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VIEWPOINT: PART OF A SPECIAL ISSUE ON MATCHING ROOTS TO THEIR ENVIRONMENT

A paradigm shift towards low-nitrifying production systems: the role of biological nitrification inhibition (BNI)

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• *Background* Agriculture is the single largest geo-engineering initiative that humans have initiated on planet Earth, largely through the introduction of unprecedented amounts of reactive nitrogen (N) into ecosystems. A major portion of this reactive N applied as fertilizer leaks into the environment in massive amounts, with cascading negative effects on ecosystem health and function. Natural ecosystems utilize many of the multiple pathways in the N cycle to regulate N flow. In contrast, the massive amounts of N currently applied to agricultural systems cycle primarily through the nitrification pathway, a single inefficient route that channels much of this reactive N into the environment. This is largely due to the rapid nitrifying soil environment of present-day agricultural systems.

• *Scope* In this Viewpoint paper, the importance of regulating nitrification as a strategy to minimize N leakage and to improve N-use efficiency (NUE) in agricultural systems is highlighted. The ability to suppress soil nitrification by the release of nitrification inhibitors from plant roots is termed 'biological nitrification inhibition' (BNI), an active plant-mediated natural function that can limit the amount of N cycling via the nitrification pathway. The development of a bioassay using luminescent *Nitrosomonas* to quantify nitrification inhibitory activity from roots has facilitated the characterization of BNI function. Release of BNIs from roots is a tightly regulated physiological process, with extensive genetic variability found in selected crops and pasture grasses. Here, the current status of understanding of the BNI function is reviewed using *Brachiaria* forage grasses, wheat and sorghum to illustrate how BNI function can be utilized for achieving low-nitrifying agricultural systems. A fundamental shift towards ammonium (NH_4^+)-dominated agricultural systems could be achieved by using crops and pastures with high BNI capacities. When viewed from an agricultural and environmental perspective, the BNI function in plants could potentially have a large influence on biogeochemical cycling and closure of the N loop in crop–livestock systems.

Key words: AMO, ammonia mono-oxygenase, biological nitrification inhibition, BNI, BNI capacity, brachialactone, fatty acids, HAO, hydroxylamine oxidoreductase, high-nitrifying production systems, low-nitrifying production systems, nitrification, *Nitrosomonas*, nitrate leaching, synthetic nitrification inhibitors, nitrous oxide emissions, sustainability.

INTRODUCTION

The biological oxidation of ammonium (NH_4^+) to nitrate (NO_3^-), a critical aerobic process, termed 'nitrification', evolved about 2·5 billion years ago (Berner, 2006). It is carried out by two groups of chemo-lithotrophic bacteria – ammonia-oxidizing bacteria (AOB; mainly *Nitrosomonas* spp. and *Nitrobacter* spp.) and ammonia-oxidizing archaea (AOA), that are ubiquitous components of the soil microbial population (Leninger *et al.*, 2006; Taylor *et al.*, 2010). In most agricultural soils, AOB and AOA are largely responsible for nitrification. Other soil bacterial spp. such as *Nitrosocystus* and *Nitrosospira*, and some heterotrophic fungi such as *Aspergillus flavus*, can play a significant role in the nitrification of selected forest ecosystems (Sommer *et al.*, 1976). Inorganic N forms, i.e. NH_4^+ or NO_3^-, are predominantly the major source of nitrogen (N) uptake in agricultural systems. In some N-limited natural ecosystems of the Arctic, however, organic N forms, particularly free amino acids, can be absorbed directly by plant roots (Kielland, 2001). Nitrification and subsequent denitrification that reduce nitrate are critical parts of the processes used for removing excess N from organic wastes and aquatic ecosystems. Conversely, in agricultural systems, rapid and unregulated nitrification results in inefficient N use by crops, leading to increased N leakage and environmental pollution (Clark, 1962; Likens *et al.*, 1969; Schlesinger, 2009). Most

plants have the ability to use either NH_4^+ or NO_3^- as their N source, and thus are not dependent solely on NO_3^- (Haynes and Goh, 1978; Salsac *et al.*, 1987; Boudsocq *et al.*, 2012). Consequently, reducing nitrification rates in agricultural systems does not alter the intrinsic ability of plants to absorb N, but increases N retention time in the root zone as NH_4^+ is much less mobile than NO_3^-, providing additional time for the plants to absorb N. This in turn reduces the amount of N lost through leaching and denitrification (Hodge *et al.*, 2000; Subbarao *et al.*, 2012*a*).

Nitrogen cycle in intensified agricultural systems

Nitrogen fixation, organic matter (OM) mineralization, ammonification, nitrification and denitrification are all important components of the soil N cycle in terrestrial ecosystems; however, their relative importance may vary. To limit N leakage, natural ecosystems exploit these multiple pathways to regulate N flows through the suppression of nitrification and by utilizing various N forms (both organic and inorganic forms) as N sources, restricting N flow through the nitrification path (Hari and Kulmala, 2008; Smolander *et al.*, 2012). In contrast, nitrification is the process that dominates the N cycle in typical agricultural systems (i.e. neutral upland aerobic soils; Fig. 1), and NO_3^- accounts for >95 % of the total N uptake by crop plants, making N cycling in these systems inefficient and extremely leaky to the environment (Fig. 1; Sahrawat, 1982; Galloway *et al.*, 2008; Schlesinger, 2009).

High-nitrifying agricultural systems impact the global environment

The Green Revolution, largely based on the use of massive amounts of industrially fixed N for semi-dwarf rice and wheat cultivars, quadrupled global food grain production during 1960–2009, but at a large environmental cost (Broadbent and Rauschkolb, 1977; Matson *et al.*, 1999; Tilman *et al.*, 2001, 2002; Hungate *et al.*, 2003; Sutton *et al.*, 2011). Worldwide, chemical fertilizer consumption has increased 4-fold during the last 50 years (FAO, 2011). The high-nitrifying nature of these intensive production systems results in loss of nearly 70 % of the overall N-fertilizer inputs (Peterjohn and Schlesinger, 1990; Raun and Johnson, 1999; Vitousek and Howarath, 1991). With the worldwide N-fertilizer applications reaching 150 Tg $year^{-1}$ (Galloway *et al.*, 2008) and the cost of urea N ranging recently from US$0·80 to 0·54 kg^{-1} N, the direct annual economic loss is estimated at nearly US$81 billion (Subbarao *et al.*, 2012*a*). Fertilizer N use is expected to double by 2050 to reach close to 300 Tg $year^{-1}$ (Galloway *et al.*, 2008; Schlesinger, 2009). This will further increase N leakage from agricultural systems, placing an even greater pollution load on the environment (Ju *et al.*, 2009; Schlesinger, 2009; Tilman *et al.*, 2001). The loss of NO_3^- from the root zone and NO_3^- contamination of ground and surface waters are major environmental concerns associated with nitrification (Tilman *et al.*, 2001; Galloway *et al.*, 2008; Schlesinger, 2009). Current estimates indicate that N lost by NO_3^- leaching from agricultural systems could reach 61·5 Tg N $year^{-1}$ by 2050 (Schlesinger, 2009). Globally, agricultural systems contribute almost 30 % of nitric oxide (NO) and 70 % of N_2O atmospheric emissions (Bremner and Blackmer, 1978; Smith *et al.*, 1997, 2007; Hofstra and Bouwman, 2005). N_2O is a powerful greenhouse gas having a GWP (global warming potential) 300 times greater than that of CO_2 (Kroeze, 1994; IPCC, 2007), while the Earth's protective ozone layer is damaged by NOs that reach the stratosphere (Crutzen and Ehhalt, 1977). Current estimates indicate that nearly 17 Tg N $year^{-1}$ is emitted into the atmosphere as N_2O (Galloway *et al.*, 2008; Schlesinger, 2009). By 2100, the global N_2O emissions are projected to be four times greater than the current estimations, due largely to an increase in the use of N-fertilizers (Hofstra and Bouwman, 2005; Galloway *et al.*, 2008; Burney *et al.*, 2010; Kahrl *et al.*, 2010).

Greenhouse gas (GHG) emissions (CO_2, N_2O and CH_4) associated with N-fertilizer production

Nitrogen-fertilizers are largely responsible for N_2O emissions in agricultural systems when they undergo nitrification/denitrification, as described earlier. In addition, substantial amounts of GHGs (eg. CO_2, N_2O and CH_4) are emitted during the production of N-fertilizers, which can be expressed as CO_2 equivalents per unit mass of fertilizer (g CO_2-e kg^{-1} N-fertilizer) based on their GWP (IPCC, 1996). Synthesis of ammonia is a very energy-intensive process, and requires about 25–35 GJ t^{-1} of ammonia (Patyk, 1996; Kongshaug, 1998; Davis and Haglund, 1999). In addition, the production of nitric acid from ammonia, the feedstock for synthesis of complex N-fertilizers (such as NPK formulations), results in large-scale N_2O emissions (IPCC, 2012). This emission factor is about 4 kg CO_2-e kg^{-1} urea N and about 10 kg CO_2-e kg^{-1} N in complex fertilizers (i.e. NPK; Kuesters and Jenssen, 1998; Davis and Haglund, 1999; Kramer *et al.*, 1999). With the current levels of annual N-fertilizer application in agriculture (i.e. 150 Tg-N), this amounts to GHG emissions of 600–1500 Tg CO_2-e associated with global N-fertilizer production (excluding GHG emissions during transportation of N-fertilizers from factory to farm). These GHG emissions are similar in magnitude to annual CO_2-e emissions from motor vehicles, which are at 900 Tg CO_2-e (Schafer and Victor, 1999; DeCicco and Fung, 2006). By the year 2050, GHG emissions from global N-fertilizer production will reach 1200–3000 Tg CO_2-e (based on 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 currently accounts for about 2–4 % of global CO_2 emissions.

Switching to low-nitrifying agricultural systems?

Nitrification plays a relatively minor role in many natural climax plant communities where only a small portion of the total N goes through the nitrification pathway (Rice and Pancholy, 1972, 1973, 1974; White, 1991; Nasholm *et al.*, 1998; Paavolainen *et al.*, 1998). In contrast, >90 % of the total N flow is through the nitrification pathway in most agricultural systems (Fig. 1; Sahrawat, 1982; Vitousek *et al.*, 1997; Smolander *et al.*, 1998; Subbarao *et al.*, 2006*a*). Most

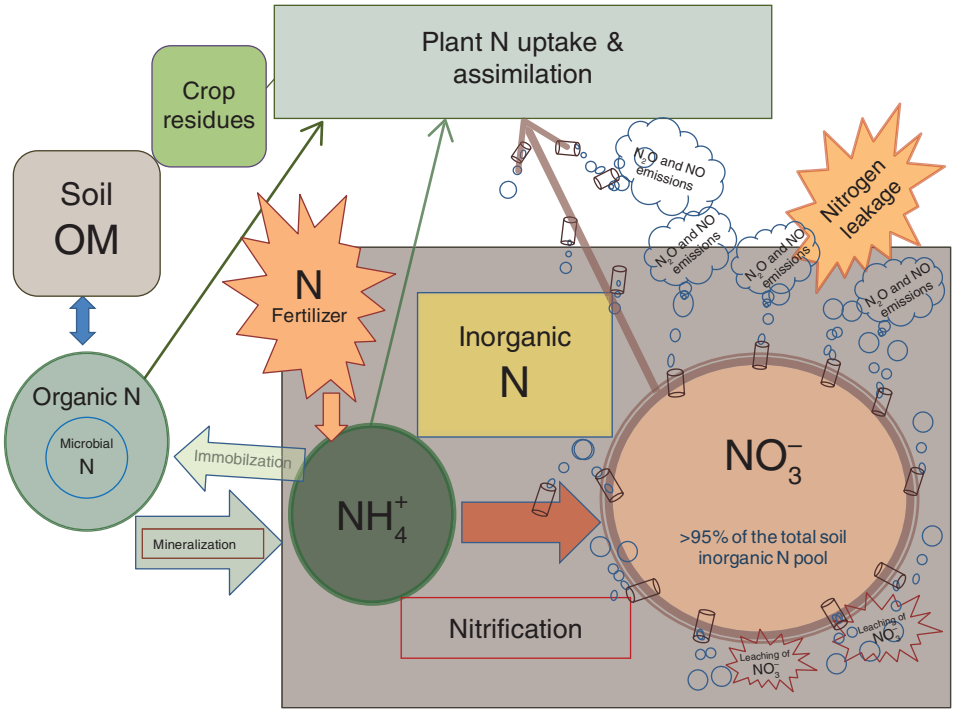

FIG. 1. The nitrogen cycle in a typical agricultural system (i.e. upland aerobic soil with neutral reaction) dominated by the nitrification pathway in which >95 % of the N flows through, and NO_3^- remains the major inorganic N form for absorption and assimilation by plants (adapted from Subbarao *et al.*, 2012*a*).

modern agricultural systems have become high-nitrifying environments where nitrification is so rapid that most of the NH_4^+ that enters either from OM mineralization or from external N inputs (i.e. chemical N-fertilizer such as urea) is nitrified within a few weeks (often <2 weeks), making it vulnerable to loss through either leaching or denitrification (Sahrawat, 2008; Subbarao *et al.*, 2012*a*).

