two primary biological drivers for the production of NO_3^-, N_2O and NO (i.e. the reactive N forms largely responsible for environmental pollution), suppressing nitrification is critical for the development of low N_2O-emitting and low NO_3^--producing agricultural systems (Subbarao *et al*., 2012 and 2013b). This is a major challenge and requires a new paradigm of approaches on how to manage N in agricultural systems. A fundamental shift from the current NO_3^--dominated production systems to NH_4^+ as the preferred crop nutrient for uptake and assimilation is a must to create such N-efficient production systems. The BNI function in plants is such a biological mechanism that could facilitate a shift in N nutrition toward NH_4^+ form in production systems. The BNI function in forage grasses and field crops can be exploited using both genetic and crop and/or production system management to design low-nitrifying agronomic environments to improve NUE of agricultural systems. Better integration of crop and livestock production to recycle C and N wastes through agricultural systems is critical for sustaining soil fertility and to minimize N losses from livestock production. A paradigm shift is needed to steer N management from the current high-nitrifying environments to low-nitrifying and low N_2O-emitting production systems that are sustainable both from ecological and economic perspective.

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Supplementary materials

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