Several changes in agricultural management practices during the 20th century have led to high-nitrifying agronomic environments. Current production systems depend heavily on industrially fixed N (i.e. N-fertilizer), and they have replaced the earlier production systems that relied primarily on N_2-fixing legumes and/or animal wastes as their N sources (Dinnes *et al.*, 2002; Foley *et al.*, 2005). The separation of crop production from animal production has led to an even greater dependence on mineral N-fertilizers, bypassing classical agricultural systems for OM recycling. This has also resulted in the reduction of soil OM (SOM) levels in agricultural systems worldwide (Elliott, 1986; Ross, 1993; Tiessen *et al.*, 1994; Celik, 2005; Foley *et al.*, 2005; van Wesemael *et al.*, 2010). The heavy dependence of modern agriculture on mineral N-fertilizers has contributed to the stimulation of nitrifier activity and the development of high-nitrifying soil environments (Poudel *et al.*, 2002; Bellamy *et al.*, 2005). In addition, installation of sub-surface drainage systems has further accelerated N losses from NO_3^- leaching and denitrification, leading to further declines in N-use efficiency (NUE; Clark, 1962; Pratt and Adriano, 1973; Dinnes *et al.*, 2002).

As a cation, NH_4^+ is held electrostatically by negatively charged clay surfaces and functional groups of the SOM (Sahrawat, 1989). This bonding is sufficiently strong to reduce the leaching loss of NH_4^+ N. In contrast, NO_3^-, with its negative charge, does not readily bond to the soil, and is much more liable to be leached out of the root zone. Several heterotrophic soil bacteria denitrify NO_3^- under anaerobic or partially anaerobic conditions. This often coincides 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 N loss during and following nitrification reduces the effectiveness of N fertilization and at the same time causes serious N pollution (Clark, 1962; Jarvis, 1996).

Rapid conversion of NH_4^+ to NO_3^- in the soil results in inefficient use of both soil N and applied N, and, as soil organic N is also subject to nitrification, this makes it liable to N loss by the same pathways as fertilizer N (Clark, 1962; Barker and Mills, 1980; Dinnes *et al.*, 2002). In addition, the assimilation of NH_4^+ is energetically more efficient than that of NO_3^- (20 mol of ATP per mol of NO_3^- vs. 5 mol of ATP per mol of NH_4^+; Salsac *et al.*, 1987). Moreover, assimilation of NO_3^-, but not of NH_4^+, results in the direct emission of N_2O from crop canopies, further reducing NUE (Smart and Bloom, 2001). Maintaining soil N in the NH_4^+ form thus is advantageous even after taking into consideration the potential negative effects of rhizosphere acidification from its uptake and assimilation (caused by H^+ excretion; Britto *et al.*, 2001). Also, by slowing the soil nitrification rates, NH_4^+ can move into the microbial pool where it becomes a slow-release N source (Vitousek and Matson, 1984; Hodge *et al.*, 2000). Better utilization of NH_4^+ also depends on the N preference of plant species or cultivars. Many of these advantages associated with inhibiting nitrification in improving crop yield,

grain quality and environmental quality have been demonstrated using chemical inhibitors (Huber *et al.*, 1977; Slangen and Kerkhoff, 1984; Sahrawat, 1989; Subbarao *et al.*, 2012*a*).

Options for nitrification control

Various N management strategies have been developed both to synchronize fertilizer N application with crop requirements to facilitate rapid uptake and to reduce N residence time in soil, thereby limiting losses associated with leaching and denitrification of NO_3^- (Newbould, 1989; Dinnes *et al.*, 2002). There are a number of agronomic strategies involving rates and/or timing of fertilizer application (such as autumn vs. spring, basal vs. broadcast, deep vs. surface applications, point injection placement of solutions, foliar applications of urea) that have been used to minimize N losses from agricultural systems, with varying degrees of success in production agriculture. Often these agronomic strategies have intrinsic limitations such as additional labour requirements/costs that make them less desirable to adopt (Dinnes *et al.*, 2002). In addition, specialty fertilizers with controlled N release in the soil such as polythene-coated urea (PCU) have been demonstrated to reduce nitrification (Shaviv and Mikkelsen, 1993; Zvomuya *et al.*, 2003). Such specialty fertilizers can be used more effectively, and the rate of N application can be reduced up to 40 % without yield loss (Shoji and Kanno, 1994); however, these fertilizers are nearly 8-fold more expensive than normal urea, and thus have not been widely adopted in production agriculture.

Use of synthetic chemical inhibitors. Nitrification inhibitors (NIs) are compounds that delay the bacterial oxidation of NH_4^+ by suppressing nitrifying soil bacteria, which should improve NUE (Hendrickson *et al.*, 1978; Bremner *et al.*, 1981; Rodgers, 1986). Reducing nitrification rates during the initial crop establishment phase and increasing it during the rapid growing phase will reduce the risk of NO_3^- leaching from the rooting zone (Dinnes *et al.*, 2002; Liao *et al.*, 2004). Though several NIs have been proposed for use in production agriculture, only a few compounds, including nitrapyrin, DCD (dicyandiamide) and DMPP (3, 4-dimethyl pyrazole phosphate), have reached the agronomic evaluation stage (Guthrie and Bomke, 1980; Weiske *et al.*, 2001; Zerulla *et al.*, 2001; Subbarao *et al.*, 2006*a*, 2012*a*). Nitrapyrin has been adopted for certain niche production systems such as winter wheat in North America. However, NIs are not widely used in production agriculture due to their limited biological stability, the non-availability of technology to deliver them to the sites of nitrification (due to differences in mobility of NIs and fertilizer N) and, equally important, their lack of cost-effectiveness (Sahrawat and Keeney, 1985; Subbarao *et al.*, 2012*a*).

In the field, the effects of NIs are most likely to be greater on soils which are N rich and where the N losses due to leaching and denitrification are large. However, the expression of these effects through plant growth will depend on the soil N status, as limiting N losses on N-rich soils may have little effect on plant production. To determine the effectiveness of NIs, it is therefore important also to take into account other soil (texture, temperature, moisture content, OM content and pH) and climatic (temperature, rainfall intensity and frequency) parameters impacting the size of N losses. In particular, OM also releases NH_4^+ through mineralization, which goes through nitrification in the same way as does fertilizer N; targeting such OM-derived NH_4^+ for NIs could be even more challenging as this OM-derived NH_4^+ could be evenly distributed in the soil profile unlike fertilizer N (which is normally banded close to plant roots).

Lessons from natural systems for managing nitrification in agricultural systems

Unlike most agricultural systems, the climax ecosystems retain large amounts of N through its incorporation into SOM (immobilization), but the underlying mechanisms remain poorly understood (Jordan *et al.*, 1979; Magill *et al.*, 2000). Natural ecosystems have evolved a range of mechanisms allowing multiple pathways for N uptake and conservation (by closing the cycle; Vitousek and Matson, 1984; Hari and Kulmala, 2008; Smolander *et al.*, 2012). They include direct uptake of organic N by plants (essentially short-circuiting mineralization) and suppressing nitrification to facilitate NH_4^+ uptake (Cooper, 1986; White, 1991; Steltzer and Bowman, 1998; Kielland, 2001; Weigelt *et al.*, 2005; Barot *et al.*, 2007; Harrison *et al.*, 2007; Houlton *et al.*, 2007; Aanderud and Bledsoe, 2009; Hewins and Hyatt, 2009; Ashton *et al.*, 2010; Smolander *et al.*, 2012). For example, in certain pine forest systems, polyphenols released from leaf litter form complexes with dissolved organic N (DON; Baldwin *et al.*, 1983), and DON–polyphenol complexes resist mineralization, but can be taken up by certain ecto-mycorrhizae (that colonize pine root systems), where it is mineralized and supplied to the pine host, thereby tightly regulating the N flow within these ecosystems without much N loss (Northup *et al.*, 1995; Smolander *et al.*, 2012).

Researchers have observed substantial differences in soil nitrification potential among several ecosystems that are not associated with soil physical and chemical characteristics (Clark *et al.*, 1960; Robertson *et al.*, 1982*a*, *b*; Montagnini *et al.*, 1989; Steltzer and Bowman, 1998; Lata *et al.*, 2004). Often the levels of NH_4^+ exceed NO_3^- levels by a factor of ten, indicating that the availability of NH_4^+ is not the limiting factor for nitrification (Schimel and Bennett, 2004). The influence of vegetation in inhibiting nitrification has long been suspected, but not directly proven (Basaraba, 1964; Bate, 1981; Donaldson and Henderson, 1990*a*, *b*; Erickson *et al.*, 2000; Smits *et al.*, 2010*a*, *b*; Smolander *et al.*, 2012). Certain forest trees, such as *Arbutus unedo*, have been reported to suppress soil nitrification and N_2O emissions by releasing gallocatechin and catechin during the decomposition of leaf litter (Castaldi *et al.*, 2009).

Since NH_4^+ assimilation in plants requires a quarter as much metabolic energy as that of NO_3^-, it is hypothesized that inhibition of nitrification could be an ecological driving force for the development of low-nitrifying climax ecosystems (Rice and Pancholy, 1972; Salsac *et al.*, 1987; Lata *et al.*, 2004). Slow rates of nitrification have been observed in several tropical grassland and forest ecosystems, and are often considered to be an indicator of ecosystem maturity (Vitousek and

Matson, 1984; Cooper, 1986; Sylvester-Bradley *et al.*, 1988; Lata *et al.*, 1999; Ishikawa *et al.*, 2003; Castaldi *et al.*, 2009; Smits *et al.*, 2010*a*, *b*; Smolander *et al.*, 2012). This led to the hypothesis that plants may influence nitrification by releasing certain phytochemicals that interfere with the activity of soil nitrifiers (Subbarao *et al.*, 2006*a*, 2012*a*; Fillery, 2007).

The hypothesis that plants can suppress or stimulate nitrification has been debated since the 1960s, and experimental evidence has proved elusive due to the lack of a suitable methodology to detect and assess any inhibitory activity of roots unequivocally (Munro, 1966*a*, *b*; Meiklejohn, 1968; Moore and Waid, 1971; Purchase, 1974; Rice and Pancholy, 1974; Lodhi, 1979, 1982; Sylvester-Bradley *et al.*, 1988; Stienstra *et al.*, 1994; Lata *et al.*, 1999, 2004; Fillery, 2007; Smits *et al.*, 2010*a*, *b*). By controlling nitrification, plants could increase N availability for their own survival in N-limiting environments (Hodge *et al.*, 2000; Weigelt *et al.*, 2005; Hewins and Hyatt, 2009). From an evolutionary viewpoint, the question remains of whether such nitrification inhibition ability [i.e. biological nitrification inhibition (BNI)] would provide a sufficient competitive advantage to outcompete other plants (Mouquet *et al.*, 2002; Lata *et al.*, 2004; Hawkes *et al.*, 2005; Hewins and Hyatt, 2009; Rossiter-Rachor *et al.*, 2009). Modelling studies on BNI function and *in situ* observations provide additional support to the hypothesis that BNI capacity may offer a sufficient competitive advantage to the observed invading/introduced tropical grasses from Africa into South America and Australia (Lata *et al.*, 2004; Hawkes *et al.*, 2005; Barot *et al.*, 2007; Boudsocq *et al.*, 2009, 2011, 2012; Hewins and Hyatt, 2009; Maire *et al.*, 2009; Rossiter-Rachor *et al.*, 2009).

BIOLOGICAL NITRIFICATION INHIBITION

The concept

The ability to suppress soil nitrification through the release of nitrification inhibitors from plant roots is termed 'biological nitrification inhibition' (Subbarao *et al.*, 2006*a*, *b*, 2009*a*, *b*, 2012*a*; Fig. 2). Nitrification is one of the most important processes determining N cycling efficiency (i.e. the proportion of N that stays in the ecosystem during a complete recycling loop); controlling nitrification thus will help in minimizing N leakage and facilitating N flow through NH_4^+ assimilation pathways.

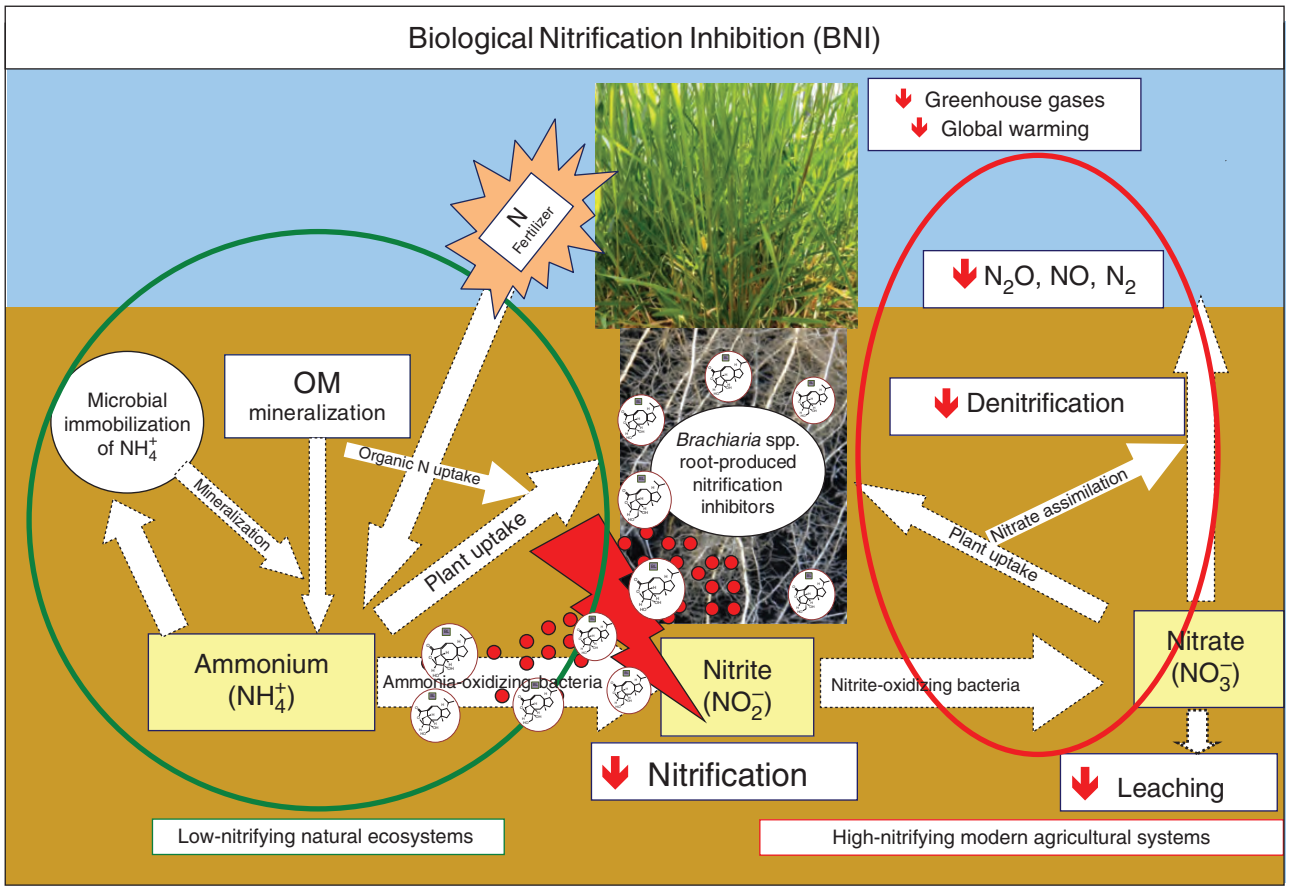

FIG. 2. Schematic representation of the biological nitrification inhibition (BNI) interfaces with the N cycle. The BNI exuded by the root inhibits nitrification that converts NH_4^+ to NO_2^-. In ecosystems with large amounts of BNI (e.g. brachialactone) such as *Brachiaria* grasses, the flow of nitrogen from NH_4^+ to NO_3^- is restricted, and it is NH_4^+ and microbial N rather than NO_3^- that accumulates in the soil and root systems. 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 by denitrification and leaching from the system (adapted from Subbarao *et al.*, 2012*a*).

Nitrogen-use efficiency ($NUE_{agronomic}$ = grain yield per unit of applied N) is a function of both intrinsic NUE ($NUE_{intrinsic}$ = dry matter produced per unit N absorbed), harvest index (HI) and N uptake. The $NUE_{intrinsic}$ of a plant is a physiologically conserved function (Glass, 2003), and thus would not necessarily be easy to manipulate genetically. Improvements in $NUE_{agronomic}$ can therefore only come from improvements in crop N uptake (Finzi *et al.*, 2007), which is largely related to recovery of applied N-fertilizer. Consequently, BNI function can positively influence $NUE_{agronomic}$ by improving N recovery by reducing N losses associated with nitrification and denitrification (Subbarao *et al.*, 2012*a*). Recent modelling studies support this hypothesis, and are linked to previous *in situ* measurements in savanna systems indicating that grasses that inhibit nitrification exhibit a 2-fold greater productivity in above-ground biomass than those that lack such ability (Lata, 1999; Boudsocq *et al.*, 2009, 2012).

Methodology to detect and determine nitrification inhibitory activity (i.e. BNI activity) in plant–soil systems

Lack of a suitable methodology to detect and quantify BNI activity in plant–soil systems has been a major hurdle for characterizing BNI function in plants (Subbarao *et al.*, 2006*a*). With the development of a bioluminescence assay using a recombinant strain of *Nitrosomonas europaea*, it is now possible to detect and quantify BNI activity released from roots, a plant function termed 'BNI capacity' (Iizumi *et al.*, 1998; Subbarao *et al.*, 2006*b*). The recombinant strain of *N. europaea* carries an expression vector for the *Vibrio harveyi lux AB* genes (Fig. 3), and produces a distinct two-peak luminescence pattern during a 30 s analysis period (Subbarao *et al.*, 2006*b*). The functional relationship between bioluminescence emission and nitrite production in the assay has been shown to be linear using a synthetic nitrification inhibitor, allylthiourea (AT; Subbarao *et al.*, 2006*b*). The inhibition caused by 0·22 μM AT in the assay (about 80 % inhibition in bioluminescence and NO_2^- production) is defined as one allylthiourea unit (ATU; Subbarao *et al.*, 2006*b*). Using the response to a concentration gradient of AT (i.e. a dose–response standard curve), the inhibitory effects of test samples (e.g. root exudates or plant or soil extracts) can be expressed and compared in ATUs. These recently developed research tools facilitated the characterization of BNI function in plants (Subbarao *et al.*, 2006*b*). Soil-based assays to determine the changes in nitrification potential of rhizosphere soil complement this characterization of BNI capacity (based on BNI activity release from roots). The changes in potential soil nitrification (due to BNI function) can be determined by monitoring ammonia-oxidizing activity (Hart *et al.*, 1994), and this methodology has been successfully deployed to assess the BNI capacity of *Brachiaria* grasses and in matgrass swards in the field (Subbarao *et al.*, 2009*a*; Smits *et al.*, 2010*a*). In addition, analysis of nitrifier populations [(AOB) and AOA)] in rhizosphere soil would provide further evidence for the changes in potential soil nitrification (Subbarao *et al.*, 2009*a*).

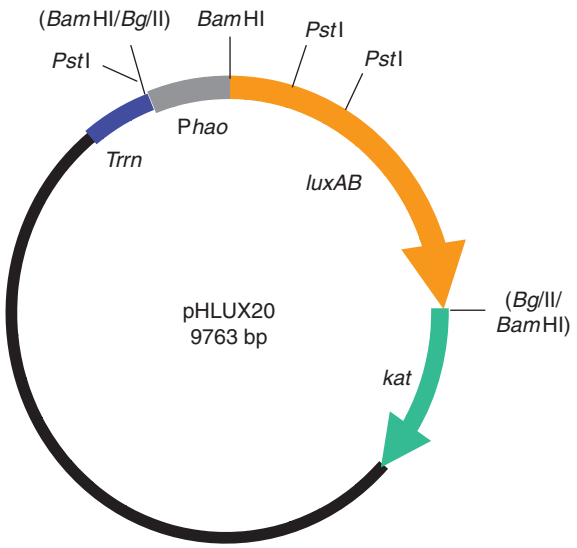

FIG. 3. Map of recombinant luminous *Nitrosomonas europaea* (pHLUX20) developed to detect and quantify nitrification inhibitors in the plant–soil system (redrawn from Iizumi *et al.*, 1998).

Evidence of BNI function in selected field crops and pasture grasses

Evaluation of selected tropical forage grasses, cereals and legume crops indicated a wide range in the BNI capacity of their root systems (Subbarao *et al.*, 2007*b*). Forage grasses of *Brachiaria humidicola* and *B. decumbens*, which are highly adapted to the low-N production environments of South American savannas (Miles *et al.*, 2004), showed the greatest BNI capacity among the tropical grasses evaluated (Subbarao *et al.*, 2007*b*). In contrast, *Panicum maximum*, which is adapted to high-N availability environments, showed the least BNI capacity (Rao *et al.*, 1996; Subbarao *et al.*, 2007*b*). Among the cereal crops evaluated, only sorghum (*Sorghum bicolor*) showed significant BNI capacity (Subbarao *et al.*, 2007*b*; 2012*b*). Other crops including rice (*Oryza sativa*), maize (*Zea mays*), wheat and barley (*Hordeum vulgare*) were found to lack BNI capacity in their root systems during initial screening studies (Subbarao *et al.*, 2007*b*, 2012*a*; Zakir *et al.*, 2008). Most legumes evaluated showed negative BNI activity in root exudates, indicating that they are likely to stimulate nitrification (Subbarao *et al.*, 2007*b*). Inhibition of nitrification is likely to be part of an adaptation mechanism to conserve and use N efficiently in natural systems that are N limiting (Lata *et al.*, 2004; Subbarao *et al.*, 2007*a*). N-limiting environments thus could be one of the dominant forces driving the evolution of BNI function (Rice and Pancholy, 1972; Lata *et al.*, 2004). It is not surprising then that legumes did not show much BNI capacity in their roots as it is likely that the BNI attribute may have no adaptive value due to their ability to fix N symbiotically; in addition, conserving N may not offer much of an advantage for legumes as it may attract non-legumes as competitors (Subbarao *et al.*, 2009*b*, 2012*a*).

Field validation of BNI function in suppressing soil nitrification and N_2O emissions

Based on conservative estimates that the live root biomass from a long-term grass pasture is 1·5 Mg ha^{-1} (Fisher *et al.*,

1994) with a BNI capacity of 17–70 ATU g^{-1} root d. wt d^{-1} (Subbarao *et al.*, 2007*a*), it was estimated that BNI activity of $2{\cdot}6 \times 10^6$–$7{\cdot}5 \times 10^6$ ATU ha^{-1} d^{-1} can potentially be released from *B. humidicola* roots (Subbarao *et al.*, 2007*a*, 2009*a*). This estimate amounts to nitrification inhibitory potential equivalent to the application of about 6·2–18 kg of nitrapyrin ha^{-1} $year^{-1}$ (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 populations and nitrification rates (Subbarao *et al.*, 2009*a*). Subsequently, field studies (Mollisol) at the International Center for Tropical Agriculture (CIAT) indicated a 90 % decline in soil ammonium oxidation rates (Fig. 4A) due to extremely small populations of nitrifiers [(AOB and AOA); determined as *amoA* genes] in *B. humidicola* plots within 3 years of establishment. Nitrous oxide emissions were also suppressed by >90 % in field plots of *B. humidicola* (CIAT 16888) compared with plots of soybean, which lacks BNI capacity in its roots, or control plots (plant-free field plots; Fig. 4B). Two other pasture grasses, *P. maximum* and *Brachiaria* spp. hybrid 'Mulato' that have a low to moderate level of BNI capacity (3–10 ATU g^{-1} root d. wt d^{-1}), showed only an intermediate level of inhibitory effect on soil ammonium oxidation rates (Fig. 4A). A negative relationship was observed between the BNI capacity of roots of a species and N_2O emissions, based on field monitoring of N_2O emissions over a 3-year period in tropical pasture grasses having a wide range of BNI capacity in roots (Fig. 5).

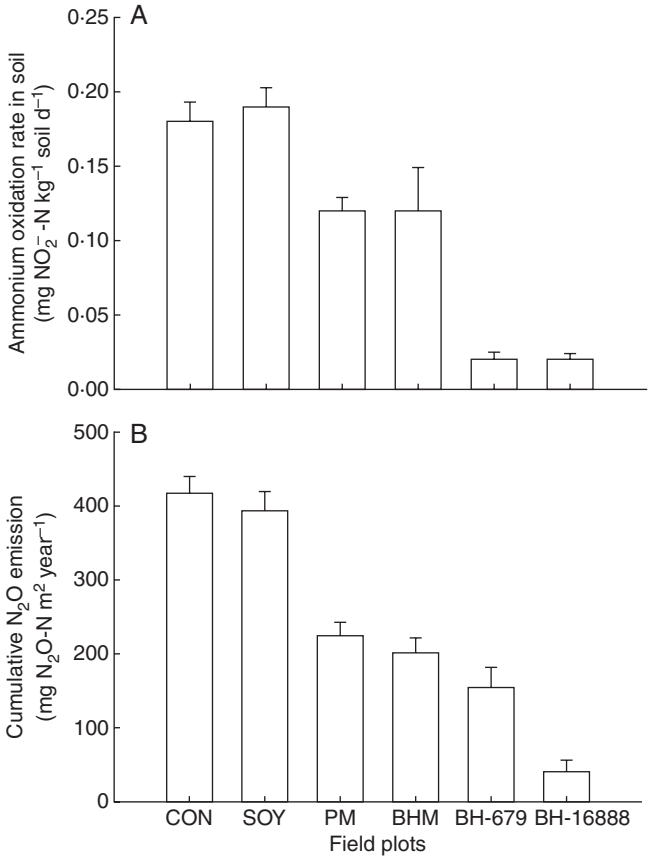

FIG. 4. (A) Soil ammonium oxidation rates (mg of NO_2^- N kg^{-1} soil d^{-1}) in field plots planted to tropical pasture grasses (differing in BNI capacity) and soybean (lacking BNI capacity in roots) [covering 3 years from establishment of pastures (September 2004–November 2007); for soybean, two planting seasons every year and after six seasons of cultivation]. CON, control (plant-free) plots; SOY, soybean; PM, *P. maximum*; BHM, *Brachiaria* hybrid 'Mulato'; BH-679, *B. humidicola* CIAT 679 (standard cultivar); BH-16888, *B. humidicola* accession CIAT 16888 (a germplasm accession). Values are means ± s.e. of three replications (adapted from Subbarao *et al.*, 2009*a*). (B) Cumulative N_2O emissions (mg of N_2O N m^{-2} $year^{-1}$) from field plots of tropical pasture grasses (monitored monthly over a 3-year period, from September 2004 to November 2007). Values are means ± s.e. of three replications (adapted from Subbarao *et al.*, 2009*a*).

Regulation of BNI function

Synthesis and release of BNIs is a highly regulated attribute (Subbarao *et al.*, 2007*a*; Zhu *et al.*, 2012). The form of N applied (i.e. NH_4^+ or NO_3^-) has a major influence on the synthesis and release of BNIs in *B. humidicola*, sorghum and *Leymus racemosus*, wild wheat (Subbarao *et al.*, 2007*a*, *c*, 2009*c*, 2012*a*, *b*; Zakir *et al.*, 2008). Plants grown with NO_3^- as N source did not release BNIs from roots, whereas BNIs were released from plants grown with NH_4^+ as their N source (Subbarao *et al.*, 2007*a*, 2009*a*; Zakir *et al.*, 2008; Zhu *et al.*, 2012). Despite high levels of BNIs detected in the root tissues of NH_4^+-grown plants, the release of BNIs was observed only when plant roots were directly exposed to NH_4^+ (Subbarao *et al.*, 2007*a*, *c*, 2009*a*, *b*; Fig. 6A, B). In addition to the presence of NH_4^+ in the root zone, the rhizosphere pH may also influence the release of BNIs from roots. Recent results suggest that sorghum plants do not release BNIs from their roots in the presence of NH_4^+ when the rhizosphere pH is 7 or higher; maximum release of BNI activity was observed only at a rhizosphere pH ranging from 5·0 to 6·0 (Subbarao *et al.*, 2012*b*). Such a tight control of rhizosphere pH on BNI release has implications regarding in which soils the BNI function is likely to be effective (Subbarao *et al.*, 2012*b*). For example, the heavy black soils (Vertisols), which generally exhibit a soil pH of >7·0, have a large buffering capacity and thus resist changes in rhizosphere pH

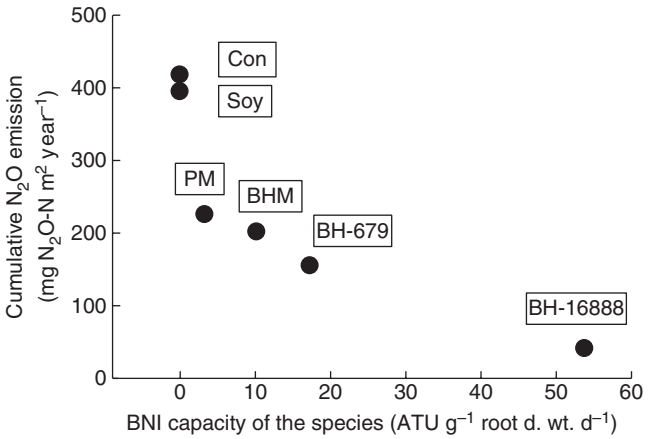

FIG. 5. Relationships of the BNI capacity of plant species to N_2O emissions from field plots (based on data from Fig. 4B). The N_2O emissions were monitored over a period of 3 years (adapted from Subbarao *et al.*, 2012*a*; see Fig. 4 for abbreviations and treatment details).

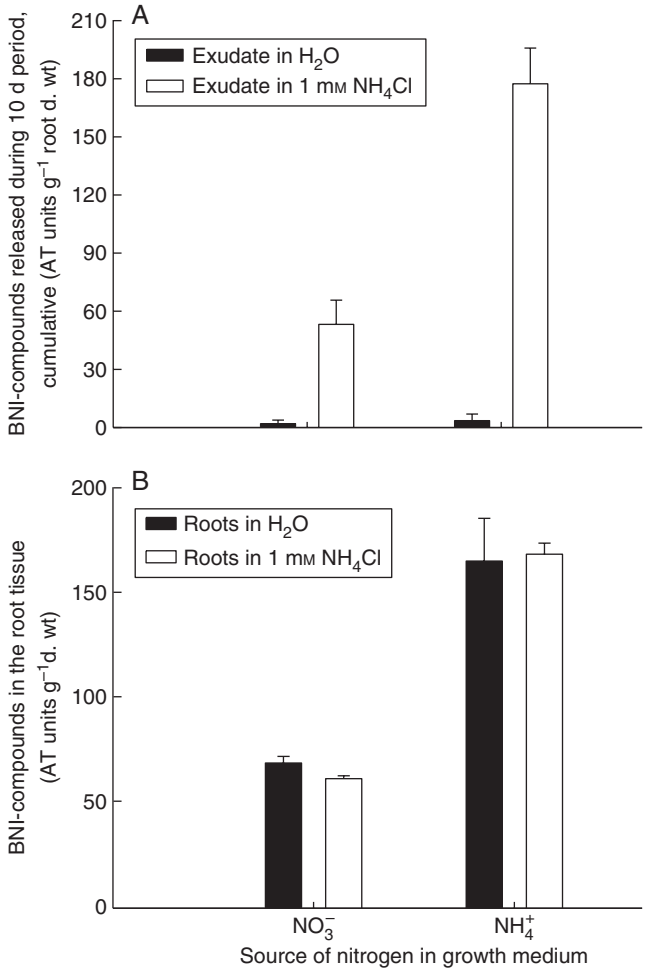

FIG. 6. (A) BNI activity released from *B. humidicola* roots during a 10 d period (adapted from Subbarao *et al.*, 2007*a*). (B) The BNI activity in the root tissue (ATU g^{-1} root d. wt) at the end of the 10 d root exudate collection (redrawn from Subbarao *et al.*, 2007*a*).

(Burford and Sahrawat, 1989). Sorghum grown on soils with pH in the alkaline range might thus not release BNIs, and hence such soil types might not be suitable for the expression of BNI function in sorghum. Perhaps light-textured soils with low buffering capacity and with moderate acidity (pH <6·0) might be better suited for the expression and exploitation of BNI function in sorghum (Subbarao *et al.*, 2012*a*, *b*).

Further, the release of BNIs from plant roots appears to be a highly regulated physiological function. The physiological consequences associated with the uptake of NH_4^+, such as activation of H^+ pumps in the plasmalemma and acidification of the rhizosphere, facilitate BNI release from sorghum roots (Zhu *et al.*, 2012). In addition, the release of BNIs from roots is a localized phenomenon (Subbarao *et al.*, 2009*a*). The release of BNIs was confined to parts of the root system exposed to NH_4^+, and was not extended to the remaining parts of the root system. Such localized release of BNIs by roots ensures a relatively high concentration of BNIs in soil microsites where nitrifiers are active, which is often associated with the presence of NH_4^+ (Subbarao *et al.*, 2009*a*). The availability of NH_4^+ in the soil either from soil organic N mineralization or through the application of N-fertilizers can thus enhance nitrifier activity (Robinson, 1963; Woldendorp and Laanbroek, 1989). The regulatory role of NH_4^+ in the synthesis and release of BNIs suggests a possible adaptive role in protecting NH_4^+ from nitrifiers, a key factor for the successful evolution of the BNI capacity as an adaptive trait (Subbarao *et al.*, 2007*a*, 2009*a*, 2012*a*).

Stability of the BNIs in soil systems

The inhibitory activity of the BNIs was initially determined in an assay lasting only for a 30 min incubation exposure period to pure cultures of *Nitrosomonas* sp. (Subbarao *et al.*, 2006*b*). For the BNIs to function effectively in the soil-based systems, the persistence of these compounds in the soil is a pre-requisite for the effectiveness of BNI function under field environments. This hypothesis was tested by adding extracted BNI activity (from root exudates of *B. humidicola*) to soil at different levels (0–20 ATU g^{-1} soil) along with an NH_4^+ source (200 mg N kg^{-1}) and then incubating for 55 d at 20 °C. Results from these studies indicated that for the inhibitory activity to be effective, a threshold level of 5 ATU g^{-1} soil was needed; nearly 50 % inhibition was observed when the BNI activity level was 10 ATU g^{-1} soil and a nearly complete suppression of soil nitrification was achieved at 20 ATU g^{-1} soil (Subbarao *et al.*, 2006*a*; Gopalakrishnan *et al.*, 2009). Further, it was shown that certain BNIs [such as linoleic acid (LA) and α-linolenic acid (LN)] partially lost their effectiveness in the soil after 80 d, and their inhibitory effect was completely lost after 100 d (Subbarao *et al.*, 2008). Preliminary measurements on mixed tropical savanna soils showed that the inhibitory effect in the soil can persist for a long period during natural air drying and storage of the soil in the dark (Lata, 1999). There is, however, still a paucity of information on the fate and efficacy of BNIs in the soil–plant systems. Thus, intensification of research is justified to generate information on emerging BNIs in different soil types under varying agro-climatic conditions relative to their persistence and effectiveness in soil–plant systems. Such knowledge will be helpful in targeting the use of BNIs to the most appropriate agro-ecosystems (Sahrawat, 1996; Wolt, 2004; Subbarao *et al.*, 2006*a*).

Biological nitrification inhibitors and their modes of action

Several BNIs that belong to different chemical groups have been successfully isolated and identified from plant tissues or root exudates using bioassay-guided purification approaches (Fig. 7; Bremner and McCarty, 1988; Subbarao *et al.*, 2006*b*, 2008, 2009*a*; Gopalakrishnan *et al.*, 2007; Zakir *et al.*, 2008). The compounds with BNI activity in the aerial parts of *B. humidicola* are unsaturated free fatty acids, LA and LN (Fig. 8; Subbarao *et al.*, 2008). They are relatively weak inhibitors of nitrification, with IC_{50} values of 3×10^{-5} M; while the IC_{50} value of the synthetic nitrification inhibitor AT is 1×10^{-7} M. However, other free fatty acids differing in chain lengths or numbers of double bonds, e.g. stearic, oleic, arachidonic and *cis*-vaccenic acids, did not show much inhibitory activity (Subbarao *et al.*, 2008). The inhibitory effect of LA was increased by its conversion to its methyl

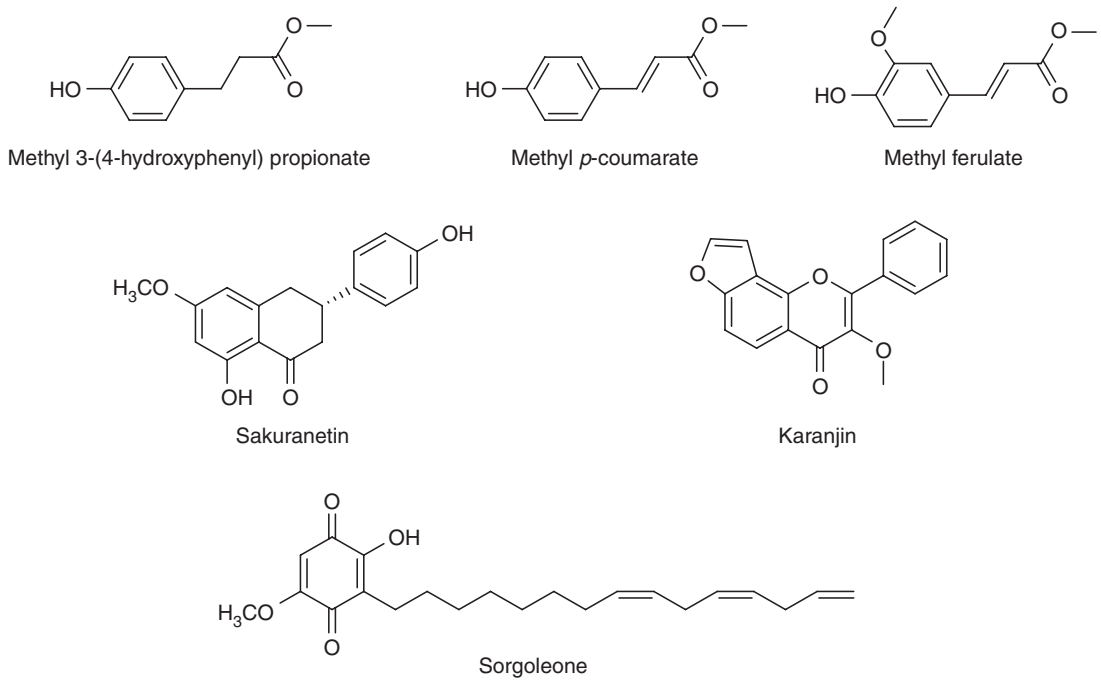

FIG. 7. Chemical structure of BNIs belonging to different chemical functional groups isolated from plant tissues or root exudates.

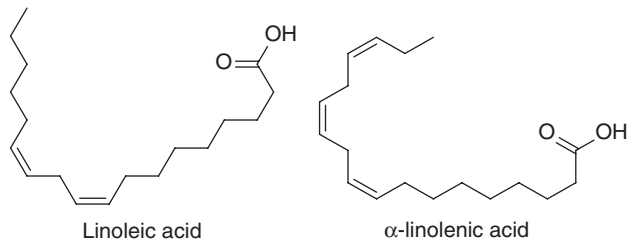

FIG. 8. Chemical structure of linoleic acid and α-linolenic acid, the BNIs isolated from the aerial parts of *B. humidicola.*

ester (LA-ME), but the activity was lost when converted into its ethyl ester (LA-EE; Table 1). 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 (Subbarao *et al.*, 2008). Both LA and LN inhibit *Nitrosomonas* through blocking of both AMO (ammonia mono-oxygenase) and HAO (hydroxylamine oxidoreductase) enzymatic pathways, which catalyse the essential reactions of ammonia oxidation (Subbarao *et al.*, 2008). In addition, BNIs could also disrupt the electron transfer pathway from HAO to ubiquinone and cytochrome (which needs to be maintained to generate reducing power, i.e. NADPH), which is crucial to the metabolic functions of *Nitrosomonas* (Subbarao *et al.*, 2009*b*). Most synthetic nitrification inhibitors (e.g. nitrapyrin, dicyandiamide, and DMPP) suppress *Nitrosomonas* activity by suppressing the AMO enzymatic pathway (McCarty, 1999; Subbarao *et al.*, 2006*a*; Table 1). Also, our knowledge of the mechanisms of inhibition by BNIs is entirely based on AOBs (i.e. *Nitrosomonas* sp.). It is increasingly evident that

TABLE 1. *Relative effectiveness of free fatty acids, fatty acid esters and standard chemical nitrification inhibitors on* Nitrosomonas *in an* in vitro *bioassay (based on Subbarao* et al., *2008)*

Compound	ED^{*}_{80}
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)	>2000·0

*Effective dose (μg mL^{-1}) for 80 % inhibition of *Nitrosomonas* function (i.e. bioluminescence) in an *in vitro* bioassay system.

AOA play a significant role in nitrification of most ecosystems and a dominant role in nitrification of certain ecosystems (Leninger *et al.*, 2006). The AMO enzymatic pathway operates in both AOB and AOA; it is assumed that most BNIs that suppress nitrifier activity by blocking the AMO pathway (as mentioned above) are likely to be effective in inhibiting AOB and AOA. However, the second enzymatic pathway in nitrification (i.e. the HAO pathway) is only established for AOB; the existence of this second enzymatic pathway remains to be proven and established for AOA.

A phenyl propanoid from root exudates of hydroponically grown sorghum, methyl 3-(4-hydroxyphenyl) propionate (MHPP), has been identified as the BNI component of the

inhibitory activity released from roots (Fig. 7; Zakir *et al.*, 2008). The IC_{50} value for MHPP is 9×10^{-6} M (Zakir *et al.*, 2008). The mode of inhibitory action for MHPP is solely through the AMO enzymatic pathway, and it has no inhibitory effect on the HAO enzymatic pathway in *Nitrosomonas* (Zakir *et al.*, 2008). It was discovered more recently that sorgoleone, a *p*-benzoquinone (Fig. 7) exuded from sorghum roots, has a strong inhibitory effect on *Nitrosomonas* sp. activity and that this compound contributes significantly to the BNI capacity in sorghum (Subbarao *et al.*, 2009*c*). Two phenyl propanoids, methyl-*p*-coumarate and methyl ferulate, were identified as responsible for the BNI activity in root tissues of *B. humidicola* (Fig. 7; Gopalakrishnan *et al.*, 2007). The IC_{50} values for methyl-*p*-coumarate and methyl ferulate are 2×10^{-5} and 4×10^{-6} M, respectively (Gopalakrishnan *et al.*, 2007). The corresponding free phenolic acids, namely *p*-coumaric acid and ferulic acid, which are involved in lignin biosynthesis, showed no inhibitory activity at concentrations $<1 \times 10^{-2}$ M (Gopalakrishnan *et al.*, 2007). It was suggested that nitrification inhibitors released through exudation and from the root tissues during decomposition/turnover can potentially play a major role in modifying the soil nitrification potential in *B. humidicola* pasture ecosystems (Subbarao *et al.*, 2006*b*; Gopalakrishnan *et al.*, 2007).

Karanjin (3-methoxy furano-2,3,7,8-flavone or 3-methoxy-2-phenyl furo-[2,3-h]chromen-4-one; Fig. 7), isolated from seeds of *Pongamia glabra* Vent., shows a strong inhibitory effect on soil nitrifier activity, and karanjin was found to be effective in suppressing soil nitrification (Sahrawat and Mukerjee, 1977; Sahrawat, 1981). The furan ring present in the molecule appears to be critical for the biological activity (i.e. nitrification inhibition; Sahrawat *et al.*, 1977). Several isothiocyanates (2-propenyl-glucosinolate, methyl-isothiocyanate, 2-propenyl-isothiocyanate, butyl-isothiocyanate, phenyl-isothiocyanate, benzyl-isothiocyanate and phenethyl-isothiocyanate; Fig. 9) are formed during the degradation of cruciferous tissues reported to have inhibitory effects on soil nitrification (Fenwick *et al.*, 1983; Bending and Lincoln, 2000). Preliminary evaluation of these compounds shows inhibitory activity in the bioassay (G. V. Subbarao, unpubl. res.), indicating the possibility of incorporating cruciferous crop residues as a means to control nitrification.

Discovery of brachialactone as a BNI compound. The major nitrification inhibitor released from roots of *B. humidicola*, a cyclic diterpene (Fig. 10), has been discovered and named 'brachialactone'. 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 ring (Subbarao *et al.*, 2009*a*). Similarly, 5-8-5 tricyclic terpenoids (ophiobolanes and fusicoccanes) are found in both fungi and plants (Muromtsev *et al.*, 1994; Toyomasu *et al.*, 2007). However, to the best of our knowledge, a compound or a derivative having a lactone ring appears to be a novel addition to the nitrification inhibitory groups. Fusicoccin-type cyclic diterpenes are biologically synthesized from geranylgeranyl diphosphate by a two-step cyclization catalysed by terpene cyclases (Toyomasu *et al.*, 2007). The inhibition of nitrification in an *in vitro* assay with pure cultures of *N. europaea* was linearly related to the brachialactone concentrations in the range of 1·3–13·3 μM (Fig. 11A). Brachialactone with an ED_{80} (effective dose for 80 % inhibition) of 10·6 μM should be considered as one of the most potent nitrification inhibitors comparable with nitrapyrin or dicyandiamide, two of the synthetic nitrification inhibitors most commonly used in agriculture (ED_{80} of 5·8 μM for nitrapyrin and 2200 μM for dicyandiamide). Brachialactone inhibits *Nitrosomonas* sp. by blocking both AMO and HAO enzymatic functions, but appears to have a relatively stronger effect on the AMO than on the HAO pathway (Subbarao *et al.* 2009*a*). About 60–90 % of the inhibitory activity released from roots of *B. humidicola* is due to brachialactone (Fig. 11B); its release from roots is triggered by NH_4^+ in the rhizosphere. Also, brachialactone release is confined to root regions where NH_4^+ is present, and is mostly localized in nature (Subbarao *et al.*, 2009*a*; Fig. 12A–C). Currently efforts are underway to understand the brachialactone biosynthetic pathway (K. Nakahara, JIRCAS, pers. comm.).

Potential for genetic manipulation of BNI capacity in cereals and pasture grasses

Several biologically active molecules with diverse chemical structures that belong to phenolic acids, hydroxamic acids,

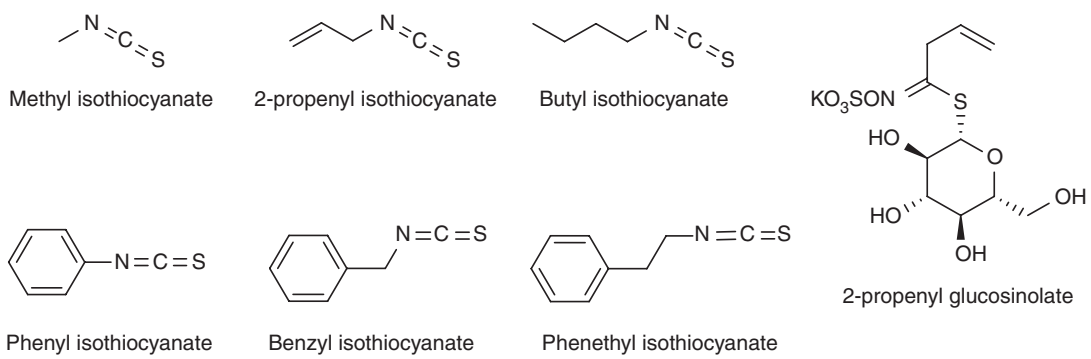

FIG. 9. Chemical structure of isothiocyanates (mostly found in crucifer tissues) that show BNI function (based on Fenwick *et al.*, 1983; Bending and Lincoln, 2000).

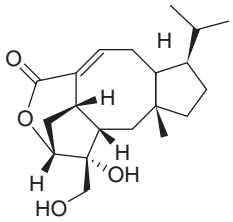

FIG. 10. Chemical structure of brachialactone, the major nitrification inhibitor isolated from root exudates of *B. humidicola* (from Subbarao *et al.*, 2009*a*).

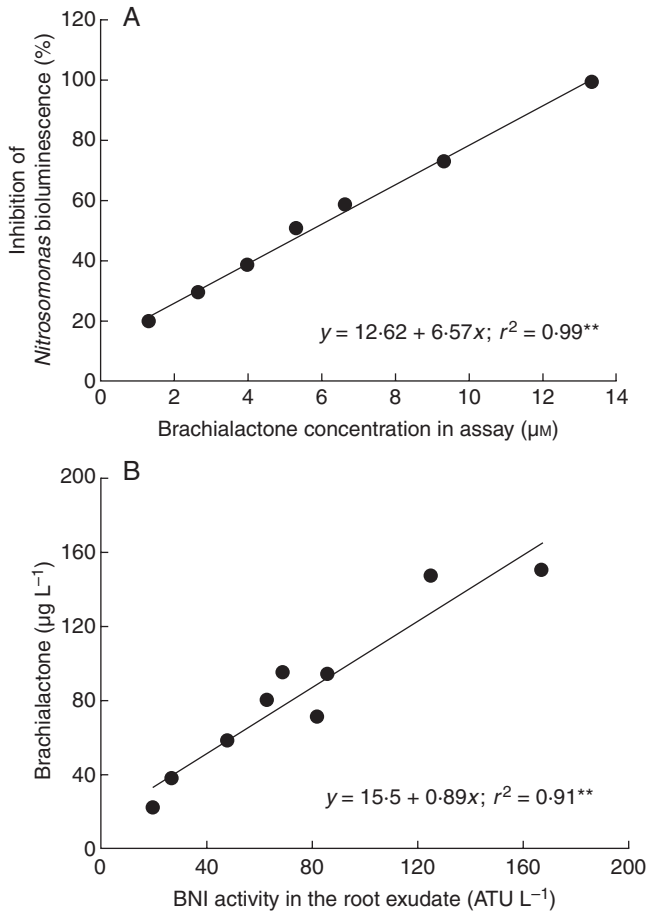

FIG. 11. Inhibition of nitrification by brachialactone and the contribution of brachialactone to the BNI activity released from roots. (A) Inhibitory effect of brachialactone on *N. europaea* in an *in vitro* assay. (B) Contribution of brachialactone to the BNI activity released from roots (i.e. in root exudates) of *B. humidicola*. Root exudates were collected from intact plants using 1 L of aerated solution of 1 mM NH_4Cl with 200 μM $CaCl_2$ over 24 h. Each data point represents root exudates collected from hydroponically grown plants in a glasshouse during March–May of 2007 and 2008 (adapted from Subbarao *et al.*, 2009*a*).

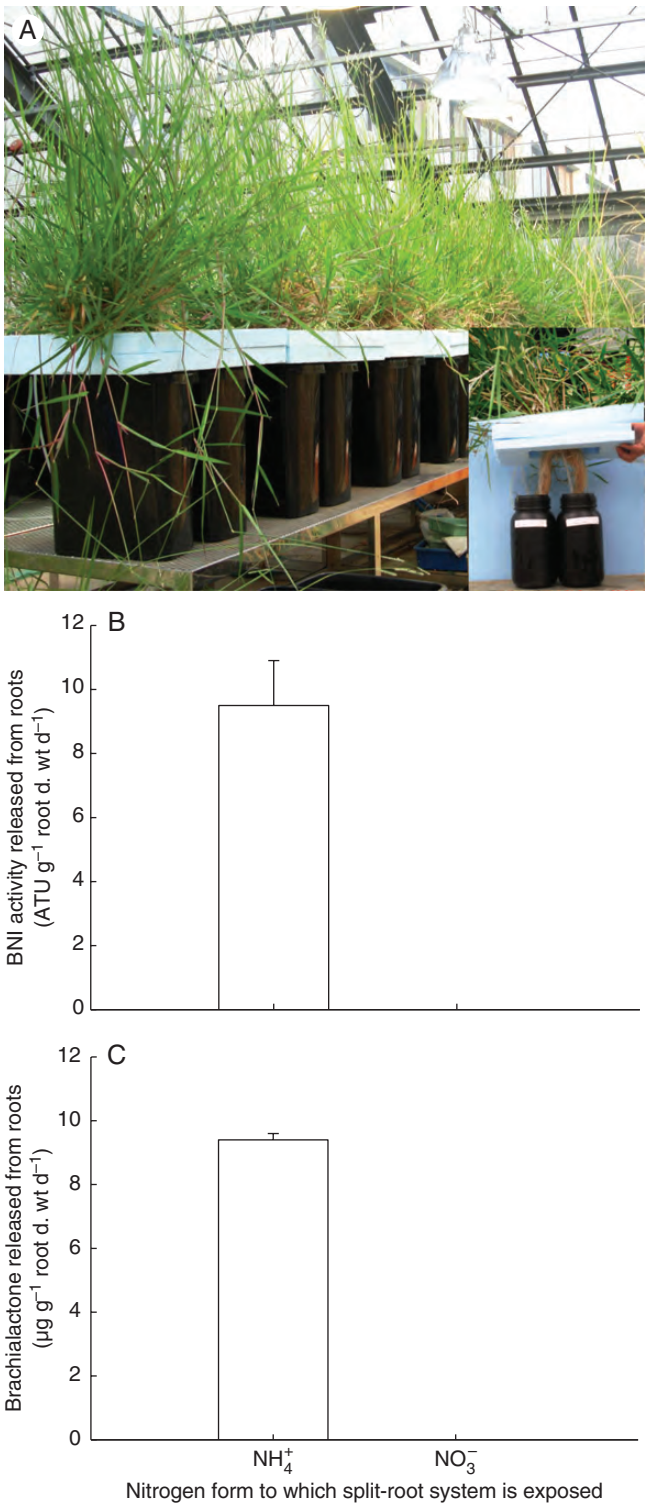

FIG. 12. (A) Split-root system of *B. humidicola.* (B) Influence of nitrogen form (NH_4^+ vs. NO_3^-) in the exudate collection solutions on the release of BNI activity. (C) Release of brachialactone from the roots of *B. humidicola* in a split-root system (adapted from Subbarao *et al.*, 2009*a*).

alkaloids, quinines, mono-terpenoids, di-terpenoids, fatty acids and isothiocyanates are released from root systems of cereal crops, crucifer members and tropical pasture grasses through exudation (Bertin *et al.*, 2003; Frank and Groffman, 2009; Raaijmakers *et al.*, 2009). These biologically active compounds exuded from roots have a wide range of functions that define the rhizosphere chemical and biological environment, and their biological roles range from nutrient acquisition, facilitating symbiotic associations with bacteria and fungi, to defending roots from pests and pathogens (Walker *et al.*,

2003; Rengel and Marschner, 2005). Some of these compounds (such as the recently discovered di-terpenoid, brachialactone, released from *Brachiaria* roots, sorgoleone released from sorghum roots, and isothiocyanates released from degraded cruciferous tissues) have BNI function and thus could become important targets for genetic improvement through conventional and molecular breeding approaches. Such efforts in the future should become an important part of breeding programmes developing genetic and management strategies to exploit biologically active molecules with BNI function to regulate nitrification in production agriculture, where most of the fertilizer N is applied worldwide (Philippot and Hallin, 2011). Some examples of deployable genetic tools for improvement of the BNI capacity follow below

Tropical pasture grasses. Availability of genetic variability is a prerequisite for the genetic improvement of any plant trait using a conventional and/or molecular breeding programme. Significant genetic variability exists for the BNI capacity in *B. humidicola* (Subbarao *et al.*, 2007*b*). Specific BNI activity (ATU g^{-1} root d. wt d^{-1}) ranged from 7·1 to 46·3, indicating a significant potential for genetic improvement of BNI capacity by selection and recombination (Subbarao *et al.*, 2007*b*). Using two ecotypes of a tropical grass *Hyparrhenia diplandra* (high- and low-nitrification ecotype), it was shown that nitrification can be stimulated or suppressed depending on the ecotype, suggesting that the suppression of soil nitrification in these grasses could be a genetic attribute (Lata *et al.*, 2000, 2004). The ongoing *Brachiaria* breeding programme at CIAT in collaboration with JIRCAS plans to identify genetic regions associated with BNI function through quantitative trait locus (QTL) analysis, using a mapping population derived from crosses between apomictic and sexual germplasm accessions of *B. humidicola*, that have contrasting BNI capacity. Also, recent findings indicate substantial genetic variability for brachialactone release among germplasm accessions of *B. humidicola*, and several genetic stocks with contrasting ability (nearly 10-fold differences) for brachialactone release have been identified (G. V. Subbarao and K. Nakahara, JIRCAS, unpubl. res.), suggesting the possibility of breeding for high brachialactone release in *B. humidicola* (i.e. high BNI capacity).

Sorghum. Preliminary investigations indicate a substantial variability in the release of sorgoleone (a major component of hydrophobic root exudate determining the BNI capacity in sorghum) among sorghum genotypes (G. V. Subbarao and C. T. Hash, unpubl. res.). This is in agreement with earlier reports of nearly 30-fold variation in sorgoleone among 25 sorghum varieties (Nimbal *et al.*, 1996). Sorgoleone is the stable product of auto-oxidation of dihydrosorgoleone, an unstable *Striga* seed germination stimulant present in the sorghum rhizosphere. Sorgoleone is highly phytotoxic as it disrupts photosystem II electron transfer, is thought to be responsible for the well-known allelopathy of sorghum, and has been the subject of considerable studies due to its potential as a bio-herbicide. Several genes controlling the biosynthetic pathway of sorgoleone are known (Baerson *et al.*, 2007; Pan *et al.*, 2007) as well as their positions on the aligned genomic sequences of sorghum chromosomes SBI-04, SBI-05, SBI-06 and SBI-08 (Ramu *et al.*, 2010; Satish *et al.*, 2011). Genomic regions associated with production of sorgolactone may also be involved in regulating sorgoleone production (Haussmann *et al.*, 2004; Ejeta, 2007). The immediate precursor of sorgoleone, dihydrosorgoleone, is now thought to be a minor component of the germination stimulant for seeds of parasitic weeds (*Striga* sp.) that is exuded by sorghum root hairs (Rich and Ejeta, 2007). Sorgoleone itself has no *Striga* germination stimulant activity (Rich and Ejeta, 2007), but was once thought to be a key factor in the mechanistic basis for resistance to *Striga* infection (Chang *et al.*, 1986; Netzly *et al.*, 1988). However, Hess *et al.* (1992) found very little variation in levels of sorgoleone between many high and low *Striga* germination stimulant sorghum accessions.

Genomic regions associated with production of strigalactones might also be involved in regulating sorgoleone production (Haussmann, *et al.*, 2004; Ejeta, 2007). Sorghum produces at least five different strigalactones, 5-deoxystrigol, sorgolactone, strigol, strigyl acetate and sorgomol (Cook *et al.*, 1972; Hauck *et al.*, 1992; Siame *et al.*, 1993; Awad *et al.*, 2006; Xie *et al.*, 2008), and these are chemically distinct from sorgoleone. Subsequently it was discovered that the strigolactone 5-deoxystrigol, that is released from sorghum roots in response to N and phosphorus deficiency stress, apparently attracts vascular arbuscular mycorrhizae symbionts – largely responsible for sorghum stimulation of *Striga* seed germination (Bouwmeester *et al.*, 2007; Yoneyama *et al.*, 2007). More recent studies support this important role for strigalactones as germination stimulants for *Striga*, e.g. the discovery of a major recessive gene for low *Striga* germination capacity *lgs* that causes a 35-fold reduction in the level of 5-deoxystrigol in the *Striga*-resistant sorghum line SRN39 compared with the *Striga*-susceptible sorghum line Tabat (Yoneyama *et al.*, 2010), and is associated with *Striga* resistance in sorghum. This gene maps to a distal region of the long arm of sorghum chromosome SBI-05 that may be associated with one or more genes controlling strigalactone biosynthesis (Satish *et al.*, 2011), and not with those previously identified as controlling the final steps of sorgoleone biosynthesis (Baerson *et al.*, 2007; Pan *et al.*, 2007; Cook *et al.*, 2010). Future research will hopefully unravel the interconnectivity in the biosynthetic pathways and regulation of sorgoleone and strigalactone exudation (Akiyama and Hayashi, 2006; Gomez-Roldan *et al.*, 2008) and their functional relationships to both *Striga* germination stimulation and BNI capacity in sorghum. The discovery of sorgoleone's BNI function adds a new dimension to the functional significance of its release from sorghum roots (Subbarao *et al.*, 2012*b*). The International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) has recently developed several mapping populations of random inbred lines based on crosses of sorghum parental lines that differ in sorgoleone exudation (G. V. Subbarao and C. T. Hash, unpubl. data), and these are being 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. Association mapping approaches for sorgoleone could be explored by evaluating the mini-core sub-set (10 % of the

core collection and 1 % of the entire collection, which amounts to 242 accessions) of ICRISAT'S global sorghum germplasm collection, a large portion of which were included in the recently developed Generation Challenge Programme's sorghum reference germplasm set of 384 wild and cultivated accessions. The association panel is being subjected to genotyping-by-sequencing (Elshire *et al.*, 2011) to provide the very high density single nucleotide polymorphism marker fingerprints necessary for whole-genome scan approaches to association mapping (C. T. Hash, pers. comm.). This in turn will facilitate allele mining of traits linked to sorgoleone exudation (Brown *et al.*, 2008; Casa *et al.*, 2008). The basic tools for the identification of alleles that accelerate sorgoleone exudation as a strategy to improve BNI capacity in sorghum are thus available. Once superior alleles that control sorgoleone exudation are identified, they can be rapidly transferred to genetic backgrounds of elite sorghum hybrid parental lines and/or open-pollinated varieties by marker-assisted backcrossing. With the introgression of favourable alleles of one or two major genes (to accelerate the exudation of sorgoleone) into elite genetic backgrounds, it should be possible to improve the BNI capacity in sorghum.

Wheat and barley. Wild progenitors of crop species and traditional varieties/landraces often possess traits that do not exist in the elite germplasm (Manske *et al.*, 2000). Wild progenitors and wild relatives have been used extensively as the source of traits for disease resistance and tolerance to abiotic stresses in wheat breeding (Friebe *et al.*, 1996; Munns *et al.*, 2000). The discrepancy between wild relatives and elite germplasm is often attributed to the impact of decades of breeding and selection under favourable agronomic conditions (Buso and Bliss, 1988). Preliminary results suggested a lack of significant BNI capacity in cultivated wheat (Subbarao *et al.*, 2006*b*). However, subsequent evaluation of wild wheats indicated that roots of *L. racemosus* possess high BNI capacity (Fig. 13; Subbarao *et al.*, 2007*c*). The BNI activity released from *L. racemosus* effectively suppressed soil nitrification for >60 d (Subbarao *et al.*, 2007*c*). Using chromosome addition lines derived from the hybridization of *L. racemosus* with cultivated wheat (Kishii *et al.*, 2004), it was shown that the genes conferring high BNI capacity were located on chromosomes Lr#n, Lr#I and Lr#J, and could be successfully introduced into and expressed in cultivated wheat (Fig. 14; Subbarao *et al.*, 2007*c*). These results indicate that there exists a potential for developing future wheat cultivars with sufficient BNI capacity to suppress soil nitrification in wheat production systems (Subbarao *et al.*, 2007*c*; Zahn, 2007).

Currently wheat production uses a third of the global N-fertilizer production (Raun and Johnson, 1999). Introducing a sufficient level of BNI capacity into cultivated wheat thus would have a large impact on reducing N leakage. However, the alien chromosome of this chromosome addition line may also carry many undesirable traits that could reduce yield. For example, preliminary field evaluations indicate that the introduction of the Lr#n chromosome into Chinese Spring (i.e. DAL#r) made them susceptible to rust (M. Kishii, unpubl. res.). It will be necessary therefore, to transfer to wheat only small segments of this *L. racemosus* chromosome containing favourable alleles of genes linked to the BNI trait to minimize the negative linkage drag that is normally associated with introgressions from wild relatives of wheat.

Various chromosomal manipulations can be deployed to induce a translocation between wheat and alien chromosomes, including the use of a gametocidal chromosome system (Endo, 2007), irradiation and mutants such as *ph1b* that reduce the stringency of pairing control mechanisms to allow pairing of homeologous chromosomes (Sears, 1977, 1993). Reciprocal exchange of alien chromosome segments with the corresponding wheat chromosomes without disrupting the genetic balance would be desirable. Centromeric or Robertsonian translocations could provide reciprocal or near-reciprocal translocations in which half of the target *L. racemosus* chromosome (short or long arm) replaces the corresponding wheat chromosome arms. Since the Lr#n chromosome of *L. racemosus* that controls BNI function has homoeology to both of wheat homoeologous groups 3 and 7 (Kishii *et al.*, 2004) and the fact that we

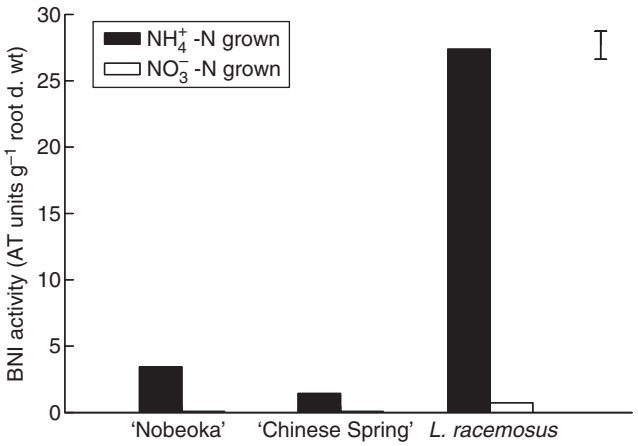

FIG. 13. BNI activity released from roots of two cultivars of cultivated wheat and its wild relative *L. racemosus*. Plants were grown with either NH_4^+ or NO_3^- as their N source. Root exudate was collected from intact roots in aerated distilled water with 200 μM Ca over a 24 h period. The vertical bar represents Fisher's l.s.d. ($P < 0{\cdot}001$) for the interaction term (N source × species; adapted from Subbarao *et al.*, 2007*c*).

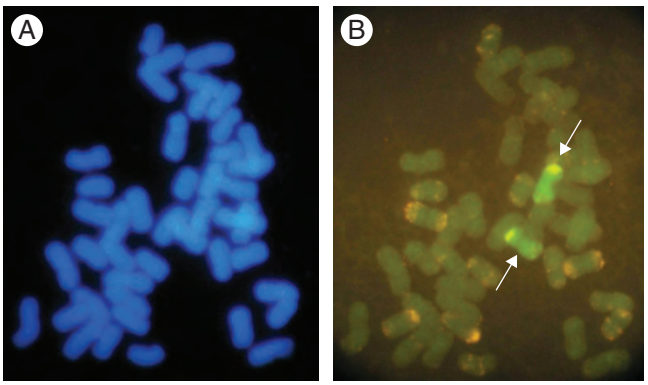

FIG. 14. Karyotype analysis of DALr#n, a chromosome addition line derived from *L. racemosus* × *T. aestivum*. (A) DAPI (4′,6-diamidino-2-phenylindole) staining revealed 44 chromosomes. (B) The probe of *L. racemosus* genomic DNA (green) and TaiI and Afa family repetitive sequences showed the presence of two Lr#n chromosomes. The arrows indicate Lr#n chromosomes (adapted from Subbarao *et al.*, 2007*c*).

have obtained a naturally occurring substitution line with the group 3 chromosome of wheat, it will be desirable to generate translocations with wheat chromosomes of the corresponding groups. The production of such translocations has been achieved by crossing the Lr#n chromosome addition line with the 3B and 7B chromosome monosomic lines of wheat, in which one of the 3B or 7B chromosomes is missing, to produce an F_1 hybrid where chromosome breakage and re-fusion at centromeric regions could be induced between Lr#n and 3B or 7B chromosomes during meiosis (Kishii, 2011).

Crosses of Lr#n addition and translocation lines with the Chinese Spring *ph1b* mutant have also been made by the International Center for the Improvement of Maize and Wheat (CIMMYT) in an effort to generate additional translocations incorporating smaller segments of Lr#n that carry the BNI trait but with a reduced risk of problems associated with linkage drag. Homozygous translocation lines are currently available in the Chinese Spring background but, due to the poor agronomic background of this line, these translocations are being transferred into elite CIMMYT bread wheat. This should allow realistic evaluation of BNI potential to reduce N leakage from wheat systems and increase grain yields at lower N-fertilizer applications. While a range of translocations including some with smaller segments of Lr#n are being produced, small segments are not always needed, as history has shown that one good centromeric translocation can have a large impact on wheat breeding (Lukaszewski, 2000; Singh *et al.*, 2006). The best example of this is the 1BL.IRS translocation involving the short arm of chromosome 1R from rye (*Secale cerale*), which is present in most wheat cultivars in the Middle East and West Asia (most of these are CIMMYT derived), and in a significant proportion of cultivars in China, the USA and East Europe (Stokstad, 2007). The wide distribution of these wheats can be attributed to their high yield in diverse environments, despite all of the known disease resistance genes in the 1RS segment no longer being completely effective. However, if the original translocation is accompanied by many undesirable traits, it will be necessary to perform further reduction of the introgressed *L. racemosus* chromosome segment, a process currently underway through use of the *ph1b* mutant that permits homoeologous recombination between wheat and alien chromosomes (Sears, 1977; Lukaszewski, 2000). As N is an increasingly expensive input in agricultural systems, both yield at low N and responsiveness to added N are important in simultaneously reducing environmental pollution, increasing food production and reducing input costs. Field evaluations of the response of chromosome addition lines of Lr#n, Lr#I and Lr#J lines to N fertilization (250 kg N ha^{-1}) at two locations at CIMMYT indicate that the Lr#I line did not respond to N fertilization, i.e. there was no difference in grain yield between N-fertilizer and no-N field plots. Also, TA7646, which is carrying a homologous chromosome to Lr#I (Qi *et al.*, 1997; Kishii *et al.*, 2004), did not respond to N fertilization during these yield evaluation trials at two field locations at CIMMYT (M. Kishii and I. Ortiz-Monasterio, CIMMYT, unpubl. res.). Currently, efforts are underway at CIMMYT to introduce Lr#n, Lr#I and Lr#J into various high-yielding backgrounds to assess the value of the BNI trait for yield maintenance under sub-optimum N fertilization levels. The material under development in elite backgrounds could be utilized to develop new cultivars rapidly if field trials indicate that translocations show high BNI capacity.

Introduction of the BNI trait from *L. racemosus* to barley would be more challenging as diploid barley is sensitive to chromosome manipulation [compared with tetraploid durum wheat (*Triticum turgidum*) or hexaploid bread wheat]. Also, a gene to induce homeologous recombination like that found in wheat has not been reported for barley. One possible method to introduce an *L. racemosus* chromosome into barley could be through the use of a tetraploid barley line, which has its chromosome number doubled with colchicine, as this would allow a better tolerance to the addition of alien chromosomes. The utilization of barley chromosome addition lines of wheat is an alternative. A set of these addition lines has been produced (Islam *et al.*, 1975), and it may be possible to manipulate the homoeologous barley and *L. racemosus* chromosomes in wheat; first, by crossing the corresponding barley and *L. racemosus* chromosome addition lines to generate the required centromeric translocation, and then transferring the translocation into barley by crossing the tetraploid barley chromosome substitution line with cultivated diploid barley.

Deploying BNI function in production agriculture

Soil physical, chemical and biological properties influence the rhizosphere environment and thus the release of BNIs from roots; in addition, these factors also may determine the effectiveness and stability of the released BNIs in suppressing soil nitrification. For example, alkaline pH may limit the expression and stability of BNI function, thus heavy clay soils such as Vertisols that are generally alkaline in pH may not be suitable for the expression of BNI function. Also high bacterial activity in soils with relatively high OM content (such as organic soils) may enhance the degradation of BNIs, thus making the BNI function less effective. There is little information on how soil temperature and moisture status (linked to inter- and intraseasonal variability or to stresses due to excess or insufficient moisture) modulate the BNI function. For instance, when modelling the rhizosphere and its associated gradients with exudates from roots, it was shown that adsorption properties, solute life time and soil water contents are the key determinants of both the extent of the rhizosphere and the time to reach a steady state, indicating their fundamental roles in the interactions between roots and soil organisms (Raynaud, 2010). Also, the impact of *H. diplandra* ecotypes (stimulation or suppression) on nitrification in tropical savannas has been shown to be highly heterogeneous at both inter- and intra-annual scales, but could be partially explained by a climatic parameter such as average T° or total precipitations in the months prior the date of measurement (Lata, 1999).

For annual crops the crop duration, often $\leq$120 d, may not be adequate (given the BNI activity release rates observed for sorghum and other major food crops; Subbarao *et al.*, 2007*b*, *c*, 2009*c*, 2012*b*, Zakir *et al.*, 2008; Zhu *et al.*, 2012) to reach the critical threshold levels needed to reduce the bulk soil nitrification potential. It is likely that the impact of BNI may be confined to the rhizosphere–soil environment. Tropical pastures with high BNI capacity and extensive root

systems coupled with their perennial habits (e.g. *Brachiaria* spp.) can significantly reduce the soil nitrification potential and nitrifier population (i.e. low-nitrifying production environments). This could be exploited for the benefit of annual crops, such as maize, wheat and rice which receive most of the fertilization, but at present have little inherent BNI capacity, by integrating pastures with crop production using agro-pastoral systems or mixed crop–livestock systems. The pasture component could provide the required BNI activity to suppress soil nitrifier activity and thus the nitrification potential to improve the 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 the soil NH_4^+ oxidation rate, where an annual crop such as maize or soybean is grown after *Brachiaria* pasture is not yet known, but this could determine the interval between pasture phases in such agro-pastoral systems (Subbarao *et al.*, 2012*a*).

For crops that produce BNIs in their plant tissue, but do not release them from their root systems, e.g. crucifers (Bending and Lincoln, 2000), the incorporation of plant residues into the soil could be an alternative way to control soil nitrification. In addition, *Brachiaria* pastures could also be used as cover crops to use their biomass as a mulch after 3–4 months of growth, followed by direct sowing of maize or soybean into the mulch. This is an emerging agronomic practice that is being developed by progressive farmers for controlling nitrification in maize and soybean cultivation in Llanos, Colombia. Such novel agronomic practices supplement the addition of BNIs by *Brachiaria*'s shoot tissues (Subbarao *et al.*, 2008), in addition to that added by the root systems. Thus multidisciplinary efforts through crop genetic improvements of BNI capacity, proper agronomic practices in appropriate cropping systems could be combined to utilize the BNI function to design and promote low-nitrifying production systems in agriculture. In addition, the boundaries of the agro-ecosystems where BNI function can be effectively deployed will have to be defined with the help of agro-ecologists and agronomists. This will help breeders and molecular biologists targeting the desired BNI traits in crops and pastures from a genetic improvement perspective for an entire agro-ecosystem.

PERSPECTIVES

As we race to satisfy the growing global demand for food through massive injections of reactive N (i.e. N-fertilizer) into agricultural systems, the unintended (eutrophication of lakes and NO_3^- contamination of ground water) and unknown (N_2O and NO emissions and their impact on global warming) consequences on the environment have become of great concern (Canfield *et al.*, 2010). Never in the recent history of planet Earth (i.e. during the last 10 000 years) have such major perturbations in the N cycle occurred similar to those that the Earth has experienced since the 1930s (Dansgaard *et al.*, 1993; Petit *et al.*, 1999; Rioual *et al.*, 2001; Rockstrom *et al.*, 2009; Canfield *et al.*, 2010). Current annual N-fertilizer inputs into agricultural systems have reached close to 150 Tg, a level one and a half times greater than Earth's N-fixing capacity (Vitousek *et al.*, 1997; Tilman *et al.*, 2001). This suggests that humans have nearly doubled the reactive N load of Earth in just over 50 years (1960–2010); most of this additional reactive N is routed through just 11 % of the Earth's surface (Newbould, 1989). This has created serious environmental problems, and there is great concern as to how we can protect our environment, while meeting the food demand of the growing world population (Rockstrom *et al.*, 2009). This is a major challenge and requires a new paradigm of approaches on how to manage N in agricultural systems.

The Green Revolution quadrupled the world food production largely through the development of high-yielding, fertilizer-responsive wheat, rice and maize cultivars along with increased N-fertilizer inputs and major changes in agronomic practices (Matson *et al.*, 1999; Tilman *et al.*, 2001; Dinnes *et al.*, 2002; Hungate *et al.*, 2003). 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 drawing force, largely responsible for an inefficient use of N, and for the resulting N pollution associated with agricultural systems.

As discussed in this Viewpoint paper, it is not necessary and prudent that most N be cycled through the nitrification pathway to achieve higher productivity. Nature has shown us that by routing the reactive N through multiple pathways and restricting the flow through the nitrification path, N can be cycled more effectively with limited leakage into the environment (Schimel and Bennett, 2004; Weigelt *et al.*, 2005; Barot *et al.*, 2007; Harrison *et al.*, 2007; Houlton *et al.*, 2007; Hewins and Hyatt, 2009; Ashton *et al.*, 2010). As nitrification and denitrification are the two major biological drivers for the production of NO_3^-, N_2O and NO (i.e. 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.

The BNI capacity in field crops and pastures can be genetically enhanced using both conventional and molecular genetics tools to develop the next-generation cultivars that have the ability to suppress nitrification (Philippot and Hallin, 2011; Subbarao *et al.*, 2012*a*). Wild relatives of wheat seem a promising source of the BNI trait needed to improve the BNI capacity of cultivated wheat (Subbarao *et al.*, 2007*c*; Zahn, 2007). Efforts are under way to transfer the high BNI capacity trait located on Lr#n, Lr#I and Lr#J to cultivated wheat. Molecular breeding approaches such as marker-assisted breeding and metabolic engineering can be deployed to introduce the biosynthetic pathway for brachialactone (a powerful BNI) synthesis and release from root systems of major food crops – wheat, maize and rice. We have demonstrated the effectiveness of BNI function in tropical *Brachiaria* grasses in suppressing soil nitrification and N_2O emissions. Also, potential exists to select and breed for the high BNI trait in other tropical pasture grasses such as *P. maximum* (that have high productivity and forage quality but lack BNI capacity in their root systems) using both conventional and molecular breeding tools.

For an effective deployment of BNI function as a strategy to control nitrification, a multidisciplinary effort using a systems approach is necessary to understand the interactions among crops/pastures, the relative contributions of BNIs from root

exudation, and residue incorporation on N cycling in production systems. Exploiting the BNI function using both genetic and crop/system management approaches is the first step towards designing a low-nitrifying agronomic environment in agricultural systems. A paradigm shift thus is needed to steer N management from the current high-nitrifying environments to low-nitrifying production systems, that are sustainable from both an ecological and an economic perspective, without jeopardizing the ability to meet the global food demand.

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